Enzymes in Food Technology

Second edition

Edited by

Robert J. Whitehurst

and

Maarten van Oort

A John Wiley & Sons, Ltd., Publication

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Contributors

Soottawat Benjakul
Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, Thailand

Caroline H.M. van Benschop
DSM Food Specialties, Delft, The Netherlands

Andreas Bruchmann
Technical Service Fruit Processing Ingredients & Alcohol, DSM Food Specialties Germany GmbH, Germany

Johanna Buchert
VTT Technical Research Centre of Finland, Espoo, Finland

Yves Coutel
DSM Food Specialties, Montpellier Cedex, France

David Cowan
Novozymes UK, Chesham, UK

Céline Fauveau
DSM Food Specialties France SAS, France

Declan Goode
Kerry Ingredients, Kerry Group, County Cork, Ireland

Catherine Grassin
DSM Food Specialties, Montpellier Cedex, France

Hanne Vang Hendriksen
Novozymes, Bagsværd, Denmark

Jan D.R. Hille
DSM Food Specialties, Delft, The Netherlands

Kaisu Honkapää
VTT Technical Research Centre of Finland, Espoo, Finland

Sappasith Klomklao
Department of Food Science and Technology, Faculty of Technology and Community Development, Thaksin University, Phattalung, Thailand

Beate A. Kornbrust
Novozymes, Dittingen, Switzerland

Kristiina Kruus
VTT Technical Research Centre of Finland, Espoo, Finland

Eoin Lalor
Kerry Ingredients, Kerry Group, County Cork, Ireland

Niels Erik Krebs Lange
Novozymes, Bagsværd, Denmark

Raija Lantto
VTT Technical Research Centre of Finland, Espoo, Finland

Barry A. Law
R & D Consultant, Melbourne, Australia

Xiaoli Liu
Nagase ChemteX Corporation, Kobe, Japan

Marc J.E.C. van der Maarel
TNO Quality of Life, The Netherlands, and Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, The Netherlands

Per Munk Nielsen
Novozymes Bagsværd, Denmark

Maarten van Oort
Baking Technology Group, AB Mauri
The Netherlands

Eero Puolanne
Food Technology, University of Helsinki, Finland

Katariina Roininen
VTT Technical Research Centre of Finland, Espoo, Finland

Benjamin K. Simpson
Department of Food Science and Agricultural Chemistry, McGill University, Quebec, Canada

Mary Ann Stringer
Novozymes, Bagsværd, Denmark

Robert J. Whitehurst
Baking Technology Group, AB Mauri,
UK

Preface

In 1833, Payen and Persoz treated an aqueous extract of malt with ethanol and precipitated a heat-labile substance which promoted the hydrolysis of starch. We may say that this was the point at which enzymes were 'discovered'. They named their fraction 'diastase' from the Greek word meaning 'separation'. Having been identified as the component of yeast cell which causes fermentation, the term 'enzyme' was first used by Kühne in 1878, derived from the Greek term meaning 'in yeast'. Enzymes played an important role in foods as early as 2000 BC when the Egyptians and Sumerians developed fermentation for use in brewing, bread baking and cheese making, and then more 'recently' around 800 BC when calves' stomachs and the enzyme chymosin were used for cheese making.

At the end of the first quarter of the twentieth century, enzymes were shown to be proteins, and industrial production and commercial use soon commenced. In 1982, the first application of gene technology to the production of enzymes took place, producing an a-amylase. Six years later, recombinant chymosin was approved and introduced in Switzerland, signalling the introduction of a product of gene technology for a food use.

This technology was introduced to the US food enzyme market in 1990. The preface to the first edition of this book described enzymes as 'functional catalytic proteins' and, furthermore, as useful and targeted workforces which, like the more familiar human workforces, have preferred working conditions, may be trained (cultured) to carry out very specific tasks, and cannot function when their food (substrate) runs out. This volume is again intended to provide both a basic grounding for those not experienced in the use of enzymes and to update the technology published in the first edition. This volume therefore aims to provide a state-of-the-art account of today's enzyme technology as applied to food and drink.

New chapters in the second edition look at the use of enzymes in the reduction of acrylamide, fish processing and in non-bread cereal applications such as flour confectionery. An important further addition is the chapter on GMO and protein engineering (PE), whereby modifications are made to the DNA in the cells of the enzyme-producing organisms. This technology mimics mutations which may occur in nature but at a faster and more targeted rate and has the ability to deliver highly specialized and purer products.

Authors have been selected not only for their practical, working knowledge of enzymes but also for their infectious enthusiasm for the subject.

Enzymes are introduced first according to their nomenclature and then by their nature and mode of action. An explanation of GMO and PE precedes that of enzyme production processes. Chapters go on to describe the basic theory and practical applications of both endogenous and exogenous enzymes in food and drink technology and how enzymes improve raw materials and influence and modify the biochemical and physical events that we describe as 'food processing'. Endogenous enzymes in food raw materials have long played a role in food production. Today, however, enzymologists and geneticists, working together with food development technologists and with a view to legislation and market requirements, have helped and improved upon nature to bring us varieties of food and drink that were unheard

of a relatively short time ago. Newer examples of these are the replacement of emulsifiers, reduction in acrylamide and better utilization of food by-products.

Our thanks go to all the contributors to this book for sharing their practical approach to the subject. We hope that the reader finds the volume as rewarding as we did its preparation.

Robert J. Whitehurst
Maarten van Oort

1 Enzymes in food technology – introduction

Maarten van Oort

Enzymes are proteins that enhance (or accelerate) chemical reactions. This process is called catalysis and enzymes thus catalyze chemical reactions.[1] In enzymatic reactions, the molecules present at the beginning of the reaction are called substrates. Enzymes convert substrates into different molecules, called products. All processes in nature require enzymes in order to occur at significant rates. Enzymes are selective for their substrates and therefore catalyze only a few reactions from among many possibilities.

Like all catalysts, enzymes work by lowering the activation energy for a reaction. This is illustrated in Fig. 1.1.

Catalysts, like enzymes, act by lowering the energy difference between the reactants (A, B) and the transition state. This lowers the activation barrier for the reaction, allowing it to proceed more rapidly.

When the energy ΔG is lowered by a relatively small amount of 5.71 kJ mol^{-1} (a typical H-bond in water has an energy of 20 kJ mol^{-1}), a 10-fold rate enhancement can be obtained. Lowering of the activation energy ΔG by 34.25 kJ mol^{-1} leads to a 10^6-fold rate enhancement.

Since lowering of the kinetic barrier also accelerates the reverse reaction, the equilibrium of the reaction remains unchanged.

As with all catalysts, enzymes are not consumed by the reactions they catalyze, nor do they alter the equilibrium of these reactions. However, enzymes do differ from most other catalysts by being much more specific. Although almost all enzymes are proteins, not all biochemical catalysts are enzymes, since some RNA molecules called ribozymes also catalyze reactions.[2]

1.1 HISTORY

In the nineteenth century, Pasteur studied the fermentation of sugar to alcohol by yeast. He concluded that this fermentation was catalyzed by 'ferments' within the yeast cells. Pasteur believed that these ferments displayed their activity only within living organisms.

Near the end of the nineteenth century, Kühne first used the term enzyme, which comes from Greek 'in yeast', to describe the activity already investigated by Pasteur. The word enzyme was used later to distinguish non-living substances, such as the enzymes we know today, from ferments, whereby 'ferment' is used to describe chemical activity produced by living organisms.

Also at the end of the nineteenth century, Buchner made a significant contribution to enzymology by studying the ability of yeast extracts, lacking living yeast cells, to ferment

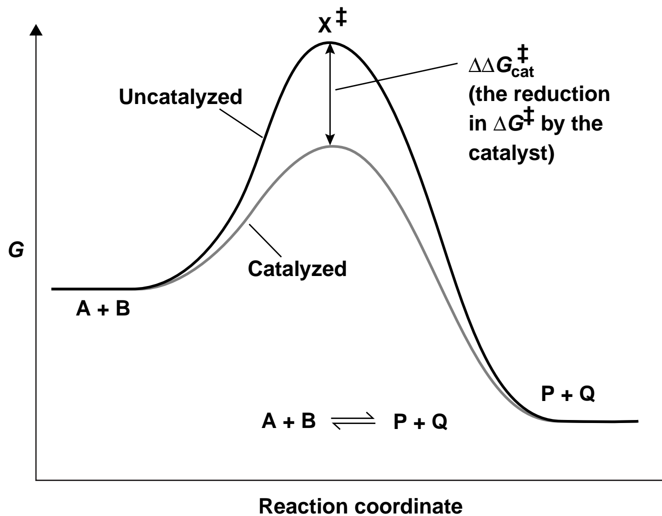

Fig. 1.1 Lowering the activation energy of a reaction.

sugar. He found that the sugar was fermented even when there were no living yeast cells present.

In 1926, Sumner was able to obtain the first enzyme in pure form. He isolated and crystallized the enzyme urease from jack beans.

In the middle of the nineteenth century, Northrop and Stanley developed a complex procedure for isolating pepsin. Their precipitation technique has since been used to crystallize many enzymes. A few years later, for the first time, an enzyme (a protease) was produced by fermentation of *Bacillus licheniformis*. In this way, large-scale production of enzymes became possible, thus facilitating the industrial application of enzymes.

In 1969, the first chemical synthesis of an enzyme was reported. Since then thousands of enzymes have been studied by X-ray crystallography and nuclear magnetic resonance (NMR). Application of genetic engineering techniques improved enzyme production efficiency and even allowed improvement of the properties of specific enzymes by means of protein engineering and evolutionary design. In 2004, the first computer-designed enzymes were reported.

1.2 NOMENCLATURE OF ENZYMES

Enzymes are usually named according to the reaction they carry out. Typically, the suffix '*ase*' is added to the name of the substrate (e.g. glucose-oxidase, an enzyme which oxidizes glucose) or the type of reaction (e.g. a polymerase or isomerase for a polymerization or isomerization reaction). The exceptions to this rule are some of the enzymes studied originally, such as pepsin, rennin and trypsin. The International Union of Biochemistry (IUB) initiated standards of enzyme nomenclature which recommend that enzyme names indicate both the substrate acted upon and the type of reaction catalyzed. Detailed information on nomenclature can be found on the IUB homepage.[3]

Enzymes can be classified by the kind of chemical reaction catalyzed. Officially, six groups of enzymes have been classified:

- **EC 1 oxidoreductases**: catalyze oxidation/reduction reactions which generally involve the transfer of electrons. Examples are oxidases or dehydrogenases.
- **EC 2 transferases**: transfer a functional group (e.g. a methyl or phosphate group) and these generally involve the transfer of a radical. Examples are: transglycosidases, e.g. of monosaccharides; transphosphorylases and phosphomutases, e.g. of a phosphate group; transaminases, e.g. of an amino group; transmethylases, e.g. of a methyl group; and transacetylases, e.g. of an acetyl group.
- **EC 3 hydrolases**: catalyze the hydrolysis of various bonds. The hydrolase reaction generally involves addition or removal of water. Examples are: hydrolases, including esterases, carbohydrases, nucleases, deaminases, amidases and proteases; hydrases such as fumarase, enolase, aconitase and carbonic anhydrase.
- **EC 4 lyases**: cleave various bonds by means other than hydrolysis and oxidation. This reaction involves the splitting or forming a C=C bond. Examples are desmolases.
- **EC 5 isomerases**: catalyze isomerization changes within a single molecule and involve changing the geometry or structure of a molecule. An example is glucose-isomerase.
- **EC 6 ligases**: join two molecules with covalent bonds.

1.3 ENZYMOLOGY

Any living cell inside human beings, animals, microorganisms, plants, etc., is the site of enormous biochemical activity called metabolism. Metabolism is the process of chemical and physical change which goes on continually in a living organism; build-up of new tissue, replacement of old tissue, conversion of food to energy, disposal of waste materials, reproduction – all the activities that we characterize as 'life'. However, hardly any of these biochemical reactions take place spontaneously. Enzymatic catalysis is needed to make these biochemical reactions possible. In this way, enzymes are responsible for bringing about all chemical reactions in living organisms. Without enzymes, chemical reactions would take place at a rate far too slow for the pace of metabolism.

1.3.1 The function of enzymes in nature

Enzymes serve a wide variety of functions inside living organisms. They are indispensable for signal transduction and cell regulation, often via kinases and phosphatases.[4] They also generate movement, with myosin hydrolyzing adenosine triphosphate (ATP) to generate muscle contraction and also moving cargo around the cell as part of the cytoskeleton.[5] Other ATPs in the cell membrane are ion pumps involved in active ion transport. Generally, it can be said that the metabolic pathways in a cell are determined by the types and amount of enzymes present in that cell.

Enzymes have an important function in the 'digestive systems' of mammals and other animals. Enzymes such as amylases break down large starch molecules; proteases break down large protein molecules. The result of these breakdown reactions is the formation of smaller fragments, which can easily be absorbed by the intestines of animals. Starch molecules, for example, are too large to be absorbed as such, but enzymes hydrolyze starch

chains into smaller molecules such as dextrins, maltose and eventually glucose, which can then be absorbed. Different enzymes digest different food substances. In ruminants, which have herbivorous diets, microorganisms in the gut produce enzymes like cellulase to break down the cellulose cell walls of plant fibres.[6]

Several enzymes can work together in a specific order, creating metabolic pathways. In a metabolic pathway, one enzyme takes the product of another enzyme as a substrate. After the catalytic reaction, the product is then passed on to another enzyme. Sometimes more than one enzyme can catalyze the same reaction in parallel; this can allow more complex regulation: with, for example, a low constant activity being provided by one enzyme but an inducible high activity from a second enzyme.

Enzymes determine what steps occur in these pathways. Without enzymes, metabolism would neither progress through the same steps, nor be fast enough to serve the needs of the cell. A metabolic pathway such as glycolysis could not exist without enzymes. Glucose, for example, can react directly with ATP to become phosphorylated at one or more of its carbons. In the absence of enzymes, phosphorylation is insignificant. However, if the enzyme hexokinase is added, phosphorylation at carbon 6 of the glucose molecule occurs extremely fast, leading to a large excess of glucose-6-phosphate versus the products formed by the slow non-catalyzed reactions. Consequently, the network of metabolic pathways within each cell depends on the set of functional enzymes that are present.

1.3.2 Chemistry of enzymes

Enzymes are generally globular proteins, having a size range from just over 60 to more than 2500 amino acids, that is, a MW of ±6000–250 000. The activities of enzymes are determined by their three-dimensional structure.[7] Most enzymes are much larger than the substrates they act on. It is therefore even more remarkable that only a small part of the enzyme molecule is directly involved in catalysis.[7] This small section is called the active site and this site usually contains not more than a few (3–4) amino acids which are directly involved in the catalytic process. The substrate is normally bound by the enzyme in close proximity to, or even in, the active site.

1.3.3 Specificity of enzymes

One of the most relevant and also intriguing properties of enzymes is their specificity. Some enzymes exhibit absolute specificity. This means that these enzymes catalyze only one particular reaction. Other enzymes will be specific for a particular type of chemical bond or functional group. In general, there are four distinct types of specificity:

- **Absolute specificity**: highly specific enzymes catalyze only one reaction.
- **Group specificity**: group specific enzymes act only on molecules that have specific functional groups, such as amino, phosphate or methyl groups.
- **Linkage specificity**: such enzymes act on chemical bonds of certain nature, regardless of the rest of the molecular structure.
- **Stereochemical specificity**: stereospecific enzymes act only on a particular steric or optical isomer and not on their isomeric counterparts.

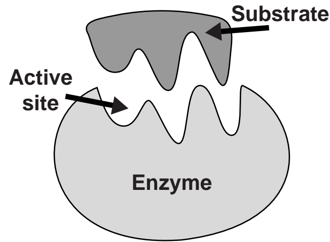

Fig. 1.2 Complementary geometric shapes.

The specificity of enzymes is determined by complementary shape, charge, hydrophilic/hydrophobic characteristics of the substrates and their three-dimensional organization.[8] The three-dimensional interaction has been described in various interaction models. The two most relevant are described.

1.3.3.1 *'Lock and key' model*

Emil Fischer suggested as early as 1894 that enzyme specificity was caused by specific complementary geometric shapes of both the enzyme and the substrate (see Fig. 1.2).

Thanks to these shapes, enzyme and substrate would fit exactly into one another.[9] This is often referred to as 'the lock and key' model. However, while this model explains enzyme specificity, it fails to explain the stabilization of the transition state that enzymes achieve. The 'lock and key' model has proven inaccurate and the induced fit model is the most currently accepted model for enzyme–substrate–coenzyme interaction.

1.3.3.2 *Induced fit model*

In 1958, Koshland[10] suggested a modification to the lock and key model: since enzymes are rather flexible structures, the active site is continuously reshaped by interactions with the substrate as the substrate interacts with the enzyme. As a result, the substrate does not simply bind to a rigid active site; the amino acid side chains which make up the active site are moulded into the precise positions that enable the enzyme to perform its catalytic function. In some cases, such as glycosidases, the substrate molecule also changes shape slightly as it enters the active site.[11] The active site continues to change until the substrate is completely bound, at which point the final shape and charge are determined.[12]

1.3.4 Mechanisms

Enzymes can act in several ways, whereby each enzyme lowers the energy needed for the reaction to occur or to proceed. These mechanisms are described briefly as follows:

- Lowering the activation energy by creating an environment in which the transition state is stabilized. This can be achieved by binding and thus stabilizing the transition-state conformation of the substrate/product molecules.
- Lowering the energy of the transition state, but without distorting the substrate, by creating an environment with the opposite charge distribution to that of the transition state.

- Providing an alternative pathway. For example, temporarily reacting with the substrate to form an intermediate enzyme–substrate (ES) complex, which would be impossible in the absence of the enzyme.
- Reducing the reaction entropy change by bringing substrates together in the correct orientation to react. Considering an energy effect ($\Delta H^{\ddagger}$) alone overlooks this effect.

1.3.5 The enzyme–substrate complex

A theory to explain the catalytic action of enzymes was proposed by Arrhenius at the end of the nineteenth century.[13] He proposed that the substrate and enzyme formed some intermediate transition state which is known as the ES complex. This can be schematically represented as shown in equation (1).

$$E + S \underset{K_{-1}}{\overset{K_1}{\rightleftarrows}} ES \quad (1)$$

The existence of intermediate ES complexes has been demonstrated in the laboratory, for example, using catalase and a hydrogen peroxide derivative.

From this intermediate ES complex, the reaction proceeds with the formation of the product(s) and the enzyme then returns to its original form after the reaction is concluded.

1.3.6 Chemical equilibrium

Many chemical reactions do not go to true completion. Enzyme-catalyzed reactions do not form an exception to that chemical 'law', which is due to the reversibility of most (enzyme-catalyzed) reactions.

Equilibrium is a sort of steady-state condition which is reached when the forward reaction rates equal the backward rates. Enzyme activity studies are always based on the principle of equilibrium reactions.

1.4 ENZYME KINETICS

Enzyme kinetics is a fundamental way of describing, predicting and calculating how enzymes bind substrates, turn these into products and also how fast and efficiently this is happening.

Early last century, a quantitative theory of enzyme kinetics was proposed,[14] but the experimental data were not useful since the logarithmic pH-scale was not known yet. This scale was introduced a little later[15] together with the concept of buffering. Later on, when these early experiments were repeated, the equations were confirmed and referred to as (Henri–) Michaelis–Menten kinetics.[16] This work was further developed and resulted in kinetic equations which are still in use.[17]

The model for enzyme action, as first elucidated by Michaelis and Menten,[16] suggests the binding of free enzyme to the reactant forming an enzyme-reactant complex. This complex undergoes a transformation, releasing product and free enzyme. This is schematically shown

in equation (2).

$$ES \xrightarrow{K_2} E + P \quad (2)$$

$$E + S \underset{K_{-1}}{\overset{K_1}{\rightleftarrows}} ES \xrightarrow{K_2} E + P \quad (3)$$

When reactions (1) and (2) are combined into reaction (3), a model for enzyme catalysis is obtained. First, the enzyme (*E*) and substrate (*S*) come together to form an ES complex; the reaction occurs by which the substrate is converted into the product of the reaction and then the ES complex is broken apart, yielding enzyme (*E*) plus product (*P*).

The Michaelis–Menten model assumes that only a negligible amount of ES complex reverts to reactants (i.e. $k_1 \gg k_{-1}$ in equation (1)). The rate of formation of product (shown in equation (4)) can be determined from equation (2) in the mechanism written above:

$$\text{Rate of formation of product is } K_2\,[S] \quad (4)$$

and the rate of formation of the intermediate ES (equation (5)) can be determined from equations (1) and (2) in the mechanism written above:

$$\text{Rate of formation of } ES = K_1[E][S] - (K_2 + K_1)[ES] \quad (5)$$

Using the steady-state approximation, that is, the assumption that the concentration of intermediates (ES) stays constant while the concentrations of reactants and products change, the equation for the rate of formation of the product can be calculated as follows:

$$\frac{\delta[P]}{\delta t} = \frac{K_2[E_0][S]}{[S] + K_\mathrm{m}} \quad (6)$$

Here $[E_0]$ is the initial concentration of free enzyme, $[S]$ is the substrate concentration and K_m is a constant specific to a given enzyme known as the Michaelis–Menten constant. The value of K_m relates to the rate constants shown in equations (1) and (2), as given by the following equation:

$$K_\mathrm{m} = \frac{K_{-1} + K_2}{K_1} \quad (7)$$

The Michaelis–Menten constant (K_m) is very important, because it can be determined experimentally and describes the catalytic power of an enzyme. K_m can also be used to predict the rate of an enzyme-catalyzed reaction when the starting conditions (enzyme and substrate concentration) are known.

The major contribution of Henri[14] was to think of enzyme reactions in two stages. In the first, the substrate binds reversibly to the enzyme, forming the ES complex. In the second reaction, the enzyme catalyzes the chemical step and releases the product.

Enzymes can catalyze up to several million reactions per second. Enzyme rates depend on solution conditions and substrate concentration. Conditions that denature the protein reduce or eliminate enzyme activity. Such conditions are high temperature, extreme pH or high salt concentrations. Raising substrate concentration tends to increase activity. To

find the maximum speed of an enzymatic reaction, the substrate concentration is increased until a constant rate of product formation is seen. Saturation happens because, as substrate concentration increases, more and more of the free enzyme is converted into the substrate-bound ES form. At the maximum velocity (V_{max}) of the enzyme, all the enzyme active sites are bound to substrate, and the amount of ES complex is the same as the total amount of enzyme. However, V_{max} is only one kinetic constant of enzymes. The amount of substrate needed to achieve a given rate of reaction is also important. This is given by the Michaelis–Menten constant (K_m), which is the substrate concentration required for an enzyme to reach one-half of its maximum velocity. Each enzyme has a characteristic K_m for a given substrate, and this can show how tight the binding of the substrate is to the enzyme. Another useful constant is k_{cat}, which is the number of substrate molecules handled by one active site per second.

The efficiency of an enzyme can be expressed in terms of k_{cat}/K_m. This is also called the specificity constant and incorporates the rate constants for all steps in the reaction. Because the specificity constant reflects both affinity and catalytic ability, it is useful for comparing different enzymes against each other, or the same enzyme with different substrates. The theoretical maximum for the specificity constant is called the diffusion limit and is about 10^8–10^9 (M^{-1} s^{-1}). At this point, every collision of the enzyme with its substrate will result in catalysis, and the rate of product formation is not limited by the reaction rate but by the diffusion rate. Enzymes with this property are called catalytically perfect or kinetically perfect.

1.5 FACTORS AFFECTING ENZYME ACTIVITY

Knowledge of basic enzyme kinetic theory is important in order to understand the basic enzymatic reaction mechanism and to select methods for enzyme analysis.

Several factors affect the rate at which enzymatic reactions proceed, such as temperature, pH, enzyme concentration, substrate concentration and the presence of any inhibitors or activators.

1.5.1 Enzyme concentration

In order to study the effect of increasing the enzyme concentration upon the reaction rate, the substrate must be present in an excess amount; that is, the reaction must be independent of the substrate concentration. Any change in the amount of product formed over a specified period of time will be dependent upon the level of enzyme present. These reactions are said to be ‘zero order’ because the rates are independent of substrate concentration and are equal to some constant k. The formation of product proceeds at a rate which is linear with time. The addition of more substrate does not serve to increase the rate. In zero-order kinetics, allowing the assay to run for double time results in double the amount of product.

The amount of enzyme present in a reaction is measured by the activity it catalyzes. The relationship between activity and concentration is affected by many factors such as temperature, pH, etc. Highest enzyme activity is generally measured when substrate concentration is unlimiting.

1.5.2 Substrate concentration

It has been shown experimentally that if the amount of the enzyme is kept constant and the substrate concentration is then gradually increased, the reaction velocity will increase until

it reaches a maximum. After this point, increases in substrate concentration will not increase the velocity. It is theorized that when this maximum velocity is reached, all of the available enzyme is converted to ES, the ES complex. Michaelis constants have been determined for many of the commonly used enzymes. The size of K_m tells us several things about a particular enzyme:

1. A small K_m indicates that the enzyme requires only a small amount of substrate to become saturated. Hence, the maximum velocity is reached at relatively low substrate concentrations.
2. A large K_m indicates the need for high substrate concentrations to achieve maximum reaction velocity.
3. The substrate with the lowest K_m upon which the enzyme acts as a catalyst is frequently assumed to be enzyme's natural substrate, though this is not true for all enzymes.

1.5.3 Allostery

Allostery or allosteric regulation is the regulation of an enzyme or other protein by binding an effector molecule at the protein's allosteric site. The allosteric site is a site other than the active site of the enzyme. Effectors that enhance the enzyme's activity are referred to as allosteric activators, whereas those that decrease the protein's activity are called allosteric inhibitors. Following this mechanism, allosteric inhibition is a form of non-competitive inhibition (see Section 1.5.6.3).

The term allostery comes from the Greek allos, 'other', and stereos, 'space', referring to the regulatory site of an allosteric protein being separate from its active site. Allosteric regulation is a natural example of feedback control.

1.5.4 Cofactors

Many enzymes require the presence of other compounds, called cofactors, which are needed in order to demonstrate their catalytic activity. This entire active complex is referred to as the holoenzyme; that is, the apoenzyme (protein portion) plus the cofactor (coenzyme, prosthetic group or metal-ion-activator) together is called the holoenzyme.

A cofactor may be:

1. a coenzyme – a non-protein organic substance which is dialyzable, thermostable and loosely attached to the protein part;
2. a prosthetic group – an organic substance which is dialyzable and thermostable which is firmly attached to the protein or apoenzyme portion; and
3. a metal-ion-activator – these include K^+, Fe^{++}, Fe^{+++}, Cu^{++}, Co^{++}, Zn^{++}, Mn^{++}, Mg^{++}, Ca^{++} and Mo^{+++}.

Enzymes usually bind these cofactors in or close by the active site. Furthermore, some enzymes can additionally bind other molecules than substrates or cofactors and these are often molecules inhibiting the enzyme or are interfering with the catalytic process. In this way the enzymatic reaction can be regulated by the cell. Some enzymes do not need any additional components to show full activity. However, others require non-protein molecules called cofactors to be bound for activity. Cofactors can be either inorganic (e.g. metal ions or iron–sulphur clusters) or organic compounds, (e.g. flavin or haem). Organic cofactors can

be either prosthetic groups, which are tightly bound to an enzyme, or coenzymes, which are released from the enzyme's active site during the reaction. Coenzymes include NADH, NADPH and ATP. These molecules act to transfer chemical groups between enzymes.

The tightly bound cofactors are usually found in the active site and are involved in catalysis. For example, flavin and heme cofactors are often involved in redox reactions.

1.5.5 Coenzymes

Coenzymes are small organic molecules that transport chemical groups from one enzyme to another.[18] Some of these chemicals such as riboflavin, thiamine and folic acid are vitamins. Such compounds cannot be made in the body and must be acquired from the diet. The chemical groups carried include the hydride ion (H^-) carried by NAD or $NADP^+$, the acetyl group carried by coenzyme A, formyl, methenyl or methyl groups carried by folic acid and the methyl group carried by *S*-adenosylmethionine.

Since coenzymes are chemically changed as a consequence of enzyme action, it is useful to consider coenzymes to be a special class of substrates, or second substrates, which are common to many different enzymes. For example, about 700 enzymes are known to use coenzyme NADH.[19]

Coenzymes are usually regenerated and their concentrations maintained at a steady level inside the cell.

1.5.6 Inhibitors

Enzyme inhibitors are substances which alter the catalytic action of the enzyme and consequently slow down, or in some cases, stop catalysis.

Most theories concerning inhibition mechanisms are based on the existence of the ES complex. Substrate inhibition will sometime occur when excessive amounts of substrate are present.

There are three common types of enzyme inhibition – competitive, non-competitive and substrate inhibition. Besides these inhibitor types, a mixed inhibition exists as well.

1.5.6.1 *Competitive inhibitors*

Competitive inhibition occurs when the substrate and a substance resembling the substrate are both added to the enzyme. The 'lock–key theory' of enzyme catalysts is used to explain why inhibition occurs.

The concept holds that one particular portion of the enzyme surface has a strong affinity for the substrate. The substrate is held in such a way that its conversion to the reaction products is more favourable. However, when an inhibitor which resembles the substrate is present, it will compete with the substrate for the position in the active site. When the inhibitor is bound to the active site of the enzyme, it blocks this position in the active site, but is not converted by the enzyme. In this way, the active site remains blocked. Hence, the observed reaction is slowed down because a part of the available enzyme sites is occupied by the inhibitor. If a dissimilar substance which does not fit the site is present, the enzyme rejects it, accepts the substrate and the reaction proceeds normally.

In competitive inhibition, the maximal velocity of the reaction is not changed, but higher substrate concentrations are required to reach a given velocity, increasing the apparent K_m.

1.5.6.2 *Uncompetitive inhibition*

In uncompetitive inhibition, the inhibitor cannot bind to the free enzyme, but only to the ES complex. The EIS complex thus formed is enzymatically inactive. This type of inhibition is rare, but may occur in multimeric enzymes.

1.5.6.3 *Non-competitive inhibition*

Non-competitive inhibitors are considered to be substances which, when added to the enzyme, alter the enzyme in a way that it cannot accept the substrate.

Non-competitive inhibitors can bind to the enzyme at the same time as the substrate; that is, they never bind to the active site. In this way a complex is formed of enzyme, inhibitor and substrate (EIS). Both the EI and EIS complexes are enzymatically inactive. Because the inhibitor cannot be driven from the enzyme by higher substrate concentration (in contrast to competitive inhibition), the apparent V_{max} changes. But because the substrate can still bind to the enzyme, the K_m stays the same.

1.5.6.4 *Mixed inhibition*

This type of inhibition resembles the non-competitive, except that the EIS complex has residual enzymatic activity.

In many organisms, inhibitors may act as part of a feedback mechanism. If an enzyme produces too much of one substance in the organism, that substance may act as an inhibitor for the enzyme at the beginning of the pathway that produces it, causing production of the substance to slow down or stop when there is sufficient amount. This is a form of negative feedback. Enzymes which are subject to this form of regulation are often multimeric and have allosteric binding sites for regulatory substances. Irreversible inhibitors react with the enzyme and form a covalent adduct with the protein.

1.6 INDUSTRIAL ENZYMES

For centuries, enzymes have been employed in a variety of applications such as beer and cheese production. Both in the past and currently, enzymes have been derived from natural sources such as the tissue of plants and animals; Table 1.1 summarizes these. However, over the years, advancements in biotechnology have resulted in newer and more highly efficient varieties of enzymes.

The industrial success of enzymes can be attributed to certain key benefits that enzymes offer in comparison with chemicals. The combination of catalytic function, specificity and the ability to work under reasonably mild conditions makes enzymes the preferred catalyst in a variety of applications.

Industrial enzymes are prepared and commercialized as partly purified or ‘bulk’ enzymes, as opposed to highly purified enzymes for analytical or diagnostic use. Industrial enzymes may be derived from a wide variety of plant, animal or microbial sources, although most production processes rely on microbial sources. Microbial enzymes are either extracellular, such as the proteases and carbohydrates, which account for a large proportion of total sales, or intracellular, such as glucose oxidase. Intracellular enzymes usually remain associated with the cell and therefore have to be released, unless the microorganism itself is used as the catalyst.

Table 1.1 Enzymes widely sourced from animals and plants used in food manufacturing technology.

Enzyme	Source	Action in food	Food applications
α-Amylase	Cereal seeds, e.g. wheat, barley	Starch hydrolysis to oligosaccharides	Bread making, brewing (malting)
β-Amylase	Sweet potato	Starch hydrolysis to maltose	Production of high malt syrups
Papain	Latex of unripe papaya fruit	Food and beverage protein hydrolysis	Meat tenderization, chill haze prevention in beer
Bromelain	Pineapple juice and stem	Muscle and connective tissue protein hydrolysis	Meat tenderization
Ficin	Fig fruit latex	As bromelain	As bromelain and papain but not widely used due to cost
Trypsin	Bovine/porcine pancreas	Food protein hydrolysis	Production of hydrolyzates for food flavouring (mostly replaced now by microbial proteinases)
Chymosin (rennet)	Calf abomasum	κ-Casein hydrolysis	Coagulation of milk in cheese making
Pepsin	Bovine abomasum	As chymosin + more general casein hydrolysis in cheese	Usually present with chymosin as part of 'rennet'
Lipase/esterase	Gullet of goat and lamb; calf abomasum; pig pancreas	Triglyceride (fat) hydrolysis	Flavour enhancement in cheese products; fat function modification by interesterification
Lipoxygenase	Soya bean	Oxidation of unsaturated fatty acids in flour	Bread dough improvement
Lysozyme	Hen egg white	Hydrolysis of bacterial cell wall polysaccharides	Prevention of late-blowing defects in cheese by spore-forming bacteria
Lactoperoxidase	Cheese whey; bovine colostrum	Oxidation of thiocyanate ion to bactericidal hypothiocyanate	Cold sterilization of milk

The real breakthrough of enzymes occurred with the introduction of microbial proteases into washing powders. The first commercial bacterial, *Bacillus* protease was marketed in 1959 and the first major detergent manufacturer started to use it around 1963. The industrial enzyme producers sell enzymes for a wide variety of applications. The estimated value of world market is presently about US$ 2.2 billion. Detergents (30%), textiles (12%), starch (12%), baking (11%), biofuel (9%) and animal feed (8%) are the main industrial applications, which use about >80% of industrially produced enzymes.

Industrial enzymes represent the heart of biotechnology. Advancements in biotechnology and genomics have aided the discovery of fresh enzyme sources and production strains for commercialization. The operating conditions and performance of enzyme candidates can be tuned to provide the desired performance.

Enzymes can be used not only for chemical processes, but also for mechanical and physical processes. An example of a chemical reaction is the use of amylases to replace acid in the hydrolysis of starch. The use of cellulose degrading or modifying enzymes instead of pumice stone for the abrasion of denim is a perfect example of enzymes replacing a mechanical

process. Employing protease enzymes, one can easily perform physical processes such as high temperature resistance for laundry cleaning.

With advances in biotechnology, the horizon of enzyme applications is getting broader day by day. Enzymes are now being used in newer processes that could compete with synthetic processes which were previously not commercially viable. For example, several companies are nowadays developing newer enzymes that could convert cellulosic biomass into ethanol to be blended in fuels. Other examples include the use of enzyme technology when making sugars from starch, which helped turn high fructose corn syrup production into a multi-billion dollar industry.

Most industrial enzymes are produced by modified microorganisms (by recombinant DNA techniques) for the following reasons:

1. Higher expression levels.
2. Higher purity (% enzyme protein vs. % other components).
3. Cheaper production due to the above.
4. Recombinant DNA techniques open the door to engineering the enzyme protein.
5. Enzymes can be expressed which originate from organisms which have low expression levels or which are pathogenic.

Protein engineering (item 4 in the list above) can improve enzymes with regard to, for example, oxidation resistance, improved processing tolerance, changed substrate specificity, improved thermostability and improved storage stability, for example, in detergent systems containing bleach agents.

Recombinant DNA techniques may open the door to the application of enzymes from so-called extremophiles. These are microorganisms which can, in contrast to mesophiles, grow under extreme conditions. Such organisms grow under the following conditions:

- Thermophiles (high temperature $> 90^\circ$C stability)
- Psycrophiles (extreme low temperatures, 0°C or lower)
- Thermoacidophiles (high temperature, low pH)
- Barophiles (high pressure)
- Halophiles (high concentrations of salt)
- Alkaliphiles (high pH)
- Acidophiles (low pH)

It can be imagined that such organisms either produce a different range of enzymes than mesophiles, or produce enzymes with extreme characteristics, such as temperature or stability and activity at extreme pH values.

1.7 FOOD ENZYMES

1.7.1 Food biotechnology

The food industry is using a wide variety of crop plants and animal products as basis for their manufacturing processes, leading to an even wider variety of consumer products. Biotechnology, which has been used to manufacture food products for more than 8000 years, offers ways to improve the processing of raw materials into final products. Bread, alcoholic beverages, vinegar, cheese and yogurt and many other foods have been made using enzymes

which were found in various microorganisms. Today, biotechnology is still affecting the food industry by providing new products, lowering costs and improving processes on which food producers have long relied. Without a doubt, this will continue into the future.

Using biotechnology, improvements in functionality, nutritional value, sensoric properties, like flavour and texture have been achieved as well as improvements in the processing itself, using new tools, such as enzymes, emulsifiers and improved starter cultures. Biotechnology also offers improved ways to deal with waste problems, food safety problems, packaging issues, etc.

1.7.2 Food enzyme application

Enzymes can modify and improve the functional, nutritional and sensoric properties of ingredients and products, and therefore enzymes have found widespread applications in processing and production of all kinds of food products.

In food production, enzymes have a number of advantages. First and most important is that enzymes are used as alternatives to traditional chemical-based technology. Enzymes can thus replace synthetic chemicals in a wide range of processes. This allows advantages in environmental performance of processes by lowering energy consumption levels and biodegradability of products. Furthermore, since enzymes are more specific in their action than chemical reactants, enzyme-catalyzed processes have fewer side reactions and by-products (waste products). The result is higher quality products and less pollution. Enzymes can catalyze reactions under very mild conditions, allowing mild processing conditions which do not destroy valuable attributes of foods and food components. Finally, enzymes allow processes to be carried out which would be otherwise impossible.

The first commercial food product produced by biotechnology was an enzyme used in cheese making. Prior to biotech techniques, this enzyme had to be extracted from the stomach of calves, lambs and baby goats, but it is now produced by microorganisms that were given the gene for this enzyme.

The food industry uses more than 55 different enzyme products in food processing. This number will increase as we discover how to capitalize on the extraordinary diversity of the microbial world and obtain new enzymes that will prove important in food processing. A summary of enzymes used in various food applications is shown in Table 1.2.

1.8 GENETIC ENGINEERING

Since the early 1980s, companies which produce enzymes have been using genetic engineering techniques to improve production efficiency and quality and to develop new products. There are clear advantages here for both industry and consumers, with major improvements in enzyme production giving better products and processes. However, progress is being slowed down because the debate on some other, more controversial applications of biotechnology – such as genetic engineering in animals – is continuing throughout Europe.

1.9 ENZYME ALLERGY

To date, there have been no reports of consumer allergies to enzyme residues in food. The levels of enzyme residues appearing in foods are so low that they are highly unlikely ever to cause allergies. Like all proteins, enzymes can cause allergic reactions when people have

Table 1.2 Enzyme usage in food applications.

Enzyme	Source	Action in food	Application in food technology
α-Amylase	*Aspergillus* spp. *Bacillus* spp. *Microbacterium imperiale*	Wheat starch hydrolysis	Amylase dough softening, increased bread volume, aid in the production of sugars for yeast fermentation
α-Acetolactate	*Bacillus subtilis*	Converts acetolactate to acetoin	Reduction of wine maturation time by circumventing need of decarboxylase for secondary fermentation of diacetyl to acetoin
Amyloglucosidase	*Aspergillus niger* *Rhizopus* spp.	Hydrolyzes starch dextrins to glucose (saccharification)	One stage of high fructose corn syrup production; production of 'lite' beers
Aminopeptidase	*Lactococcus lactis* *Aspergillus* spp. *Rhizopus oryzae*	Releases free amino acids from N-terminus of proteins and peptides	Debittering protein hydrolyzates accelerating cheese maturation
Catalase	*Aspergillus niger* *Micrococcus luteus*	Breaks down hydrogen peroxide to water and oxygen	Oxygen removal technology, combined with glucose oxidase
Cellulase	*Aspergillus niger* *Trichoderma* spp.	Hydrolyzes cellulose	Fruit liquefaction in juice production
Chymosin	*Aspergillus awamori* *Kluyveromyces lactis*	Hydrolyzes κ-casein	Coagulation of milk for cheese making
Cyclodextrin glucanotransferase	*Bacillus* spp.	Synthesize cyclodextrins from liquefied starch	Cyclodextrins are food grade microencapsulants for colours, flavours and vitamins
β-Galactosidase (lactase)	*Aspergillus* spp. *Kluyveromyces* spp.	Hydrolyzes milk lactose to glucose and galactose	Sweetening milk and whey; products for lactose-intolerant individuals; reduction of crystallization in ice cream containing whey; improving functionality of whey protein concentrates; manufacture of lactulose
β-Glucanase	*Aspergillus* spp. *Bacillus subtilis*	Hydrolyzes β-glucans in beer mashes	Filtration aids, haze prevention in beer production
Glucose isomerase	*Actinoplanes missouriensis* *Bacillus coagulans* *Streptomyces lividans* *Streptomyces rubiginosus*	Converts glucose to fructose	Production of high fructose corn syrup (beverage sweetener)
Glucose oxidase	*Aspergillus niger* *Penicillium chrysogenum*	Oxidizes glucose to gluconic acid	Oxygen removal from food packaging; removal of glucose from egg white to prevent browning
Hemicellulase and xylanase	*Aspergillus* spp. *Bacillus subtilis* *Trichoderma reesei*	Hydrolyzes hemicelluloses (insoluble non-starch polysaccharides in flour)	Bread improvement through improved crumb structure

(*continued*)

Table 1.2 (*Continued*)

Enzyme	Source	Action in food	Application in food technology
Lipase and esterase	*Aspergillus* spp. *Candida* spp. *Rhizomucor miehei* *Penicillium roqueforti* *Rhizopus* spp. *Bacillus subtilis*	Hydrolyzes triglycerides to fatty acids and glycerol; hydrolyzes alkyl esters to fatty acids and alcohol	Flavour enhancement in cheese products; fat function modification by interesterification; synthesis of flavour esters
Pectinase (polygalacturonase)	*Aspergillus* spp. *Penicillium funiculosum*	Hydrolyzes pectin	Clarification of fruit juices by depectinization
Pectinesterase	*Aspergillus* spp.	Removes methyl groups from galactose units in pectin	With pectinase in depectinization technology
Pentosanase	*Humicola insolens* *Trichoderma reesei*	Hydrolyzes pentosans (soluble non-starch polysaccharides in wheat flours)	Part of bread dough improvement technology
Pullulanase	*Bacillus* spp. *Klebsiella* spp.	Hydrolyzes 1–6 bonds that form branches in starch structure	Starch saccharification (improves efficiency)
Protease (proteinase)	*Aspergillus* spp. *Rhizomucor miehei* *Cryphonectria parasitica* *Penicillium citrinum* *Rhizopus niveus* *Bacillus* spp.	Hydrolysis of κ-casein; hydrolysis of animal and vegetable food proteins; hydrolysis of wheat glutens	Milk coagulation for cheese making; hydrolyzate production for soups and savoury foods; bread dough improvement

been sensitized through exposure to large quantities. For this reason, enzyme companies take a variety of protective measures and some enzymes are produced as liquids, granules, in capsules or as immobilized preparations to limit worker exposure.

1.10 SUMMARY AND CONCLUSIONS

From the earliest application of enzymes derived from plant or animal origin, enzymologists and biotechnologists continue to develop an increasingly sophisticated range of enzymes for specific food applications. To name but a few examples, their products enable the removal or minimization of chemicals used for food processing, minimization of waste, facilitate processes which may otherwise be impractical or uneconomic, enhance nutrition, texture and extend shelf life. The more recent techniques of protein engineering of enzymes will enable technologists to drive these valuable tools into the future.

REFERENCES

1. Smith, A.D., Datta, S.P., Smith, G.H., Campbell, P.N., Bentley, R. and McKenzie, H.A. (eds) (1997) *Oxford Dictionary of Biochemistry and Molecular Biology*. Oxford University Press, Oxford.

2. Lilley, D. (2005). Structure, folding and mechanisms of ribozymes. *Current Opinion in Structural Biology* **15**(3), 313–323.
3. IUB homepage, http://www.chem.qmul.ac.uk/iubmb/
4. Hunter, T. (1995) Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. *Cell* **80**(2), 225–236.
5. Berg, J.S., Powell, B.C. and Cheney, R.E. (2001) A millennial myosin census. *Molecular Biology of the Cell* **12**(4), 780–794.
6. Mackie, R.I. and White, B.A. (1990) Recent advances in rumen microbial ecology and metabolism: potential impact on nutrient output. *Journal of Dairy Science* **73**(10), 2971–2995.
7. Anfinsen, C.B. (1973) Principles that govern the folding of protein chains. *Science* **181**(96), 223–230.
8. Jaeger, K.E. and Eggert, T. (2004) Enantioselective biocatalysis optimized by directed evolution. *Current Opinion in Biotechnology* **15**(4), 305–313.
9. Fischer, E. (1894) Einfluss der configuration auf die wirkung der enzyme. *Berichte der Deutschen Chemischen Gesellschaft* **27**, 2985–2993.
10. Koshland, D.E. (1958) Application of a theory of enzyme specificity to protein synthesis. *Proceedings of the National Academy of Sciences of the United States of America* **44**(2), 98–104.
11. Vasella, A., Davies, G.J. and Bohm, M. (2002) Glycosidase mechanisms. *Current Opinion in Chemical Biology* **6**(5), 619–629.
12. Boyer, R. (2002) *Concepts in Biochemistry*, 2nd edn (in English). John Wiley & Sons, Inc., New York, pp. 137–138.
13. Arrhenius, S. (1899) On the theory of chemical reaction velocity. *Zeitschrift für Physikalische Chemie* **28**, 317.
14. Henri, V. (1902) Theorie generale de l'action de quelques diastases. *Comptes Rendus Hebdomadaires Academie de Sciences de Paris* **135**, 916–919.
15. Sørensen, P.L. (1909) Enzymstudien {II}. Über die Messung und Bedeutung der Wasserstoffionenkonzentration bei enzymatischen Prozessen. *Biochemische Zeitschrift* **21**, 131–304.
16. Michaelis, L. and Menten, M. (1913) Die Kinetik der Invertinwirkung. *Biochemische Zeitschrift* **49**, 333–369.
17. Briggs, G.E. and Haldane, J.B.S. (1925) A note on the kinetics of enzyme action. *Biochemical Journal* **19**, 338–339.
18. Wagner, A.F. and Folkers, K.A. (1975) *Vitamins and Coenzymes*. Interscience Publishers, New York.
19. BRENDA Enzyme Information Database, http://www.brenda-enzymes.info/

2 GMO and protein engineering

Xiaoli Liu

2.1 INTRODUCTION

Although there are still intense public debates about using genetically modified organisms (GMOs), especially regarding transgenic plants and animals, general fear, due to lack of knowledge, is subsiding, especially in the scientific community. After several decades of experience gained through experiments, it is now possible to evaluate GMOs on the basis of safety, environmental issues and economical effects in a more rational manner. As a matter of fact, GMOs are playing increasingly important roles in various industries and everyday life. These include the following:

- **Pharmaceuticals**: Drug proteins (hormones, antibodies, vaccines, etc.) produced by genetically modified microorganisms, plants or animals including cells; for example human insulin produced by recombinant *Escherichia coli* (*E. coli*).
- **Materials for food or food processing**: Nutrient materials such as amino acids and vitamins produced from GMOs, enzymes as food processing aids, enriched crops such as β carotene enriched rice ('the Golden Rice'), bovine chymosin produced from genetically modified yeast and fungi for cheese manufacturing.
- **Agricultural products**: Crops and vegetables with improved properties such as pesticide resistant, insect resistant or crops with improved nutritional values; for example insect-resistant corn, slow softening tomatoes and oil seeds containing higher concentrations of polyunsaturated fatty acids.
- **Research**: Gene knockouts as tools for understanding life phenomena and for functional studies of drugs and health food ingredients.
- **General industry**: Biocatalysts taking the place of conventional catalysts in chemical reactions for better efficiency, safer working conditions and less environmental concern; genetically engineered microorganisms (GMMs) or enzymes produced from GMMs used in bioconversions for manufacturing chemicals and pharmaceutical intermediates.

The creation of GMOs involves using recombinant DNA technologies (sometimes referred to as genetic engineering, genetic modification/manipulation or gene splicing). Recombinant DNA technology is considered one of the greatest advancements of the twentieth century, which also sets the foundation for modern biotechnology encompassing protein engineering, cell engineering and metabolic engineering. Since the initial experiments of DNA recombination conducted in 1970s,[1–3] recombinant DNA technology has thrived along the way and now it has become a common practice in biological laboratories and corporate research units.

Using this technology, one can do the following: clone a single gene in order to facilitate the production of the gene product, which can be a protein or an enzyme; rearrange several genes in a fusion state for efficient bioconversions; engineer the genes of metabolic pathways of an organism in order to efficiently produce a specific product; create cloned cells which can be cultured; or generate transgenic plants or animals as the embodiment of biopharmaceutical working force. The number of isolated genes has increased explosively, as can be seen from the great number of Gene bank databases. It is too ambitious to try to review all of them. Therefore, this chapter is intended rather to show the essence of the technology and to demonstrate through examples how the technology can be applied to produce industrial enzymes in a much more efficient way.

Recombinant DNA technology was later expanded to the area of protein/enzyme engineering, the concept of which was first put forward by Ulmer.[4] Once a gene is isolated, the gene can be changed or modified by various methods at specific positions in order to 'tune' the protein it encodes, thereby giving it more desirable properties. Alternatively, a gene can be spliced randomly and recombined to generate a gene mixture (library), which contains altered versions or mutants of the original gene. The library can then be screened or selected for target products with the desired properties. In this chapter, the essence of protein engineering technology will also be demonstrated through examples, showing how it can be applied in the modification of enzymes to give them better performances.

The genus *Streptomyces* are gram-positive, soil-dwelling, filamentous bacteria, which are known as antibiotic-producing microorganisms. Different from most bacteria, *Streptomyces* show complicated morphogenic characteristics in multicellular differentiation. Therefore, these organisms are regarded as intermediates between bacteria and fungi. *Streptomyces* are known for producing secondary metabolites including active anti-tumour agents, enzyme inhibitors as well as antibiotics. They are also used as producers of industrial enzymes. For example, commercial glucose isomerases that are used in high fructose syrup production are from *Streptomyces olivaceus*, *Streptomyces olivochromogenes*, *Streptomyces rubiginosus and Streptomyces murinus.* Another enzyme of great economical impact, a transglutaminase that is used in the quality improvement of processed meat and fish products and for textural improvement of noodles, comes from *Streptomyces mobaraensis*.[5] The mycelia lifestyle of *Streptomyces* often results in viscous broths and unfavourable pellet formation during deep-liquid cultivation processes. For this reason, the application of *Streptomyces* as a general producer of industrial enzymes is, so far, still limited. However, fermentation technologies have developed rapidly, and together with the discovery of numerous unique enzymes, *Streptomyces* are attracting more and more attention in industry. *Streptomyces* are 'an enzyme treasure trove', as commented by Professor Horinouchi of Tokyo University.[6]

The genetics of *Streptomyces* was intensively studied in the 1960s and 1970s.[7] Besides cell morphology, genetic phenomena such as chromosomal recombination through transformation, conjugation and transduction within *Streptomyces* species and the presence of plasmids were discovered and investigated thoroughly. The application of recombinant DNA technology in studying genes of *Streptomyces* developed rapidly in 1980s and the first manual on genetic manipulation of *Streptomyces* was published in 1985.[8] Today, the complete genome sequences of three species, *Streptomyces coelicolor*, *Streptomyces avermitilis* and *Streptomyces griseus*, have been determined,[9–11] which provide valuable tools for studying genes and related functions. *Streptomyces* have very large genome size among bacteria and the number of genes encoding proteins exceeds some of the eukaryotic microorganisms such as *Saccharomyces cerevisiae* (*S. cerevisiae*). Gene clusters for the biosynthesis of antibiotics and other functional products are abundant. Knowledge of *Streptomyces* suggests this genus

to be a microorganism with great industrial potential. Therefore, this chapter focuses on how to take advantage of *Streptomyces* for producing industrially valuable enzymes.

Lastly, like other technologies, there are pros and cons about the application of recombinant DNA technology and protein engineering. It is important to control these technologies so that they can be used in beneficial ways. Regulations and guidelines for evaluating the use of GMOs and general issues regarding safety as well as environment are introduced and discussed.

2.2 RECOMBINANT DNA TECHNOLOGY

Recombinant DNA technologies involve isolating a target gene, connecting it with a carrier, transforming it into another organism and use that organism to propagate the gene product. This process is also called 'cloning'. There are a few elemental factors that need to be considered in order to succeed in cloning a gene:

- **Donor organism**: A donor is the organism that provides the gene of interest. It can be derived from mammalian sources, plants or, in most cases, from microorganisms. The choice of a proper donor depends on the specific requirements for the property of the gene product. The donor can be found through either a screening process designed for locating the gene of interest or through a DNA matching process performed on genome DNA with known partial gene sequence information.
- **Host organism**: A host is the organism in which the target gene can be replicated, transcribed and further translated into products of interest. In most cases, the host organism is chosen because of its easy reproduction. It can also be mammalian cells, plant cells or an insect system. Choosing the correct host organism is very important for successful expression of a target gene. Although, unfortunately, there are no definite rules in this respect, it has been generally established that homologous gene expression, that is genes of one origin expressed in the same or closely related organism, is the better choice. Nevertheless, a vast number of heterologous expressions succeeded in the bacterium *E. coli* support the conclusion that it is a rather universal host. Choosing a host cannot be separated from choosing a vector. The choice of a host–vector system as a set should be the consideration for the purpose of each target expression. Table 2.1 lists some commonly used host–vector systems such as *E. coli* (pUC18/19), *Bacillus subtilis* (pUB110) and *Streptomyces lividans* (pIJ702).
- **Vector**: A vector is the carrier, as mentioned above, which is capable of carrying the target gene and replicating it in the corresponding host cells and has the property to integrate into the chromosome of the host. The most commonly used vector is closed double-helix circular plasmid DNA. It usually contains an *ori* region for replication and typically some antibiotic-resistant genes as selection markers and multiple restriction sites for splicing and joining it with target genes. It can also be single-stranded phage DNA or cosmid DNA. Figure 2.1 shows a typical plasmid map of pIJ702, a commonly used vector for *Streptomyces* hosts.[12] A shuttle vector is a vector with the characteristic of functioning in multiple hosts of different genus.
- **Target gene**: A target gene can be isolated from the donor organism by 'shotgun cloning' (a method which will be described later) or by using primers designed from known sequences of the target gene. These sequences can be deduced from N-terminal or internal amino

Table 2.1 Commonly used vector–host systems.

Host	Vector	Promoter	Supplier
Escherichia coli	pUC18/19	*lac*	Takara Bio Inc., Toyobo
	pET	*T7*	Novagen
	pBAD	*BAD*	Invitrogen
	pCold	*csp*	Takara Bio Inc.
	pPROTet	*Ltet0-1*	Clontech Laboratories, Inc.
	pMAL	*tac*	NEB
	pTXB	*T7*	NEB
Bacillus subtilis	pUB110		ATCC37015
	pC194		ATCC37034
	pE194		ATCC37128
Brevibacillus choshinensis	pNY326	*P5*	Takara Bio Inc.
	pNCMO2	*P2*	Takara Bio Inc.
Streptomyces	pIJ702		The John Innes Foundation[a]
Pichia pastoris	pPIC	*AOX1*	Invitrogen
	pFLD	*FLD1*	Invitrogen
Pichia methanolica	pMET	*AUG1*	Invitrogen
Saccharomyces cerevisiae	pAUR101 (integration)		Takara Bio Inc.
	pYES	*GAL1*	Invitrogen
	pAUR123	*ADH1*	Takara Bio Inc.
	pLP-GADT7, pLP-GBKT7	*ADH1*	Clontech Laboratories, Inc.
	pESC	*GAL1*, *GAL10*	Stratagene
Schizosaccharomyces pombe	pAUR224	*CMV*	Takara Bio Inc.
Kluyveromyces lactis	pKLAC1 (integration)	*ADH1*	NEB
Aspergillus	pPTRI (integration)		Takara Bio Inc.
	pUC18/19[b]		Takara Bio Inc., Toyobo
Insect cells	Baculovirus	polyhedrin	Invitrogen, Novagen, Clontech
	pIEx	*ie1*	Novagen
Drosophila melanogaster	pMT	Metallothionein	Invitrogen
	pAC	Actin 5C	Invitorgen
Plant cells			
Mammalian cells	Lentivirus	CMV	Invitrogen
	Adenovirus	CMV	Invitrogen, Stratagene, Clontech
	Retrovirus	CMV, TRE	Stratagene, Clontech
	pFRET	SV40	Invitrogen
	pTriEx	CMV	Novagen

[a] Not for sale.
[b] Homologous DNA recombination.

acid sequences of the corresponding protein. A target gene can also be made synthetically on the basis of knowledge of the known sequences. If a target gene is from eukaryotic organisms such as mammalian cells, the gene should be obtained from a cDNA library in order to remove the introns that are processed in mammalian cells but not in the prokaryotic host.

- **Tool enzymes**: The discovery of a group of enzymes (endonucleases or restriction enzymes) which cleave or splice double-helix DNA molecules marked the beginning of recombinant DNA technology. Restriction enzymes are necessary tools because they can cleave DNA molecules at specific sites, producing ‘sticky ends’ or ‘blunt ends’ depending

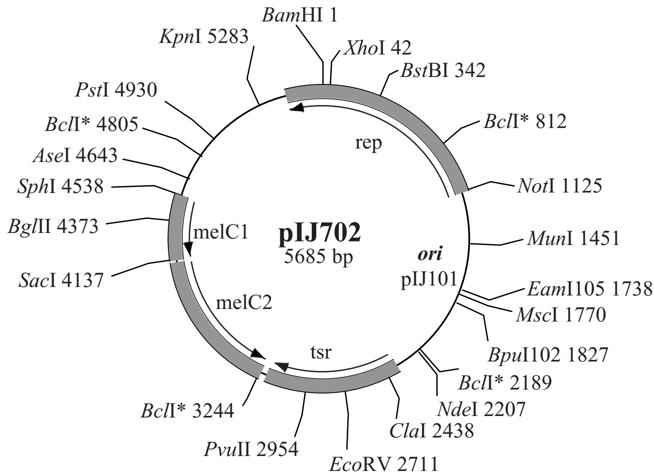

Fig. 2.1 Restriction map of pIJ702.

on the action pattern of the enzyme. DNA molecules can be rejoined by a ligase, which is able to connect different gene segments with complementary sequences together, for example to ligate the isolated target gene to plasmid carriers. Another important enzyme used in the manipulation of DNA is a polymerase, which is used in the amplification of DNA molecules by a 'polymerase chain reaction' (PCR). The starting double-strand DNA molecules are first denatured by heat to single-strand DNA molecules that serve as templates. Primers with complementary sequences are annealed to the template DNA and in the presence of the constructing units of DNA molecule, that is deoxynucleoside triphosphates (dNTPs) of four kinds and other necessary elements such as buffers and ions, the enzyme reaction is carried out repeatedly through denaturing–annealing cycles with an increased amount of DNA molecules after each cycle. The polymerases are high temperature ($>90°C$) stable enzymes, allowing the reaction to be performed automatically through a thermal cycler without having to add more enzymes through the cycles. The development of PCR made the process of cloning more precise and efficient. Table 2.2 lists some of the major enzymes used as tools to manipulate DNA molecules.

- **Introduction of recombinant DNA molecules into host cells**: Three methods are currently used to introduce foreign DNAs into host cells. The first is to use competent *E. coli* cells.[13] Host cells are cultivated and treated with $CaCl_2$, which allows the cells to take up foreign DNA at a frequency of typically 10^7–10^9 colonies per gram supercoiled plasmid DNA. Another method involves exposing the cells and DNA molecules to an electrical field (electroporation).[13] This method is used in both transforming DNA into bacteria and eukaryotic cells with a typical frequency of 10^9–10^{10} colonies per gram DNA with bacteria. The third method involves using protoplast cells generated by treating cells with polyethylene glycol or sucrose and lysozyme, allowing cells to be able to take up foreign DNA at a lower frequency of 10^6–10^7 colonies per gram supercoiled plasmid DNA.[8] The frequency of transformation depends on the state of the cells and treatment conditions.
- **Isolation of the recombinant clone**: Once a recombinant DNA (target gene in a plasmid) is introduced into the host cell, cells are cultivated and placed on agar plates, often containing chemicals such as antibiotics for easy selection of recombinant clones. Colonies

Table 2.2 Enzymes used in recombinant DNA technologies.

Restriction enzymes
Cohesive end: *Apa*I, *Bam*HI, *Eco*RI, *Hind*III, *Kpn*I, *Nco*I, *Nde*I, *Pst*I, *Sac*I, *Sal*I, *Sph*I, *Xba*I, *Xho*I, etc.
Blunt end: *Acc*II, *Dra*I, *Eco*RV, *Pvu*II, *Sma*I, *Sna*BI, etc.
DNA polymerases
For PCR: *Taq*, *Pfx*, KOD, *Tth*, *Pfu*, etc.
For modification: T4 DNA polymerase, DNA polymerase I, Klenow fragment (DNA polymerase I, large fragment)
For sequence: Δ*Tth* DNA polymerase, *Taq*
Reverse transcriptases
Moloney murine leukemia virus (MMLV), avian myeloblastosis virus (AMV)
Ligases
T4 DNA ligase, *E. coli* DNA ligase, T4 RNA ligase, *Taq* DNA ligase
Polynucleotide kinase
T4 Polynucleotide kinase
Alkaline phosphatase
Bacterial (BAP), calf instein (CIAP), shrimp (SAP)
Nucleases
S1 nuclease, mung bean nuclease, exonuclease I, DNase I, RNase I, RNase H, etc.

with the required gene can be screened, selected or confirmed by assaying enzyme activities, detecting proteins with antibodies or in situ hybridization with probes derived from known DNA sequences.

- **Fermentation or cultivation technologies for expression**: Once a recombinant DNA (target gene in a plasmid) is introduced into the host cell and the recombinant organism isolated, fermentation or cell cultivation technologies are necessary for optimal expression of the cloned gene. Understanding of the metabolism of the recombinant organism or cell system and the characteristics of regulating elements such as gene promoters and regulators are essential in this respect. Traditional techniques for growing an organism under optimum production conditions are also applicable.

2.2.1 'Shotgun' cloning

This technique was practised quite in the past at the early stage of recombinant DNA technology. It is practised even today when no or limited protein sequences of the target are available. The chromosome DNA of the donor organism is extracted with standard methods and partially digested with a restriction enzyme such as Sau3AI to create a DNA mixture with various sizes. The DNA mixture is ligated to an expression plasmid that is introduced into the host cells to create a library. Then the cells of the library are grown on agar plates with selection pressure (often antibiotics) applied for isolating the target cells that carry the plasmid integrated with the desired gene. The selection can be done by measuring the functional activities of the desired clone or by detecting it with antibodies raised against the protein. If the partial gene sequence is available, probes can be designed to detect the target gene at the transcription level; that is to detect the mRNA that has been transcribed from the gene, especially when the cloned gene cannot be processed into the regular enzyme or protein with the vector–host system. The host–vector system of *E. coli* (pUC18/pUC19) is

often chosen in shotgun cloning because using this system recombinant clones appear as white colonies in contrast to non-recombinant clones that appear as blue colonies, making selection of the target clone more efficient. The shotgun cloning method can be risky, but sometimes it leads to surprising results with the possibility of finding new enzymes or cloning multiple genes at one time.

2.2.2 Self-cloning

When a target gene is expressed in an organism that also served as the donor of the gene, it is defined as 'self-cloning'. The recombinant clone can be obtained by plasmid DNA as the gene carrier or by integrating the gene into chromosomes through integrative vectors. In either case, expression problems inherent in heterogeneous hosts are encountered less.

2.2.3 Cloning by PCR

The PCR has greatly changed the way of cloning to make it more efficient and precise. With known (sometimes even very limited) sequence information, the desired gene segments can be isolated efficiently. For example, if the N-terminal sequence of a desired protein is acquired, DNA segments as primers can be designed and used to bind to the genome DNA. This genome DNA is then denatured at a high temperature to serve as a template. DNA polymerases are used to elongate or amplify the corresponding DNA sequences precisely through many denaturing–annealing cycles, resulting in a product: the desired gene. It can then be isolated and joined to a vector for expression. Compared with the traditional shotgun cloning (Fig. 2.2), the process for isolating a gene is shorter and restriction enzyme sequences as tags can be introduced into the termini of the gene for later easy handling. However, because this process is more specific and directed, the chances for isolating different genes fall short of expectation.

2.2.4 Application examples

2.2.4.1 *Example 1: efficient production of a phospholipase D from Streptomyces cinnamoneum by using recombinant DNA technology*

Phospholipase D (EC 3.1.4.4) (PLD) carries out the hydrolysis of phosphatidylcholine (PC) to phosphatidic acid as well as the synthesis reaction between PC and alcohols, for example the trans-phosphatidylation reaction between PC and glycerol to produce phosphatidylglycerol (PG). This enzyme is important in synthesizing phospholipids that are used widely in the pharmaceutical, food and agricultural industries. Recently, phosphatidylserine (PS), one of the rarely existing natural phospholipids, which can be produced by this enzyme from soya or egg lecithin, has attracted much attention as a functional food material on the market because of accumulating evidence on functional effectiveness. Therefore, efficient production of PLD has become the task of several research groups.[14,15] *Streptomyces* are known to be able to secrete this enzyme into the culture medium. Fukuda *et al.*[16] screened several actinomycetes as production strain of this enzyme and found highest activity in a strain of *Streptoverticillium cinnamoneum* (reclassified as *S. cinnamoneum*). The enzyme was purified to homogeneity and a partial amino acid sequence was determined.[17] In order to clone the gene, a partially digested (Sau3AI) chromosome DNA library was constructed. The partial amino acid sequences were used to design probes for screening the DNA library by a radio-labelled in situ colony hybridization technique. Unfortunately, this process

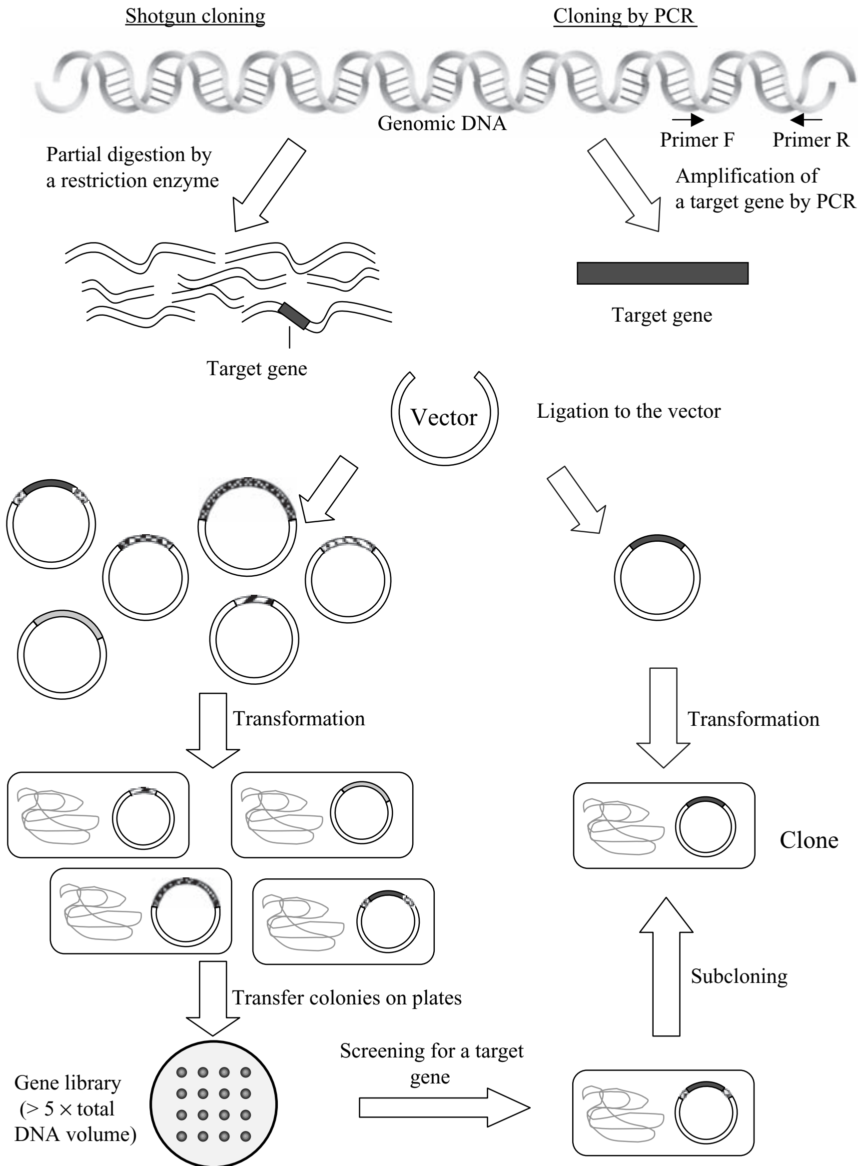

Fig. 2.2 Comparison of shotgun cloning and cloning by PCR.

only yielded a truncated gene. The PCR technique was then employed to 'walk' the chromosome to obtain the complete gene. Because the determined partial amino acid sequences showed high similarity with the PLD from *Streptomyces antibioticus*, the gene information of *S. antibioticus* was also used for designing primers. The isolated gene and its regulatory elements are introduced into a shuttle vector of *E. coli* and *S. lividans* (pUC702) and introduced into *S. lividans* protoplast cells for expression. The enzyme activity from the recombinant microorganism was found 15 times higher than the original production strain and this high production is due to the strong promoter of the native *pld*.[18] When the constructed expression vector was introduced into *E. coli* and yeast cells, however, the expression level was only

one-twentieth and one-quarter, respectively, of that of the original strain. Zambonelli *et al.*[15] reported that when the *pld* gene from *Streptomyces* PMF was cloned and expressed in *E. coli*, dramatic decrease in the recombinant *E. coli* cell growth accompanied by plasmid instability was observed, suggesting possible toxic effect caused by the PLD enzyme on cell membrane. These experiences demonstrated the importance of selecting a proper vector–host system in order to successfully express a target gene. The genus *Streptomyces* has a very high G+C content in its genomic DNA (>70%). So far, the best expression system in *Streptomyces* is that established by Hopwood *et al.*,[8] generally involving the use of *Streptomyces* originated vector (pIJ702) with *S. lividans* as the host. Ogino *et al.*'s[18] new expression system with the strong promoter of native *pld* stimulated the development of other industrially important enzymes.

2.2.4.2 *Example 2: expression of a phospholipase A_2 from Streptomyces violaceoruber*

Phospholipase A_2 (EC3.1.1.4) (PLA_2) is an enzyme that specifically acts on the fatty acid in sn-2 position of phospholipids. When used as a processing aid in treatment of egg yolk or lecithin, it is kept at a constant temperature for a certain period of time. After the treatment, the temperature of the yolk is raised in order to inactivate the enzyme, or in the case of lecithin, further purification procedures are followed. The treated egg yolk and lecithin have improved emulsifying properties and are widely used in food processing. Phospholipases, including PLA_2, are found in most cells and tissues including animal tissues that are historically consumed by humans. A commercial PLA_2 enzyme is extracted and purified from porcine pancreas. However, in recent years, enzymes from animal origins are less favoured because of very high production costs and their relatively high risk of disease potency in manufacturing and handling. Besides that, supply problems have caused some turmoil in the food industry in several regions of the world. These problems can be prevented by producing the enzyme using microorganisms which can be grown in a fermenter, thus preventing regional supply limitations. There are two approaches in this respect: one is by cloning and expressing the gene of pancreas origin into eukaryotic microorganisms such as the fungus *Aspergillus*, and the other is to produce a real microbial enzyme.

A PLA_2 enzyme from *S. violaceoruber* was purified and cloned by Sugiyama *et al.*[19] The expression efficiency of the system of Ogino as described in Section 2.4.1 was evaluated for expression of the gene. The cloning strategy is shown in Fig. 2.3. The vector containing the *pld* expression cassette (promoter, signal peptides, *pld* gene and terminator) was spliced and rejointed to produce pUC702-EX with the *pld* gene and the signal peptide removed (details not shown). The process involves using restriction enzymes to digest and ligate the DNA and PCR techniques to combine different DNA molecules. The *pla2* gene and its 5′ upstream sequences including a site for peptidase recognition (encoding a signal peptide) were isolated from the chromosome DNA of *S. violaceoruber* by using the PCR technique, and an *SphI* restriction site was introduced at both ends of the gene in order to ligate it to the expression plasmid pUC702-EX to construct pUC702-EX-PLA_2. After transformation into host cells of *S. violaceoruber*, the enzyme activity of the cultivated cells was found more than 30 times higher than that of the original host. Compared with the result reported on the expression of a second PLA_2 gene from *S. violaceoruber* into *E. coli* host, in which case the enzyme has been produced in *E. coli* as inclusion bodies within the cell,[20] the vector–host system of *pld* promoter of *S. lividans* (reclassified as *S. violaceoruber*) proves powerful in expressing genes of the *Streptomyces* origin.

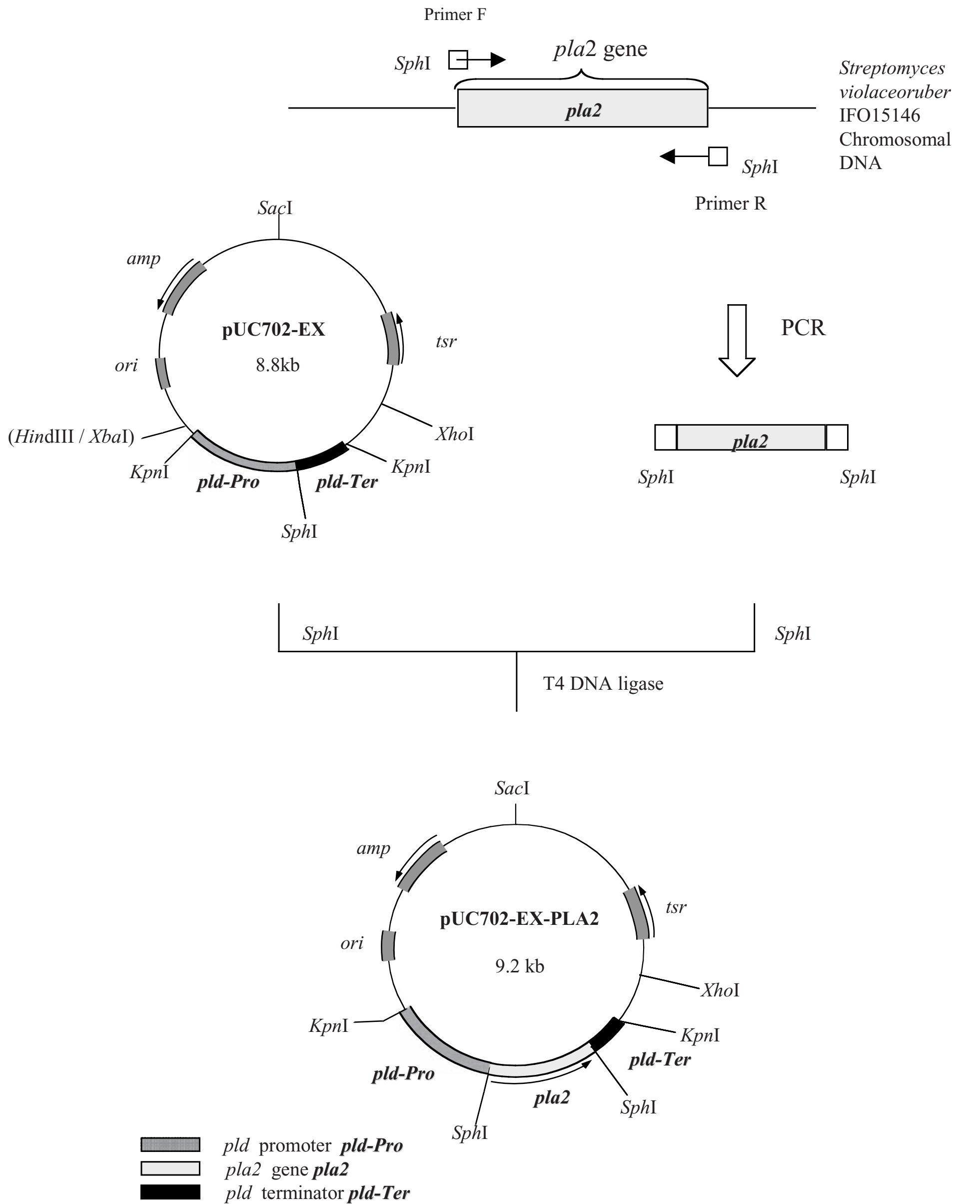

Fig. 2.3 Cloning of *pla2* in an expression vector, pUC702-EX.

2.2.4.3 *Example 3: cloning and expression of a sphingomyelinase gene from Streptomyces cinnamoneus by PCR*

Sphingomyelinase (E.C.3.1.4.12) (SMase) is an enzyme that hydrolyzes sphingomyelin to produce ceramide and PC. This enzyme is clinically important because it has been reported to be involved in cell differentiation, ageing and apoptosis in mammalian cells. Industrially, the enzyme can be used in production of ceramide, an excellent moisturizer for skin care

products. Some sphingomyelinases are reported to have phospholipase C activities, which hydrolyze phospholipids into diacylglycerol and phosphate ester. Therefore, the enzyme can also be used in the processing of lecithin, for example in the oil degumming process. Several *Streptomyces* strains were screened by measuring the production of diacylglycerol in the culture medium after incubation with PC from egg yolk. It was found that a strain of *S. cinnamoneus* is likely producing this enzyme. Using partitioning with ammonium sulphate, DEAE-toyopearl and Resource Q ion-exchange chromatography, the enzyme was purified to homogeneity and its N-terminal amino acid sequence was determined. In order to clone the gene, a pair of primers was designed according to the N-terminal amino acids sequence and the sequence of a conservative region near the C-terminal.[21] The PCR technique was employed to amplify the gene with the chromosome DNA from *S. cinnamoneus* as templates. The cloning strategy is shown in Fig. 2.4. Luckily a fragment of around 800 bp was obtained. The fragment was sequenced and primers were designed to 'walk' the remaining sequences outwards the fragment along the chromosome by the inverse PCR technique. A fragment was obtained of 999 bp encoding the total 333 amino acids of the enzyme. After cloning this fragment into the vector–host system of pIJ702–*S. lividans*, the recombinant cells produced increased sphingomyelinase enzyme activities, thus confirming that the complete gene was isolated successfully.

The gene was again expressed into the host–vector system of pUC702-EX–*S. lividans* with the vector containing the *pld* promoter and terminator of *S. cinnamoneum*, and approximately more than 1000 times higher enzyme activities were detected compared with that of the original strain in which the activity of the enzyme is barely detectable.

An original gene coding a new sphingomyelinase was successfully isolated and expressed. This enzyme has shown potential for a new process of oil refining. Other applications such as removing trace amounts of sphingomyelin in egg yolk lecithin to produce a highly purified PC for the pharmaceutical industry are under investigation.

2.3 PROTEIN ENGINEERING

Recombinant DNA technology has proven so far to be a powerful tool in the efficient production of a target enzyme. Practices of the technology have evidently brought merits in reducing energy requirement and raw materials used in the manufacturing of enzymes, hence reducing production costs. As a result, enzymes as biocatalysts have now become quite easily available and this in turn facilitates enzyme applications in various industries. However, besides cost factor and availability, other problems with enzymes are encountered. Originated from living forms, enzymes usually only work on specific substrates under specific conditions that do not always fit the real (industrial) situation; for example, an enzyme is often not robust enough: it will not work at the required pH conditions or it loses its functionality easily at an elevated temperature, extreme pH or when in contact with solvents. Sometimes, a reaction does not perform well, simply because the enzyme is not the right candidate, meaning it cannot recognize the substrate in an efficient way (K_m value of the enzyme being too large) or the reaction speed (turnover number of the enzyme) is too low. To solve these problems, one approach is to embark on the task of screening natural resources of microorganisms – though often tedious and stressful – until the right enzyme is found. In this case, a variety of reliable microbial resources and suitable cultivation conditions are needed. Another approach is to try to modify what is already at hand in order

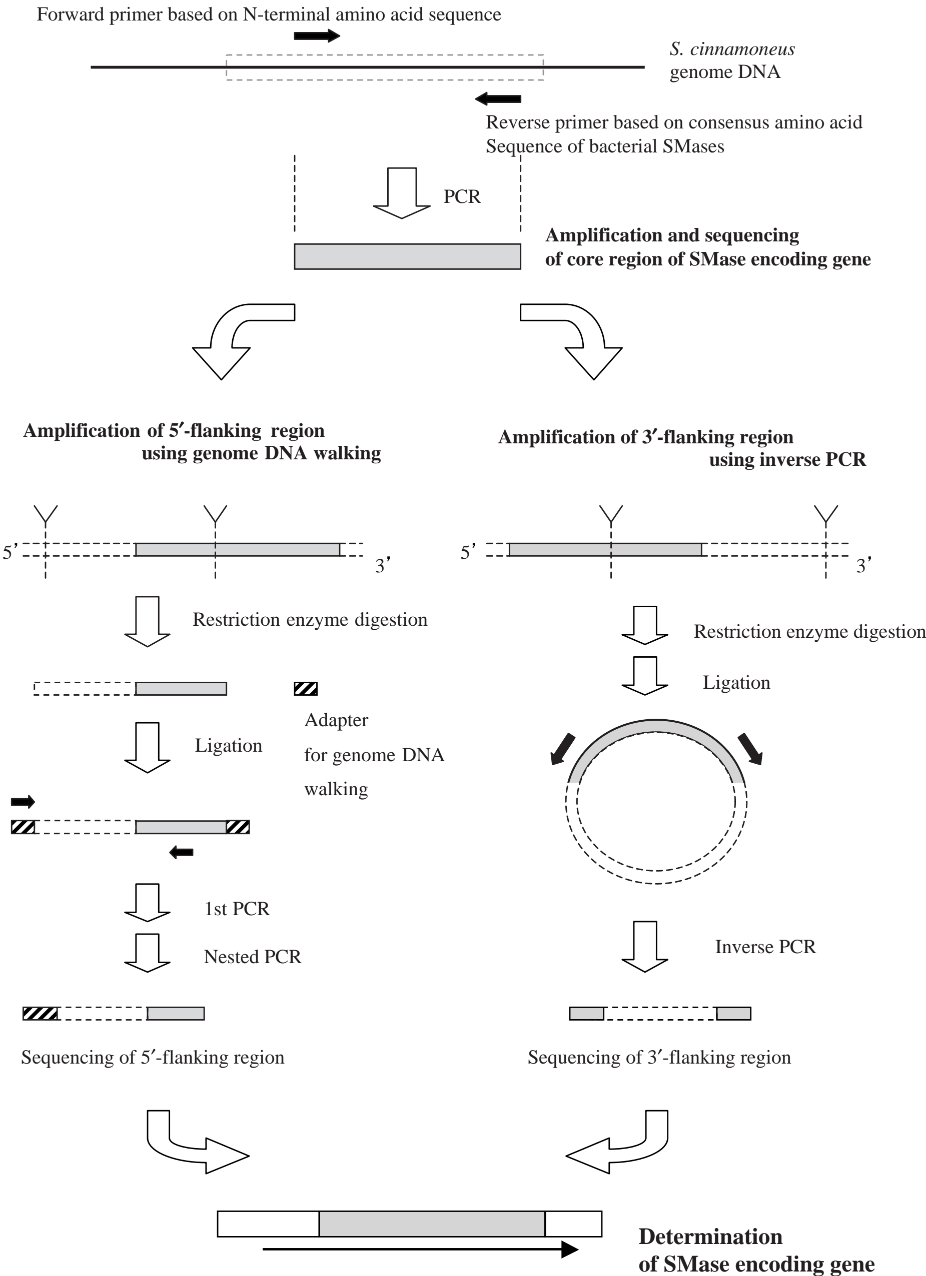

Fig. 2.4 Cloning strategy of SMase encoding gene.

to improve the properties. This modification process is called ‘protein engineering’. Because of the characteristics specific to enzymes, it is not feasible to manipulate either a protein at its conformational state or its primary structure freely without losing physical functions. However, thanks to recombinant DNA technologies, the modification of a protein or an

enzyme can be done at the genetic level. The gene encoding the protein can be changed in such a way that a specific amino acid or acids is/are exchanged against other amino acids. Alternatively, new amino acids can be inserted or deleted. The altered gene is then expressed in a proper host and mutants carrying the modified gene can be screened or selected for suitable properties.

2.3.1 Strategies of protein engineering

The choice of strategies and methods for obtaining an enzyme with desired properties relies both on how much is known about the existing protein and what target property is required. If an enzyme has been purified, its structural information is obtained (through X-ray crystallography or NMR) and the active site is clearly understood, one could postulate quite precisely which amino acids are crucial for carrying out the catalytic reactions or keeping the enzyme stable. In that case, rational mutagenesis methods are best employed. The most commonly used rational mutagenesis methods are also called site-directed mutagenesis, including saturation and cassette mutagenesis. On the other hand, if the tertiary structure of an enzyme has not been determined, random mutagenesis methods will be the only choice. Practically, this is often the case. Random mutagenesis methods include both non-recombinative and recombinative, with the use of techniques involving error-prone PCR, bacterial mutator strains and DNA shuffling, to name just a few.

2.3.1.1 *Rational mutagenesis methods*

Efforts to answer questions such as how to rationally improve the thermostability or the catalytic efficiency of an enzyme towards a substrate were made several decades ago. For example, in trying to increase the thermostability of an enzyme, initial studies were carried out by comparing homologous enzymes with very similar properties but from different sources, such as enzymes from thermophiles and mesophiles, in order to elucidate possible rules of amino acid exchanges for better thermostability. These studies have led to the discovery of amino acid substitutions like Lys to Arg, Ser to Ala, Gly to Ala, Ser to Thr, etc., that most likely contribute to increased thermostability.[22] It was found later that increased thermostability was related more to increased compactness and rigidity of the tertiary structure of an enzyme.[23] This is caused by the inherent force in driving a protein sequence to its physiological conformation. It becomes clear that increased hydrophobic forces, resulting from amino acid substitution from Ala to Gly in α-helical structures of an enzyme, likely result in increased thermostability. Other forces such as hydrogen bonds, long-range electrostatic and ion binding also have some effects. The increased number of disulphide bridges between cysteine residues does not have an obvious effect, although it does stabilize an enzyme in a different way (see Section 3.4.1). Thus, in order to increase the stability of an enzyme, substitution of amino acids in helical structures aiming at enhanced hydrophobic activities is one of the rational measures to take. Trying to improve other properties, such as increasing the catalytic efficiency of an enzyme towards a specific substrate, however, is much more complicated. It depends on the structure and size of the substrate, the structure and surrounding environment of the substrate-binding site, possible interactions between amino acids at the reaction site with the substrate, and so on. Rational design of the mutation site sometimes needs the help of computer modelling and mathematical simulations.

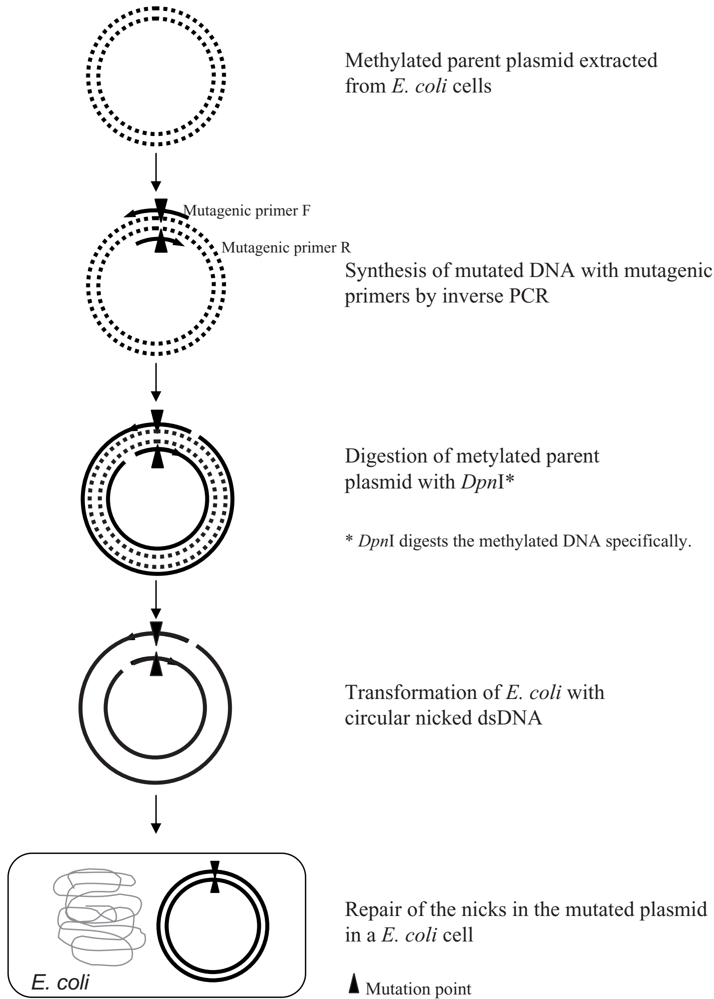

Fig. 2.5 Diagram of the site-directed mutagenesis method. *Source*: QuickChange® II site-directed mutagenesis kit (Stratagene).

Site-directed mutagenesis

This is the most commonly used technique in rational mutagenesis. Once an amino acid (or amino acids) to be changed has been decided, primers are designed which include the DNA sequence encoding for the amino acid to be substituted (with the rest of the sequences intact). These are applied for PCR amplification of the gene that has been integrated on a vector and served as the template. Figure 2.5 shows the diagram of the method of site-directed mutagenesis with the PCR technique. With this method, amino acids located anywhere on the gene can be exchanged against the other 19 naturally occurring amino acids. The site-directed mutagenesis method in which one crucial amino acid is exchanged against all other 19 amino acids, in order to find the best mutation, is called saturation mutagenesis. The method of site-directed mutagenesis on a region (with several amino acids) including all possible combinations (cassettes) is called cassette mutagenesis.

2.3.1.2 *Random mutagenesis*

Since the technique for determining the tertiary structure of an enzyme is not yet efficient enough and often no information is available on which amino acid should be changed, random mutagenesis is the obvious choice. A library containing as many mutants as possible is created and mutants with desired properties are then selected or screened. There are many methods developed for efficiently creating mutant libraries with a size large enough for finding the potential mutants. These include non-recombinative and recombinative methods. Non-recombinative methods include error-prone PCR and the use of bacterial mutator strains in order to introduce mutations randomly within a gene sequence. Recombinative methods involve splicing and rejoining of identical or very similar DNA sequences (homologous recombination) or distinctively different DNA sequences (non-homologous recombination). The libraries constructed are then screened or selected with appropriate methods for the targeted enzyme mutants. The recombinative method is also called directed evolution, which can be performed both in vivo and in vitro.

Error-prone PCR

The enzyme DNA polymerase, which carries out DNA amplification reactions, usually has very high fidelity. However, occasionally wrong nucleotides may be incorporated at a frequency in the order of 10^{-4} (such as *Taq*-polymerase in Ref. [24]. The reaction conditions, such as $MgCl_2$ concentration or the substrate concentrations of dCTP and dTTP or the amount of the *Taq*-enzyme, can be adjusted in order to obtain a desired error rate, ideally resulting in one or two amino acid changes per gene.

Bacterial mutator strains

Some bacteria like wild-type *E. coli* have the ability of spontaneous mutation at a rate of 2.5×10^{-4} mutations per 1000 nucleotides of DNA carried on a plasmid after 30 generations of growth. These strains are used as mutagenesis agents. The gene of a target enzyme is cloned into a plasmid and the plasmid is then introduced into the mutagenesis strain. After cultivation, a population of the strain with various mutations is created and is then screened for desired mutants.

DNA shuffling

Stemmer[25] first brought forward the concept of this technique. A population of starting DNA sequences is randomly fragmented and reassembled into full-length, chimerical sequences by PCR. The starting DNA sequences can be from the same family and are highly homologous (also called family shuffling). The so-called single gene shuffling is to fragment the starting gene and to introduce mutations while reassembling the gene by taking advantage of the error function of polymerases during PCR reactions under specific conditions. Compared with error-prone PCR, single gene shuffling likely results in more mutations while family shuffling allows large segment exchanges of sequences between genes, thereby significantly accelerating the efficiency of enzyme modification. This method has been extended to recombination of non-homologous DNA sequences including whole genome shuffling which more or less corresponds to the classical mutagenesis by breeding. Figure 2.6 shows a diagram of DNA shuffling.

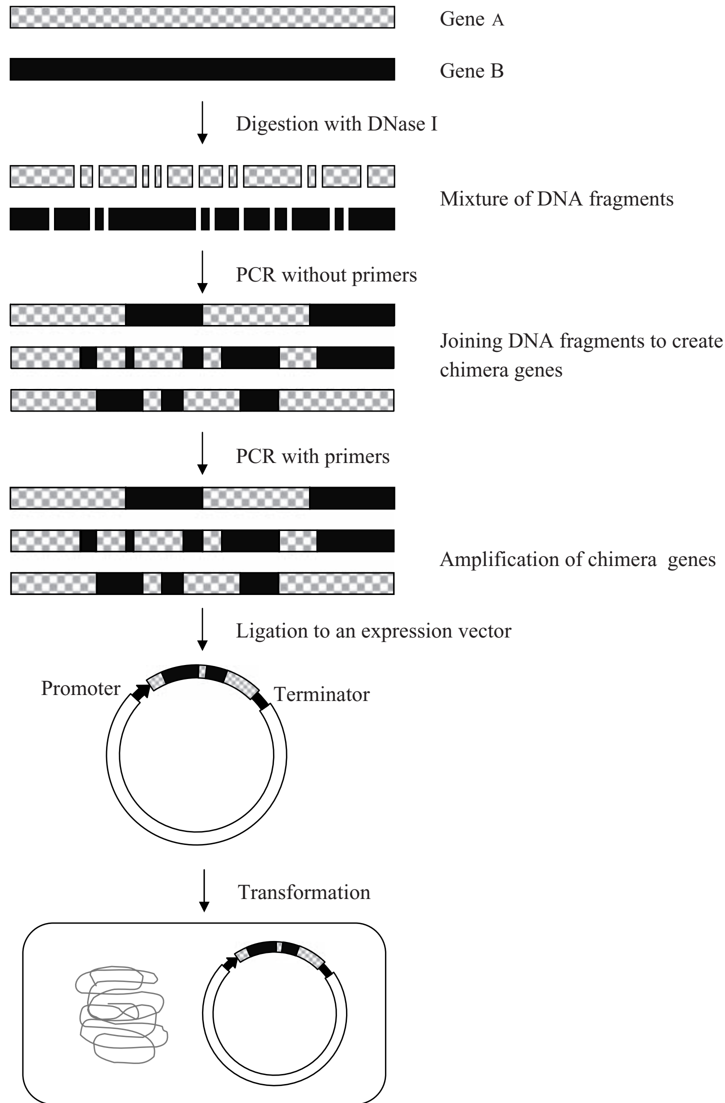

Fig. 2.6 Diagram of DNA shuffling.

2.3.2 Gene expression systems

Modified genes generated by all kinds of methods need to be expressed in suitable hosts so as to be screened or selected for the desired properties. The most commonly used host is a gram-negative bacterium *E. coli* because a vast amount of knowledge has been accumulated about this system. Other systems include gram-positive bacteria *Bacillus*, *Clostridium*, *Lactococcus*, *Staphylococcus* and *Streptomyces*, and eukaryotic systems such as yeast and the adenovirus system. The principle for choosing a suitable expression host is the same as described in Section 2.2 on host organism.

2.3.3 Selection and screening

In order to sort out the mutant protein with properties of interest, a strategic selection or screening method is crucial. Selection involves applying one or more procedures in the assay system so that only desired mutants are left over. Screening puts all the expressed mutants under an assay system for the desired property. The results of these assays are compared and mutants with the best performance are chosen. Very often the selected mutants are not good enough, therefore mutagenesis needs to be repeated. During each round of mutagenesis, small changes are accumulated until eventually a mutant stands out as a satisfactory candidate.

There can be rules applicable to selection methods for certain desired properties, for example thermostability. Mutants can be treated at a required temperature for a certain period of time so that, after the treatment, only surviving mutants have the desired thermostability. In most cases, however, selection or screening methods have to be tailored for individual enzymes for specific requirements of their properties to be improved.

2.3.3.1 *High-throughput screening*

Because most mutations are negative and only small portions of subtle changes lead to real improvement of an enzyme, a library with a sufficient number of mutants should be constructed and screened in order to find the desired mutants. If an easy detection system is available, such as determining positive mutants with a colour reagent, an automatic apparatus can be applied for efficient screening. Nowadays, high-throughput screening has become a routine practice in research facilities involved in developing industrially feasible enzymes.

2.3.4 Applications of protein engineering – a powerful tool for the development of enzymes as applied biocatalysts

2.3.4.1 *Examples for improving thermostability and altering pH optimum*

Industrial enzymes need to be stable in their working environments. To improve an enzyme's stability at elevated temperatures is one of the earliest attempts of protein engineering. A review by Goodenough[26] has summarized the factors related to thermostability. By understanding the chemistry of folding and unfolding of proteins, hydrophobic forces are found to be the major driving force for folding, thus having a major role in thermostability. Protein engineering, by increasing the hydrophobic forces of secondary helical structures, has proven a feasible strategy for increasing the thermostability of a known protein structure. Liu and Wang[27] have reported on engineering a glucoamylase from *Aspergillus awamori* for increased thermostability. Glucoamylase (EC3.2.1.3) is one of the most important enzymes in the food industry. It catalyzes the hydrolysis reaction of starch molecules from non-reducing ends. The condition for using the enzyme requires a high temperature, which improves the reaction rate, reduces viscosity of the syrup for easy processing and reduces the risk of bacterial contamination. The tertiary and the secondary structure of the enzyme have been determined, but their structure–function relationship remained unclear. Liu and Wang[27] have performed a so-called 'molecular dynamics simulation' and found out that the 11th α-helix out of the 13 α-helixes, which is located on the surface of the catalytic domain, is

very unstable. When the enzyme unfolded at the denaturing temperature, the position of the catalytic base was shifted from the hydrophobic interior provided by 12th and 13th helixes to the surface. The sites (amino acid residues) that needed to be altered for engineering the enzyme in order to increase its thermostability were determined. According to previous studies, which showed that the introduction of disulphide bonds between flexible regions most likely contributes to thermostability, the mutants were obtained that formed disulphide bonds across the 11th helix and increased the enzyme's thermostability. Exchanging several Gly residues near the 12th and 13th helixes into Ala, in order to increase the hydrophobicity of this region, also resulted in increased thermostability.

The food enzyme industry continually tries to improve the total process of hydrolyzing starch to high fructose syrup. This process involves the actions of at least three enzymes: an α-amylase for liquefying starch, the above-mentioned glucoamylase for releasing glucose units and a glucose isomerase (xylose isomerase to be precise) to convert glucose to fructose. These enzymes are naturally occurring and have pH optima at 5.5–6.0, 4–4.5 and 7.0–8.0, respectively. Therefore, traditionally these three steps have to be performed separately, and between reactions the pH has to be adjusted for the best performance of each enzyme. This causes an unnecessary increase of salt concentration and extra procedures have to be followed for removal or purification. Since the tertiary structures of several glucoamylase enzymes were determined, protein engineering was a convenient method for changing the enzyme into a more suitable biocatalyst. Fang and Ford reported on engineering the glucoamylase from *A. awamori* to alter its pH optimum.[28] The enzyme has two Glu residues (at 179 and 400) at the catalytic site. One of these acts as the catalytic acid, the other as the catalytic base. The pH dependence of an enzyme is determined by the ionization of the catalytic groups that are influenced by various interactions around their microenvironments. With this enzyme, modifications of Glu179 and Glu400 which affect their ionization state (indicated by pK1 and pK2, respectively) will likely change its pH optimum. By comparing the structure of the enzyme with that from other sources, such as *Arxula adeninivorans*, *S. cerevisiae*, *S. diastaticus* and *Saccharomycopsis*, it was found that the amino acid at position 411 near the catalytic site was different in enzymes from different microorganisms: some enzymes had a Gly at this position, whereas a Ser was found in the enzymes from other sources. Since the enzymes from other sources have pH optima more or less shifted to the alkaline side, S411 was estimated to play an important role in determining the pH optimum of the enzyme. For that reason, this residue was chosen as the target site for mutagenesis. Mutants created include S411G, S411A, S411C, S411H and S411D with the serine residue being replaced with Gly, Ala, Cys, His and Asp, respectively. S411G, S411A and S411C were created in order to eliminate the hydrogen bonds between SER411 and Glu400 of the catalytic base so that the carboxylate ion form is destabilized, which results in an increased pK1. The mutants with His and Asp as replacement of serine aimed at eliminating the hydrogen bond and introducing positive or negative charges. As a result, S411H and S411D both showed an increased pK1 but decreased pK2. The former could be explained as that the positive charge of histidine stabilizes the electrostatic interactions between that of histidine and the catalytic acid of Glu179, the latter due to increased size of the side chain. Electrostatic force is also considered to have some effects on the pH property of the enzyme.

These examples show that understanding of protein structures is important in determining the sites for mutation and site-directed mutagenesis is an efficient way to improve an enzyme's property to suit better to industrial applications.

2.3.4.2 *Example for increasing catalytic efficiency*

An example given by Toyama *et al.*[29] demonstrates how to use directed mutagenesis to engineer a *Streptomyces* cholesterol oxidase to increase its catalytic efficiency. Cholesterol oxidase (EC1.1.3.6) catalyzes the oxidation of cholesterol to 5-cholesten-3-one as well as the isomerization of steroid with a *trans* A:B ring junction to produce 4-cholesten-3-one. This enzyme is useful for detecting the cholesterol level in human serum and for analyzing steroid contents of food materials. Comparison of the known primary sequence and tertiary structure of *Streptomyces* cholesterol oxidase with that of *Brevibacterium sterolicum* reveals that the two enzymes share very high homology (59% identity and 92% similarity). The amino acids located at the substrate-binding site are identical, but several amino acids nearby this site are different. These amino acids form a loop that seems to act as a lid over the active site. Because the *Streptomyces* cholesterol oxidase has a much higher k_{cat}/K_m than that of the enzyme from *B. sterolicum*, indicating better catalytic efficiency, these loop-forming amino acids are presumed important for substrate-binding activities. Mutants created at these sites using the site-directed mutagenesis method are evaluated for catalytic efficiency. Two mutants (V145Q and S379T) have altered activities. V145Q showed a lower activity towards all substrates while S379T showed a slightly higher k_{cat} and smaller K_m, resulting in 1.8 times higher catalytic activity towards substrate cholesterol and 6 times higher catalytic activities towards pregnenolone.

Catalytic efficiency is the determinative factor for an enzyme reaction to be able to compete with reactions carried out by chemical catalysts. The possibility of improving the catalytic properties of an enzyme has a fundamental impact on the expansion of enzyme applications as catalysts.

2.3.4.3 *Example for altering substrate specificity*

Recently, Iwasaki's group at Nagoya University in Japan reported on altering the substrate specificity of a *Streptomyces* PLD dramatically by site-directed saturation mutagenesis.[30] PLD is an important enzyme used for synthesizing functional phospholipids from natural lecithin of soya or egg yolk, in the presence of an alcohol (see Section 2.2.4.1). However, this enzyme is not capable of synthesizing phosphatidylinositol, an important phospholipid that has functions in lowering the triacylglycerol levels of serum and liver.[31] A PLD enzyme from *Streptomyces antibiotics* was purified and the tertiary structure was determined both with the intact enzyme and its inactive form (mutant H168A) in complexation with dihexanylphosphatidylcholine. The tertiary structure and knowledge on the reaction mechanism of the enzyme were used to predict three sites, W187, Y191 and Y385, which are crucial for substrate recognition. Compared with other alcohol molecules, inositol is rather bulky. Therefore, the mutagenesis strategy is designed to create mutants at these three positions that will have a broader space for easier access of bulky molecules. Saturation mutagenesis methodology was applied to create the mutant library.

Simultaneous mutation at three positions yields $20^3 = 8000$ variants (20 natural amino acids). To achieve 95% coverage in screening, it is needed to screen 23 694 colonies. The current method for detecting phosphatidylinositol by either high performance liquid chromatography (HPLC) or thin layer chromatography (TLC) is time consuming and it is almost impossible to screen this size of the library by conventional assay systems. Therefore, a tailored method for detecting mutants having phosphatidylinositol-synthesizing activity was designed. In this method, the product (phosphatidylinositol) is oxidized with periodate to

form a lipid aldehyde, which in turn reacts with 4-hydrazino-7-nitrobenzofurazan (NBD-hydrazine) to form a strong fluorescent NBD-hydrazone. By this method, high-throughput screening was employed and approximately 10 000 mutants were screened. Among them, 25 positives were detected to be able to synthesize phosphatidylinositol and its various isoforms.

This example has demonstrated not only the importance of correct prediction of amino acid residues as the target for mutagenesis but also the importance of a proper detection system for selecting or screening the desired mutants. Both are crucial for efficient modification of an enzyme. To do this, an optimal combination of knowledge of physical, structural and enzyme chemistry is necessary.

2.3.4.4 *Examples for changing selectivity*

Some enzymes recognize and act on multiple substrates while others carry out side reactions. Protein engineering can also be applied to change the selectivity of enzymes. Porcine pancreatic PLA_2 has been modified for better selectivity towards negatively charged substrates by site-directed mutagenesis.[32] PLA_2, as described in Section 2.2.4.2, is a useful enzyme not only in the food industry but also in the production of phospholipids of fine chemical grade for drug delivery systems. The enzyme recognizes and hydrolyzes multiple phospholipids with the head group being zwitterionic or negatively charged. The crystal structure of the enzyme was determined and amino acid residues in the region for binding with the head group of phospholipids were analyzed. In this region, two positively charged amino acid groups, Arg53 and Arg43, are presumed responsible for binding favourably to negatively charged phospholipids. Adjacent to this region was a negatively charged Glu46 that was further identified as a residue that might unfavourably influence the binding with the negatively charged head group of phospholipids. A mutant for exchanging the Glu to Lys (E46L) was introduced by site-directed mutagenesis. This mutant was found indeed to be able to bind more efficiently to substrates with the negatively charged head group.

Another example for changing the selectivity of enzymes by protein engineering is a reduction of the hydrolyzing activity of a malto-oligotrehalose synthase over its synthesizing activity in order to increase the yield of trehalose production. Malto-oligosyltrehalose synthase (EC 5.4.99.15, MTSase) catalyzes an intramolecular transglycosylation reaction to produce a non-reducing malto-oligosyltrehalose by converting the α-1,4-glucosidic linkage at the reducing end of malto-oligosaccharide to an α,α-1,1-glucosidic linkage. The malto-oligosyltrehalose can then be hydrolyzed by malto-oligosyltrehalose trehalohydrolase (EC 3.2.1.141, MTHase) to produce trehalose, a widely used sugar as food preservative, stabilizers and cosmetic ingredients. Besides the synthesizing reaction, MTSase also carries out the hydrolysis reaction of starch molecules to produce glucose instead of malto-oligosyltrehalose. The ratio of hydrolysis over transglycosylation influences the yield of trehalose production. One attempt to modify the enzyme in order to reduce the selectivity for hydrolysis was reported by Fang *et al.*[33–35] The enzyme was selected from *Sulfolobus solfataricus*. Enzyme kinetic studies revealed possible amino acid residues (Asp228, Glu255 and Asp443) in the active site, which are crucial for the catalytic reactions. Amino acid residues close to this site (+1 and −1) were suggested as being responsible for selecting catalytic reactions towards transglycosylation or towards hydrolysis. Therefore, site-directed mutagenesis at these regions was chosen to try to enhance the selectivity towards transglycosylation. A mutant at the +1 region, F405Y, was found to decrease hydrolysis. The exchange from Phe to Tyr decreases the hydrophobic interactions between the enzyme molecule and the substrate.

Subsequent mutagenesis at this region was performed for general understanding of the rule in order to create mutants with least selectivity for hydrolysis so that the industrial production of trehalose can be greatly improved. These mutants, including F405M, F405S and F405W, showed that the decrease of hydrophobic interactions between the enzyme and substrate indeed, generally, led to the better selectivity towards transglycosylation versus hydrolysis. However, with mutant F405Y the highest yield of trehalose was obtained, indicating that it is a potentially better suited biocatalyst. However, mutants designed for decreasing the hydrophobic interactions between the enzyme and substrate at the −1 region did not give a positive mutant, indicating the complexity of forces and interactions that exist within the microenvironment of an enzyme's three-dimensional structure.

2.3.5 Safety concerns

It has been demonstrated that protein engineering is a powerful tool for improving an enzyme's physiochemical characteristics, such as catalytic properties and selectivity, to make the enzyme better suited for its working conditions. Enzymes used as processing aids, such as those used for bioconversions, are usually separated from products after the reaction. These bioconversion products are further purified, and in such cases the safety concern of engineered enzymes is less substantial. However, where the enzyme is added to food materials and remains in the food after processing, even if denatured at the end, safety concern is the main issue for consumer judgement. Enzymes modified by protein engineering usually have high homology with the wild type and only subtle changes in conformation and structures are made. Hence, it is arguable that these enzymes will pose dramatic safety threats. However, some changes might influence the digestibility of an enzyme and the resulting peptides might impose adverse health effects such as causing allergenic reactions. Therefore, it should be kept in mind that proper safety evaluations should always be performed before putting a modified enzyme into the market. Engineered proteins or enzymes are nevertheless new materials and should be released only after stringent safety assessments.

2.4 REGULATIONS

Despite the fact that transgenic organisms generated by genetic engineering and protein engineering are still bashed in most places of the world, products originated from using these technologies are penetrating our lives. These products are under strict regulations to guarantee the well-being of our society. Countries such as Japan and those of the European Union (EU) have set strict rules for regulating products that originate from the application of these modern biotechnologies. The regulations are based on environmental concerns, as well as human and animal health.

In Japan, the Food Safety Commission was founded in 2003; one of its responsibilities is to evaluate the safety of genetically modified food or feed. The standards and guidelines that have been established since then can be found on the official home page of Food Safety Commission (http://www.fsc.go.jp/senmon/idensi/index.html):

- The safety assessment of genetically modified foods (seed plants).
- Guidelines for safety assessment of recombinant crossbred plants.
- Standard for safety assessment of food additives produced by recombinant microorganisms.

- Standard for the safety assessment of non-protein food additives such as amino acids that are produced from recombinant microorganisms and are highly purified.
- Guidelines for the safety assessment of feed and feed additives produced by recombinant technology.

In the EU, legislation provides for a single authorization procedure for food consisting of (or containing) GMOs. Act 1829/2003 regulates GMOs for food and feed use, food and feed containing GMOs, or food and feed produced from or containing ingredients produced from GMOs. Act 1830/2003 further regulates GMOs that have acquired authorization for labelling and traceability to ensure consumer's rights on making choices regarding these products. Enzymes for food use are classified as food additives in Japan, but in the EU, with the exception of lysozyme and invertase, they are classified as processing aids.

Enzymes for food processing that are produced by recombinant DNA technology and protein engineering are regulated by Ministry of Health, Labour and Welfare in Japan, but they are exempted from the current regulation Acts in EU.

2.4.1 Regulations on self-cloning

Enzymes are produced by extraction from plants or animal tissue, or by isolation from microorganisms. Enzymes produced by recombinant DNA technologies are in most cases derived from genetically modified microorganisms (GMMs). Enzyme production procedures involve separating the enzyme from its production strain or releasing the enzyme from the strain by breaking down the microbial cells. Usually, this is followed by purification steps and, at the end of the process, removal of microbial remnants. Therefore, the presence of GMMs in the final enzyme products is rare. The enzyme manufacturing process can be carried out under contained conditions with proper care not to release the GMMs into the environment. Enzyme manufacturers, not enzyme users in this case, need to guarantee the contained uses of GMMs.

Council directives 98/81/EC and 90/219/EEC (http://europa.eu) regulate the contained use of GMMs. According to these directives, GMMs 'mean a microorganism in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination'. And within the terms of this definition, the techniques listed in Annex I, Part B, are not considered to result in genetic modification. These include the following:

- in vitro fertilization;
- natural processes such as conjugation, transduction and transformation;
- polyploid induction.

In Annex II, Part A (council directive 90/219/EEC), GMMs that can be excluded from the directive are listed. These GMMs are yielded through techniques matching the following criteria:

- Mutagenesis.
- Cell fusion (including protoplast fusion) of prokaryotic species that exchange genetic material by known physiological processes.
- Cell fusion (including protoplast fusion) of cells of any eukaryotic species, including production of hybridomas and plant cell fusions.

- Self-cloning consisting in the removal of nucleic acid sequences from a cell of an organism which may or may not be followed by reinsertion of all or part of that nucleic acid (or a synthetic equivalent) with or without prior enzymic or mechanical steps. Reinsertion is done into cells of the same species or into cells of phylogenetically closely related species which can exchange genetic material by natural physiological processes. The resulting microorganism is unlikely to cause disease to human, animals or plants. It is further stated: 'Self-cloning may include the use of recombinant vectors with an extended history of safe use in the particular microorganisms.'

This implies that no special measures need to be taken with self-cloning microorganisms. This self-cloning concept has also been adopted in Japan for evaluation of GMMs for food and food enzyme production. In Japan, because enzymes are classified as food additives, enzymes produced from GMMs are subject to authorization before being launched into the market. The Food Safety Commission carries out the safety assessment on enzymes from GMMs according to 'the standard for the safety assessment of food additives from GMMs'. The enzyme to be evaluated must be already in the list of food additives. There are two occasions when enzymes produced from GMMs do not need safety assessment:

- GMMs are self-cloning strains;
- GMMs are constructed by techniques based on natural occurrence.

By definition, a self-cloning strain contains only nucleic acids from phylogenetically same species. The definition of strains based on natural occurrence is rather vague; however, the authors have constructed a strain of *Streptomyces* that harbours a plasmid of the *Streptomyces* origin with all genetic structures (promoters, terminators and the target gene, *pla2*) originating from *Streptomyces* (unpublished). The enzyme (PLA_2 Nagase) produced by this strain, which is regarded as naturally occurring, is identical to the wild type and is thus exempted from the safety assessment process. This case demonstrates that DNA recombination between closely related microorganisms, which exchange genes between species naturally, can be considered self-cloning as well. Enzymes produced by recombinant DNA technologies within the self-cloning criterion are treated no different from enzymes produced by strains that are improved by traditional mutagenesis techniques.

Public concerns on GMOs also originate from ethical reasons. A survey conducted by Bruce Small, a senior scientist in the social research unit of AgResearch in Hamilton, New Zealand, found that 58% of respondents agreed that gene transfer within species was acceptable.[36] The concept of self-cloning seems more amenable to the public.

2.4.2 Cloning and expression of genes between *Streptomyces* should be considered as *'self-cloning'*

Streptomyces are phylogenetically closely related microorganisms. A lot of species of *Streptomyces* share extremely high homology of 16s rDNA (>95%) and most of them have sex plasmid which causes natural chromosomal gene exchange through conjugation. The physiological mechanisms for gene exchange involve cell contact, formation of 'pock' and exchange of large fragments of plasmid DNA with the recipient chromosome.[37,38] Evidences of gene exchange can also be shown by the fact that many species have identical gene or gene clusters, even if they are phylogenetically different.[39] Because of these genetic characteristics

of *Streptomyces*, the British Genetic Manipulation Advisory Group (GMAG) has viewed that the whole genus should be regarded as a single entity for the purposes of the categorization of recombinant DNA. Gene transfers (cloning genes from one species into another species) between non-pathogenic *Streptomyces* should be classified as 'self-cloning'.[40]

2.5 FUTURE PROSPECTS

Recombinant DNA technology serves to obtain more efficiently what we need from nature and protein engineering aims to improve what nature has to offer. The increasing possibility of future energy crisis and environmental problems strengthens the need for reducing chemical processes and using milder enzymatic processes or processes involving microorganisms (in other words 'bioprocesses'). There is no doubt that recombinant DNA technology and protein engineering will be the driving forces in this respect.

Recently, the creation of an unnatural catalyst was reported that can catalyze the Kemp elimination – a model reaction for proton transfer from carbon by combining computer design with in vitro-directed evolution.[41] Jiang *et al.*[42] also reported the creation of an aldolase to cleave carbon–carbon bonds by computational design. These exciting developments of technologies in designing catalysts from scratch have opened a new phase towards bioprocesses taking over conventional chemical processes. Together with continuous refinement of related techniques in recombinant DNA and protein engineering, bioprocesses will become more and more feasible. The next industrial revolution indeed may not be too far away.

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REFERENCES

1. Cohen, S.N., Chang, A.C.Y., Boyer, H.W. and Helling, R.B. (1973) Construction of biologically functional bacterial plasmids *in vitro*. *Proceedings of the National Academy of Sciences* **70**(11), 3240–3244.
2. Morrow, J.F., Cohen, S.N., Chang, A.C., Boyer, H.W., Goodman, H.K. and Helling, R.B. (1974) Replication and transcription of eukaryotic DNA in *Escherichia coli*. *Proceedings of the National Academy of Sciences* **71**(5), 1743–1747.
3. Chang, A.C., Nunberg, J.H., Kaufman, R.J., Erlich, H.A., Schimke, R.T. and Cohen, S.N. (1978) Phenotypic expression in *E. coli* of a DNA sequence coding for mouse dihydrofolate reductase. *Nature* **275**(5681), 617–624.
4. Ulmer, K.M. (1983) Protein engineering. *Science* **219**, 666–671.
5. Enzyme preparations used in food processing, as compiled by members of Enzyme Technical Association, http://www.enzymetechnicalassoc.org/
6. Horinouchi, S. (2007) Mining and polishing of the treasure trove in the bacterial genus *Streptomyces*. *Bioscience, Biotechnology and Biochemistry* **71**(2), 283–299.
7. Chater, K.F. and Hopwood, D.A. (1984) Streptomyces genetics. In: *The Biology of the Actinomyces* (eds M. Goodfellow, M. Mordarski and S.T. Williams). Academic Press, London, pp. 229–286.

8. Hopwood, D.A., Bibb, M.J., Chater, K.F., Kieser, T. and Bruton, C.J. (1985) *Genetic Manipulation of Streptomyces: A Laboratory Manual*. The John Innes Foundation, Norwich.
9. Bentley, S.D., Chater, K.F., Cerdeno-Tarraga, A.-M., Challis, G.L. and Thomson, N.R. (2002) Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). *Nature* **417**, 141–147.
10. Ikeda, H., Ishikawa, J., Hanamoto, A., Shinose, M., Kikuchi, H., Shiba, T., Sakaki, Y., Hattori, M. and Omura, S. (2003) Complete genome sequence and comparative analysis of the industrial microorganism *Streptomyces avermitilis*. *Nature in Biotechnology* **21**(5), 526–531.
11. Ohnishi, Y., Ishikawa, J., Hara, H., Suzuki, H., Ikenoya, M., Ikeda, H., Yamashita, A., Hattori, M. and Horinouchi, S. (2008) Genome sequence of the streptomycin-producing microorganism *Streptomyces griseus* IFO 13350. *Journal of Bacteriology* **190**, 4050–4060.
12. Katz, E., Thompson, C.J. and Hopwood, D.A. (1983) Cloning and expression of the tyrosinase gene from *Streptomyces antibioticus* in *Streptomyces lividans*. *Journal of General Microbiology* **129**, 2703–2714.
13. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning, A Laboratory Manual*, 2nd edn (ed. N. Ford). Cold Spring Harbor Laboratory Press, New York.
14. Hatanaka, T., Negishi, T., Kubota-Akizawa, M. and Hagishita, T. (2002) Purification, characterization, cloning and sequencing of phospholipase D from *Streptomyces septatus* TH-2. *Enzyme and Microbial Technology* **31**, 233–241.
15. Zambonelli, C., Morandi, P., Vanoni, M.A., Tedeschi, G., Servi, S., Curti, B., Carrea, G., Lorenzo, R. and Monti, D. (2003) Cloning and expression in *Escherichia coli* of the gene encoding Streptomyces PMF PLD, a phospholipase D with high transphosphatidylation activity. *Enzyme and Microbial Technology* **33**, 676–688.
16. Fukuda, H., Turugida, Y., Nakajima, T., Nomura, E. and Kondo, A. (1996) Phospholipase D production using immobilized cells of *Streptoverticillium cinnamoneum*. *Biotechnology Letters* **18**, 951–956.
17. Ogino, C., Negi, Y., Matsumiya, T., Nakaoka, K., Kondo, A., Kuroda, S., Tokuyama, S., Kikkawa, U., Yamane, T. and Fukuda, H. (1999) Purification, chracterization, and sequence determination of phospholiase D secreted by *Streptoverticillium cinnamoneum*. *Journal of Biochemistry* **125**, 263–269.
18. Ogino, C., Kanemasu, M., Hayashi, Y., Kondo, A. and Shimizu, N. (2004) Over-expression system for secretory phospholipase D by *Streptomyces lividans*. *Applied Microbiology and Biotechnology* **64**, 823–828.
19. Sugiyama, M., Ohtani, K., Izuhara, M., Koike, T., Suzuki, K., Imamura, S. and Misaki, H. (2002) A novel prokaryotic phospholipase A_2. *The Journal of Biological Chemistry* **277**(22), 20051–20058.
20. Jovel, S.R., Kumagai, T., Danshiitsuoodol, N., Matoba, Y., Nishimura, M. and Sugiyama, M. (2006) Purification and characterization of the second *Streptomyces* phospholipase A_2 refolded from an inclusion body. *Protein Expression and Purification* **50**, 82–88.
21. Matsuo, Y., Yamada, A., Tsukamoto, K., Tamura, H. and Ikezawa, H. (1996) A distant evolutionary relationship between bacterial sphingomyelinase and mammalian DNase I. *Protein Science* **5**, 2459–2467.
22. Argos, P., Rossmann, M.G., Grau, U.M., Zuber, H., Frank, G. and Tratschin, J.D. (1979) Thermal stability and protein structure. *Biochemistry* **18**(25), 5698–5703.
23. Goodenough, P.W. and Jenkins, J.A. (1991) Food biotechnology: protein engineering to change thermal stability for food enzymes. *Biochemical Society Transactions* **19**, 655–662.
24. Eckert, K.A. and Kunkel, T.A. (1990) High fidelity DNA synthesis by the *Thermus aquaticus* DNA polymerase. *Nucleic Acids Research* **18**, 3739–3744.
25. Stemmer, W.P.C. (1994) Rapid evolution of a protein in vitro by DNA shuffling. *Nature* **370**(6488), 389–391.
26. Goodenough, P.W. (1995) A review of protein engineering for the food industry. *Molecular Biotechnology* **4**, 151–166.
27. Liu, H.-L. and Wang, W.-C. (2003) Protein engineering to improve the thermostability of glucoamylase from *Aspergillus awamori* based on molecular dynamics simulations. *Protein Engineering* **16**(1), 19–25.
28. Fang, T.-Y. and Ford, C. (1998) Protein engineering of *Aspergillus awamori* glucoamylase to increase its pH optimum. *Protein Engineering* **11**(5), 383–388.
29. Toyama, M., Yamashita, M., Yoneda, M., Zaborowski, A., Nagato, M., Ono, H., Hirayama, N. and Murooka, Y. (2002) Alteration of substrate specificity of cholesterol oxidase from *Streptomyces* sp. by site-directed mutagenesis. *Protein Engineering* **15**(6), 477–483.
30. Masayama, A., Takahashi, T., Tsukada, K., Nishikawa, S., Takahashi, R., Adachi, M., Koga, K., Suzuki, A., Yamane, T., Nakano, H. and Iwasaki, Y. (2008) *Streptomyces* phospholipase D mutants with altered

substrate specificity capable of phosphatidylinositol synthesis. *Chembiochem: A European Journal of Chemical Biology* **9**, 974–981.

31. Yanagita, T. (2003) Nutritional functions of dietary phosphatidylinositol. *Inform* **14**(2), 64–66.
32. Bhat, M.K., Pickersgill, R.W., Perry, B.N., Brown, R.A., Jones, S.T., Mueller-Harvey, I., Sumner, I.G. and Goodenough, P.W. (1993) Modification of the head-group selectivity of porcine pancreatic phospholipase A_2 by protein engineering. *Biochemistry* **32**, 12203–12208.
33. Fang, T.Y., Huang, X.G., Shih, T.Y. and Tseng, W.C. (2004) Characterization of the trehalosyl dextrin-forming enzyme from the thermophilic archaeon *Sulfolobus solfataricus* ATCC 35092. *Extremophiles* **8**, 335–343.
34. Fang, T.Y., Tseng, W.C., Chung, Y.T. and Pan, C.H. (2006) Mutations on aromatic residues of the active site to alter selectivity of the *Sulfolobus solfataricus* maltooligosyltrehalose synthase. *Journal of Agricultural and Food Chemistry* **54**, 3585–3590.
35. Fang, T.Y., Tseng, W.C., Pan, C.H., Chun, Y.T. and Wang, M.Y. (2007) Protein engineering of *Sulfolobus solfataricus* malto-oligosyltrehalose synthase to alter its selectivity. *Journal of Agricultural and Food Chemistry* **55**, 5588–5594.
36. Kling, J. (2008) Tony conner. *Nature Biotechnology* **26**(3), 259.
37. Kieser, T., Hopwood, D.A., Wright, H.M. and Thompson, C.J. (1982) pIJ101, a multi-copy broad host-range *Streptomyces* plasmid: functional analysis and development of DNA cloning vectors. *Molecular Genetics and Genomics* **185**, 223–238.
38. Ravel, J., Wellington, E.M.H. and Hill, R.T. (2000) Interspecific transfer of *Streptomyces* giant linear plasmids in sterile amended soil microcosms. *Applied and Environmental Microbiology* **66**(2), 529–534.
39. Metsa-Ketela, M., Halo, L., Munukka, E., Hakala, J., Mantsala, P. and Ylihonko, K. (2002) Molecular evolution of aromatic polyketides and comparative sequence analysis of polyketide ketosynthase and 16S ribosomal DNA genes from various *Streptomyces* species. *Applied and Environmental Microbiology* **68**(9), 4472–4479.
40. Chater, K.F., Hopwood, D.A., Kieser, T. and Thompson, C.J. (1982) Gene cloning in *Streptomyces*. *Current Topics in Microbiology and Immunology* **96**, 69–95.
41. Rothlisberger, D., Khersonsky, O., Wollacott, A.M., Jiang, L., Dechancie, J., Betker, J. and Baker, D. (2007) Kemp elimination catalysts by computational enzyme design. *Nature* **453**(7192), 190–195.
42. Jiang, L., Althoff, E.A., Clemente, F.R., Doyle, L., Röthlisberger, D. and Zanghellini, A. (2008) De novo computational design of retro-aldol enzymes. *Science* **319**(5868), 1387–1391.

3 Production of industrial enzymes

Tim Dodge

The use of enzymes in food applications has been around for centuries.[1] Some of the first examples were the use of naturally occurring enzymes in the source substrate, such as α-amylases that are naturally present in grains used for brewing. Enzymes were also extracted from both plant and animal sources. Then came the use of microbially produced enzymes, where the enzymes were the natural products of the microbial culture. Today, most enzymes are still produced microbially; however, many of the enzymes are no longer native enzymes, but engineered versions. Protein engineering allows the properties of the enzyme to be optimized for its specific use. Many of the applications today are only possible by the use of these specialized products.

Protein engineering is practised through the use of recombinant DNA (rDNA) technology. The same rDNA technology is also utilized to design the microbial strains used to produce the enzyme. The hosts used for enzyme manufacture are becoming more limited in number. At the same time, the source of the enzyme is coming from a wider variety of organisms. This creates a host–platform-centred approach that offers numerous advantages. Efforts can be focused on developing the molecular biology tools required for the smaller set of host organisms. More in-depth knowledge can be gathered and applied to improve these strains by conferring the properties important for industrial enzyme manufacture. These include factors such as fast and efficient growth on simple media, high extracellular enzyme expression and low level of other background proteins, among others. These advantages go beyond those just for strain development, but also for the fermentation, recovery and formulation process. Developing numerous processes for different enzymes is simplified when the same host strain is used, as knowledge of the strain is leverageable. Specifically, the requirements for growing and recovering from the same strain remain the same and this provides a basis to develop a process unique to each enzyme. Requirements for growing the strain remain the same and process conditions become more similar. This advantage continues through to the industrial production plant, where similar processes can reduce capital equipment requirements and ease plant operations.

3.1 APPLICATIONS RESEARCH AND PROTEIN ENGINEERING

Although this topic will be covered in more detail in another chapter, we will nonetheless introduce the subject here. Modern industrial biotechnology is highly integrated and not just

in the production process. The enzyme molecule itself plays a role in its own manufacture and thus the development of the production process is integrated with the development of the molecule.

Most new industrial enzymes are engineered for better performance in their applications. Progress in molecular biology has greatly improved the possibilities to generate libraries of enzyme variants, but a key challenge is to develop good screening tools that can identify the best candidates. Or popularly expressed: 'What you screen for is what you get.' A thorough understanding of the application in which the enzyme has to work is essential for selecting the right enzyme. The screening system should allow working with large variant libraries, be robust and simulate the performance in the final application. The difficulty will often be to translate the requirements for optimal effect under industrial conditions to a set of biochemical assays that can be used for screening. Typical examples are properties like thermostability, pH stability and pH profile, ionic strength requirements, protease resistance, inhibitor sensitivity and substrate or product specificity. Ideally, screening for higher specific activity and for good expression in the production host organism is included from the beginning of an enzyme-engineering programme. In many cases, high throughput screening for expression in small scale can give an indication, but systematic work with larger scale fermentations is needed to reveal yield potential of a new enzyme. Also parameters like solubility in concentrated solution and stability are important for recovery and downstream processing and should be used to select the final enzyme candidate. No matter how well the screening system has been designed, it is always necessary to include extensive full-scale application trials of the final candidates to confirm the correlation between the screening system and the final end use.

One example of a new food enzyme would be an α-amylase used in bread dough modification. It needs a certain ratio of endo to exo activity on the starch in the flour. Too much exo activity and especially maltogenic activity results in poor dough quality. The correct activity can prevent the retrogradation of starch and prolong the shelf life of baked breads. An enzyme can be engineered to give the appropriate properties to meet the demanding needs of the process.[2] But the end product needs to be easily blended into the dough. Spray-dried enzyme can be used, but enzymes tablets have also been shown to be a useful delivery method. To keep the size of the tablet small, the enzyme activity to solids ratio needs to be high, setting the requirement for enzyme purification from the fermentation broth.

As can be seen, the needs of the final product have effects on the properties of the enzyme, not only from an activity standpoint, but also from a purification and formulation standpoint.

3.2 STRAIN DEVELOPMENT

3.2.1 Introduction

Once an efficient enzyme for a given application has been identified, the next important step is to select a production host. The majority of industrially produced food enzymes are manufactured using microorganisms. With the idea of platforms, previously mentioned in this chapter, enzyme manufacturers usually have a library of less than a dozen microbial species for their expression needs. The two main ones are *Bacillus* species and filamentous fungi.[3] These hosts are preferred due to the following properties: they are good secreters (a secreted enzyme will be easier to recover from the broth than an intracellular enzyme), they are recognized as safe hosts by regulatory authorities, they can be genetically manipulated

and they can be cultivated in fermenter to reach a high biomass concentration. Rational prediction of the best host, based on the enzyme to be produced, can often be made. Homologous expression (e.g. *Bacillus* enzyme in a *Bacillus* host) is generally more efficient than heterologous expression (e.g. eukaryotic enzyme in a *Bacillus* host). However, if a new type of enzyme has to be produced, it is worthwhile to attempt expression in a battery of hosts to determine the best candidate.

It must be emphasized that the paragraph above refers to genetically modified organisms (GMOs). Non-GMOs are also used in the production of food enzymes. Often, the food enzyme manufacturers have a wide range of products as options to meet customers' specific needs.

3.2.2 GMO versus non-GMO

The main advantage of using non-GMOs for food enzymes production is their larger acceptance by the consumer.[4] On the other hand, GMOs offer numerous advantages, both for the customers and the producer. An enzyme produced by a recombinant organism is generally cheaper because it can typically be expressed at higher levels compared to non-GMOs. The final products also tend to have higher purity as GMO strains are engineered to overexpress the target enzyme per total background host protein.

An enzyme will be labelled as recombinant based on the following guidelines: (1) the enzyme amino acid sequence is the same as can be found in nature but the organism used to produce it is non-native,[5] (2) the organism used to produce it has been genetically modified (e.g. stronger promoter in front of the gene[6]) and/or (3) the enzyme itself (i.e. its amino acid sequence) has been engineered.[2] To keep with the idea of minimizing the impact of genetic engineering on the environment, a recent development in strain construction has been to remove the antibiotic marker which often accompanies the gene coding for the enzyme of interest.[7]

Product labelling is regulated by the Food and Drug Administration (FDA) in the US, and by the European Union in Europe.

3.2.3 Example: construction of a *Bacillus subtilis* production host

In order for an enzyme to be produced by a host organism, the gene coding for the enzyme of interest has to be introduced into the host, together with other DNA structures allowing its transcription, translation and secretion. Figure 3.1 shows the minimum gene components required to produce an enzyme extracellularly. In brief, the promoter initiates transcription, the ribosome binding site (RBS) is necessary to initiate translation, the signal sequence (S.S.) targets the protein to the membrane to allow its secretion, the gene X encodes the enzyme of interest and the terminator marks the end of transcription. Often an antibiotic marker is added after the gene X to permit the selection of a correct recombinant clone. If the enzyme is to

Fig. 3.1 Typical structure of an expression cassette for enzyme production by microorganisms. RBS: ribosome binding site; S.S.: signal sequence. Gene X encodes for the enzyme of interest.

Table 3.1 Examples of targets for genetic engineering in the microbial production of enzymes.

Target	Expected effect from engineering	Reference
Promoter	Stronger promoters will provide more mRNA	12
Signal sequence	Modifications in the signal sequence can improve secretion	13,14
Gene X	Protein engineering can result in an enzyme more efficient in its application	15
Host	Deletion of side activities, improvement of physiology or metabolism	16,17

be produced intracellularly, the S.S. is omitted. The cassette of interest can be introduced in the host on a replicating plasmid (an extrachromosomal DNA molecule which is capable of replicating independently) or integrated on the chromosome by single or double crossover. Integration on the chromosome is usually preferred as it results in increased stability and consistency of the strain to express the target enzyme.[8]

3.2.4 Goals of genetic engineering

Genetic engineering usually has as its first goal improvement of the target enzyme production yield. It can also be used to remove side activities, such as proteases. Proteases can degrade the target enzyme or they can alter the organoleptic qualities of the food product in which the enzyme is applied.

To improve product yields, bottlenecks in transcription, translation and/or secretion need to be eliminated. For example, transcription, which is the conversion of genomic DNA into messenger RNA (mRNA), can be improved by increasing the strength of the promoter,[7] or by introducing multiple copies of the expression cassette into the host. The mRNA itself can be stabilized by modifying its 5′ end.[9,10]

Each element shown in Fig. 3.1 can be the target for genetic engineering. Table 3.1 presents different targets and the effect expected from their engineering.

3.2.5 Food enzyme production in the 'omics' era

A significant revolution in biology in the last decade is marked by the arrival of the 'omics' era (e.g. Ref. [11]). These methods include rapid sequencing of whole genomes, monitoring of mRNA expression, analytical protein measurements (especially mass spectrometry) and small molecule identification and quantification. These have resulted in the new fields of genomics, transcriptomics, proteomics and metabolomics. Availability of these data has resulted in greater understanding of the host as a whole, which leads to even safer production systems and enzyme products.

3.3 MICROBIAL FERMENTATION

The fermentation unit operation is the step at which enzyme is produced. A microbial culture is the biocatalyst used to generate the enzyme. This culture must first be propagated to generate sufficient catalyst. Enzyme is then produced, sometimes in conjunction with the microbial culture growth and at other times in a different phase where microbial growth has slowed or even stopped.

3.3.1 Culture maintenance and storage

The most common method of culture storage is in a frozen state. Bacterial cultures are typically grown under optimal conditions. A cryopreservative agent, such as glycerol or dimethyl sulphoxide, is added and the suspension dispensed into individual vials before quick freezing on dry ice. Mechanical freezers, at either −20°C or −70°C are typically used, but for short-term storage. For longer term storage, liquid nitrogen freezers, where the vials are held in the vapour phase, are preferable. Such procedures allow for high viability and maintenance of phenotype, even when stored for years.

3.3.2 Seed preparation

Most industrial enzyme fermentations are carried out in vessels ranging in size from 30 m^3 to 300 m^3. This piece of equipment is typically one of the most expensive in the production plant. To optimize use of the production fermenter, seed vessels are used in order to reduce the time required for growth in the large fermenter.

Traditional seed trains have consisted of a series of increasingly larger vessels, typically 10-fold at each stage. This was often required for strains with fastidious growth requirements. The host–platform-based approach removes this need. Strains are designed or adapted to go from vial directly to production fermentation. However, as stated above, the actual process used will be devised to optimize the use of capital. This will typically entail a single seed fermenter that can be inoculated directly from the culture vial. A longer growth time can typically be tolerated in this smaller fermenter while time in the larger production fermenter is lowered.

Conditions in the seed fermenter are optimized for fast and efficient growth. These same conditions are usually the desired conditions for the start of the production fermenter. Transfer from the seed to production fermenter is performed while the culture is in the exponential growth stage.

3.3.3 Production fermentation

3.3.3.1 *Sterilization*

One of the unique and essential features of the fermentation operation is the need for sterilization. Microorganisms are ubiquitous in the environment, present in air, soil and water. The desire is to produce a single microbial catalyst in order to produce enzyme. In order to achieve this goal, all other microorganisms need to be killed or removed from the process. This is typically achieved by either heat inactivation or filtration. Heat sterilization can be achieved through either high temperature (near 150°C) with short contact time (3–5 min) in a continuous operation or lower temperature (near 121°C) with longer contact time (30–60 min) in a batch operation. Media with insoluble components generally require batch sterilization. Soluble media or feed solutions are better suited for continuous sterilization. Air is usually sterilized by depth filtration.[18]

3.3.3.2 *Mass and heat transfer*

Most industrial enzyme fermentations are aerobic processes; that is, they require oxygen as an electron acceptor to supply energy to the culture. This oxygen requirement results in two specific issues for the fermentation equipment: oxygen mass transfer and heat removal. A schematic of an industrial fermenter is shown in Fig. 3.2.

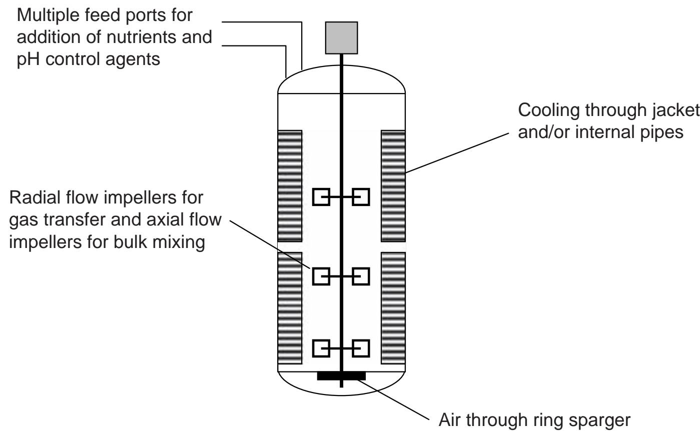


Fig. 3.2 Industrial fermenter.

Oxygen is only sparingly soluble in water solutions. Under typical fermentation conditions, solubility is around 0.5 mM. To supply the required oxygen, pressurized air is introduced at the bottom of the fermenter. As the air travels upward in the vessel, oxygen is transferred from the gas to the liquid phase. The soluble oxygen is consumed by the organisms, usually with the concomitant production of carbon dioxide, which alternatively is transferred from the liquid to the gas phase. The spent air leaves the fermenter depleted in oxygen and enriched for carbon dioxide. High pressure increases the solubility of both gases in the liquid. Increased airflow increases the oxygen content of the spent air, also increasing the solubility in the liquid. Power is added to the liquid through impellers driven by a motor. This power is used to break the incoming air into smaller bubbles increasing the surface area for mass transfer. All of these requirements (pressure, airflow and power) increase the complexity of the fermentation equipment.

In an aerobic fermentation process, heat is generated through the transfer of power from the agitator motor to the liquid (mechanical heat). Additional heat is also generated by the organism through the respiration of oxygen (metabolic heat). Metabolic heat typically dominates in aerobic fermentations. Heat must be removed through cooling surfaces in the fermenter. A jacket around the surface of the fermenter is almost always used. As the size of the fermenter increases, the volume increases faster than the surface area of the cylindrical vessel. Thus, in order to keep similar surface area for cooling, pipes can be added on the inside of the fermenter, further increasing the complexity of the equipment. The heat generated is transferred to a cooling liquid, usually water, that is circulated through the jacket and cooling pipes. Heat is dumped to the environment, typically in cooling towers. In some cases mechanical chillers are needed to give additional cooling.

High oxygen demand requires sophisticated and expensive equipment and high utility usages. Most processes, however, can be improved under these conditions. Trade-offs will be made between process performance and capital and operating costs. Most aerobic fermentation processes can be adapted to the equipment in which it must operate.

3.3.3.3 *Catalyst growth*

The first stage of the production fermenter is very similar to the conditions in the seed tank. The microbial catalyst needs to multiply in order to have sufficient concentration for maximal

enzyme production later in the fermentation. Fast specific growth rate is desired in order to maximize production of the protein production machinery within the microorganism. The number of ribosomes, the key organelle responsible for protein production, is increased at high growth rate.[19]

3.3.3.4 *Enzyme production (growth vs. non-growth associated)*

Some enzymes are best produced while the culture is actively growing and faster growth results in faster enzyme production. This is called growth-associated enzyme production. Metabolic enzymes are examples of proteins that are typically growth associated. Many hydrolytic enzymes are typically produced as secondary metabolites. Such products are produced during slow specific growth rates or even under non-growth conditions. This is called non-growth-associated production. These simplifications actually apply to only a small number of products. Most will exhibit a mixed production mode.

Platform organisms made specifically to produce enzymes most often use promoter systems that are induced under non-growth-associated conditions. Some examples are the alkaline protease promoter from *B. subtilis*, the α-amylase promoter from *Bacillus licheniformis* and the cellobiohydrolase I promoter from *Trichoderma reesei*.

3.3.3.5 *Batch versus fed-batch versus continuous*

The fermentation can be operated in three basic modes, batch, fed-batch and continuous.[20] In batch mode, all medium components are added at the beginning of the process. The culture is inoculated and allowed to grow at a maximum, but uncontrolled rate. As nutrients are consumed or waste products accumulate, growth will slow down and eventually stop. When a limiting nutrient is exhausted, growth will stop and the fermentation ends. A batch process can be effective for growth-associated enzyme production.

Continuous processes will feed nutrients and remove culture-containing medium at the same rate as the nutrient addition. A steady state can be achieved that can be maintained for weeks. Growth rate can be controlled by the rate of feed addition. Continuous fermentation is also well-suited for growth-associated enzyme production.

Fed-batch fermentation is the most dominant mode of operation for enzyme production. It is well-suited for non-growth-associated production. The typical feed is a limiting nutrient, usually the carbon-energy source. As with continuous processes, fed-batch can control growth rate through the rate of feed addition. This has the additional advantage of helping control oxygen consumption and thus heat generation. Fed-batch fermentations are easier than batch fermentations to adapt to equipment that may limit either the mass or heat transfer available to the process.

Some cultures are sensitive to gradients of substrates or waste products. In such cases, bulk mixing of the liquid in the fermenter is of great importance. Axial flow impellers force the circulation of liquid in the fermenter to reduce these gradients. However, these impellers are poor at reducing bubble size and increasing the rate of oxygen transfer. Radial flow impellers are much preferable for mass transfer. Once again, fermenter design will be a trade-off between the needs for bulk mixing and those for oxygen mass transfer.

Surface fermentation

Not all industrial fermentation processes are based on submerged (mostly liquid) cells. In fact, surface (mostly solid substrate) fermentation processes such as Japanese ‘koji’, French ‘blue

cheese', composting have longer tradition. Surface fermentations typically employ moist non-submerged particulate or non-particulate substrates (grains, legumes, agro-industrial residues, fresh cheese), preferably sterile and formed into high surface area matrices. In some cases, a mix of nutrients is also added to the solid substrate and microbial seed is grown in submerged fermentation process before inoculating the matrix. Some advanced design solid substrate fermentors have even provided, continuously or intermittently, nutrient and/or pH controlling solution to the substrate matrix. The greatest production design challenge is the supply of oxygen, removal of gases and heat of respiration from the substrate matrix, homogeneous conditions and contamination control. As a result, scale of operation of surface fermentation processes tends to be smaller than that of submerged type.

3.4 DOWNSTREAM PROCESSING

3.4.1 Bridging the gap

Fermentation broth is not fit for sale as a product. It contains live microbial cells and rDNA. Because of the high water content of the broth and the presence of proteases and peptidases, the produced enzymes are prone to degradation. The concentration of enzyme is too dilute for the application. For these reasons, fermentation broth needs to be further processed in order to refine the enzyme product such that it becomes resilient to uncontrolled storage conditions, is safe and convenient to use in the application and can deliver the benefit to the consumer in a cost-effective manner.

Collectively, the manufacturing processes used to refine the enzyme product are referred to as downstream processing. A wide variety of techniques are available to accomplish the goals laid out above. The choice of specific operations is dictated not just by the nature of the fermentation broth, the biochemical and biophysical properties of the enzyme and the refining needs of the product, but also by practical considerations such as availability of a certain unit operation in the manufacturing site, and site-specific recycling and disposal options for non-product containing side streams.[21]

Downstream processing often requires the addition of materials such as filter aids, flocculants, buffer salts, acids and bases to adjust pH. These materials need to meet food safety requirements which are put forward by regulatory and government bodies. In addition, they need to conform to religious dietary restrictions in major markets such as kosher, chometz free and halal. This constrains the source of raw materials and reduces flexibility to change materials once a product has been approved for food use. Similar constraints exist for downstream processing operations and facilities.[22]

Consumers prefer preservative-free foods. The food enzyme industry, despite being only a very minor contributor to the overall preservative content of a food product, is adapting to that need and downstream process technology is evolving to deliver on that goal. Aseptic processing techniques are increasingly adopted.[23]

3.4.2 Basic downstream process

With the exception of some lipid modifying enzymes, the majority of recombinant enzymes used in food processing are soluble in aqueous buffers. Modern microbial production strains are engineered to secrete soluble enzymes into the medium, where the enzyme accumulates during the fermentation. At its most basic level, the downstream process just needs to remove

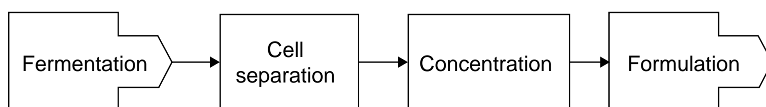


Fig. 3.3 Basic downstream process for enzyme recovery from fermentation broth.

the cells from the fermentation medium and concentrate the resulting clarified filtrate enough to facilitate formulation of the product, as shown in Fig. 3.3.[24,25]

The preferred mode of fermentation at large scale is fed-batch fermentation, which takes 2–10 days to complete. Downstream process equipment is usually sized such that the broth can be processed in about 1–2 days so as not to limit plant throughput by downstream bottlenecks. In order to make use of all downstream equipment at all times and to keep the number and size of downstream tanks small, cell separation and concentration and sometimes even formulation are performed simultaneously. As a consequence, downstream equipment operates in continuous mode or with rapid cycling on batch equipment.

There are several choices of scalable unit operations that can run in this fashion. Continuous cell separation can be achieved by centrifugation on disk stack or decanter centrifuges or by microfiltration. Rotary vacuum drum filters provide semi-continuous filtration and plate-and-frame filter presses operate in batch mode and are cycled many times to filter the whole broth volume. The choice for concentration is more limited; it is typically accomplished by ultrafiltration via synthetic membranes with a molecular weight cut-off small enough to retain all of the enzyme. In some cases, evaporation may be an option, but it is usually too expensive and does not remove dissolved salts or small molecular weight impurities.

The product of a basic downstream process is a crude enzyme concentrate. In addition to the target enzyme, this concentrate contains residual soluble and colloidal components from the fermentation feed stocks and other products from the microbial metabolic activity. Other components are residual process aids, such as antifoam oils and flocculants, non-product enzymes that are produced by the host organism and some bioburden from environmental microbes.

3.4.3 Purification

If the specifications of the enzyme product require lower concentration of the non-enzyme components in the crude concentrate, some form of purification is required. This can be as simple as a polish filtration to remove colloidal particles and bioburden, which is often integrated with the formulation of liquid products. Removal of salts and other permeable solids can be accomplished by adding a diafiltration step to the ultrafiltration. Non-UF-permeable impurities, such as biopolymers or side activities, can often be reduced by careful selection of the conditions used during cell separation: pH, salt content, flocculant type and means of addition to the process can have a significant impact.[26,27]

If these measures are not sufficient to produce an acceptable product, more specific purification process steps are needed. The first choice of bulk purification methods is precipitation of the impurities. If the soluble impurities can be induced to form a solid phase, it can simply be removed by filtration. More involved are techniques to precipitate the target enzyme, because they require the addition of two unit operations, one for collection of the precipitate, another to dissolve it again and clarify it.[28] The cleanest enzyme products are made by crystallization of the enzyme. Due to the highly selective nature of crystal formation, the solids formed in such a process are inherently very pure and the obtainable product

purity is determined by the efficiency with which the pure crystals can be isolated from the impurity-rich mother liquor.[29,30]

Chromatography is another choice for making purified enzyme products. This is usually the most expensive option and is only economical for a small number of enzyme products that are used in very low doses.[31]

3.4.4 Sustainability

Sustainability of manufacturing operations calls for all non-product side streams to be used as raw materials in other processes. The cell solids are inactivated by treatment with heat or chemicals and are used as a source of complex nutrients. Ultrafiltration is the most cost-effective way to concentrate the enzyme solution and the ultrafiltration permeate is typically rich in dissolved salts and micronutrients, which are welcome additives to biological treatment of wastewater. Ultrafiltration permeate can be concentrated by reverse osmosis (RO). The resulting RO concentrate becomes a source of complex nutrients in fermentation processes and the RO permeate is reclaimed as process water.

3.5 ENZYME FORMULATION

The final stage of enzyme production is product formulation. The resulting enzyme concentrate from the fermentation and downstream process is typically not capable of being utilized in the food applications. The enzymes need to be provided in a form that is useful for each application. In general these forms are either solids or liquids. Not only does the enzyme need formulations for use in the application, but formulation is also required to supply the desired shelf life during storage of the enzyme prior to use. It is important that the excipients and diluents utilized for product formulation are also acceptable in the food product.

3.5.1 Solid product formulation

In solid product forms, the liquid enzyme concentrate is mixed with stabilizing excipients or diluents and then converted to a dry form. The drying process can be accomplished through processing such as spray drying or encapsulation. Spray drying has been utilized for decades and is still one of the common production modes for enzyme products. Other modes of drying such as freeze drying can provide additional benefits to the products, but are typically more expensive.

3.5.1.1 *Spray drying*

In the spray drying process, the liquid enzyme concentrate is mixed with stabilizers and diluents so that the resulting product has the desired activity for the application as well as being stable. After the materials are mixed, the resulting suspension is atomized into small droplets through a nozzle either with pressurized air or through a rotary nozzle (spinning disc). The atomized liquid is introduced at the top of the spray tower and is exposed to high temperature air, up to 170°C. However, due to the evaporative cooling, the exhaust temperatures will be near 100°C and the actual enzyme powder experiences an even lower temperature. The resulting powders are very homogeneous. However, they tend to have very low particle sizes which can be very dusty. To control the dust, the powders can be blended

with low levels of oil. This is very effective in reducing any materials which may become airborne.

The spray-dried powder can also be incorporated into other product forms. These can be as simple as agglomerated powders or more sophisticated as structured particles. The powder can be agglomerated to form larger particles and reduce dust. This is done by putting the powder into mixing equipment and adding a binder material while mixing, which results in binding the powder together and forming larger particles. The agglomeration process can also be carried out in a fluid bed granulator. In this process, the powder is suspended in an air stream and, while suspended, an atomized spray of binder is applied through a nozzle. As the particles pass through the atomized liquid they will become wet, stick together and then dry in that form. This process is carried out multiple times, thus resulting in larger and larger particles. Both of these methods are somewhat uncontrolled and the resulting product has a broad size distribution.

In specialized equipment, such as multi-stage driers, the spray drying and agglomeration processes can be carried out simultaneously. In this process, the enzyme concentrate and any added stabilizers are atomized though a nozzle similar to a standard spray drier. However, any fine powders are evacuated from the drying chamber and subsequently re-introduced into the drying chamber near the point of liquid atomization. This results in the dry particle rewetting and agglomerating with other nearby particles. By controlling the airflows in the system, it is possible to target a narrow size distribution of the final agglomerated particles.

The spray-dried powders can also be incorporated into more structured particles such as extrudates, high shear granules or spray-chilled particles. These particles can offer other advantages including improved stability and particle integrity. They also have the possibility of including other food use agents and customized enzyme release mechanisms. In the extrusion process, the spray-dried powder is mixed with other materials such as sugars, starches, buffers and binders. The materials are mixed together to create a dough-like material. This mixture is pressed through a perforated plate and the resulting 'noodles' are either cut-off at regular intervals or allowed to fall-off on their own. There is also the possibility of converting these cylindrical particles into spheres via a device known as a marumerizer. Finally the particles are dried in a fluid bed drier. In the high shear process, dry ingredients including the enzyme powder are added to the process bowl. While being mixed with a plough shear mixer and a high-speed chopper, wet binding materials can be added. The shearing action generates small particles which can be dried similarly to the extrusion process. Finally, the spray-dried powder can be incorporated into a molten fat or wax. This mixture is then atomized through a rotary nozzle into a cooled chamber essentially equivalent to a spray-drier tower. The particles cool and harden as they fall, resulting in very spherical particles.

3.5.1.2 *Encapsulation*

Finally, solid enzyme products can be further encapsulated to customize delivery of the enzyme. By creating a core-shell particle where the enzyme is included in the core, a functionalized coating can be added. These coatings can not only further improve the stability of the enzyme product, but can also be triggered to release the enzyme under desired conditions. These triggers could include pH, heat, shear or water activity just to name a few. The enzyme core unit can be those already described, such as agglomerates, extrudates, high shear granules or spray-chilled particles. Additionally, the enzyme core may be generated using a fluid bed coater. In the fluid bed process, an inert carrier is suspended in a hot air

stream within a process chamber. While the particles are suspended, an air atomized enzyme liquid is introduced into the chamber. The atomized droplets are captured on the carrier and subsequently dried. The carrier/enzyme particles pass through the atomized liquid multiple times until the desired enzyme concentration is obtained. The fluid bed process can also be utilized to apply the functional coating to any of the enzyme core units described simply by changing from the enzyme solution to a solution of the desired coating material. By utilizing triggers, the enzymes can be delivered into the food application at the appropriate time, thus maximizing its effectiveness or chemistry.

3.5.1.3 *Enzyme tablets*

Another useful delivery format is to provide the enzyme in a tablet form. Tablets can offer many advantages. The enzyme can be incorporated into the tablet from any of the forms previously discussed, including spray-dried powders, agglomerates and coated particles. Similar to enzyme blends, tablets can incorporate multiple enzyme activities as well as other components that can benefit the particular application being applied. However, tablets eliminate the possibility of segregation that blends can encounter. The tablets thus provide a consistent dose of enzymes and other components compared to blends. Finally, handling and dosing of tablets into the application is far easier, less dusty and cleaner compared to a powder blend.

3.5.2 Liquid product formulation

Enzymes can also be utilized in a liquid form. These product formulations can be challenging as they are more dynamic systems compared to solids because of the presence of water. Stabilizers that are used in liquids include common carbohydrates such as sucrose and dextrose, sugar alcohols and polyols. These excipients stabilize enzymes by maintaining the protein structure and therefore prevent denaturation. Additionally upon preventing aggregation, these materials help in maintaining a soluble product. At high levels, they help to control the water activity which can play a role in controlling microbial growth. Preservatives are also added, which will be discussed in a later section of this chapter.

The liquid product formulation process is usually integrated with the downstream production process. After the enzyme has been concentrated, the enzyme stabilizers are added followed by a filtration process. The filtration is done to remove any undissolved solids and/or to reduce the level of microbial burden in the formulated product. The filtration process can be carried out using standard filtration equipment such as a rotary vacuum drum filter (RVDF), plate and frame or depth filtration. The resulting products are transparent and commonly range from clear to amber in colour.

3.5.3 Blends

Many of the products that are used in food applications contain more than one enzyme activity. Enzyme manufacturers can produce these blends through combining already formulated intermediates, such as spray-dried or encapsulated solids, or liquids in defined ratios. For the solid product blends, it is important that the intermediate products are similar in size and density. This allows for good mixing and homogeneity while also reducing the likelihood of segregation during shipping and handling. The blending operation can also be utilized to

reduce the activity of a product by blending with a non-active diluent. Liquid blends are also possible, but the compatibility of the intermediate products is an important consideration so that unstable products are not created.

The blending process can be carried out in a variety of equipment. For liquid products, it is typically carried out in a batch process using a single tank with a mixer present. It can also be done continuously utilizing in-line mixing. If necessary a filtration step can be employed post mixing. Solid product blends can be made in a variety of blending equipment, such as ribbon blenders, plough shear mixers, Nauta mixers or fluid bed apparatus.

3.5.4 Preservatives

The most common preservatives utilized for food applications to control microbial contamination are sodium benzoate and potassium sorbate. They are both effective at low pH and can control a broad spectrum of organisms. However, one of the major food trends today is to reduce or remove the level of preservatives in the products. This is being driven by consumer desire. Subsequently, all the materials that go into the production of food products, such as enzymes, need to be preservative free and this can be a challenge for enzyme products. As long as the solid products are protected from moisture, the likelihood of any microbial or fungal growth during storage is extremely low. In the case of liquids, the situation is even more challenging. To control microbial growth during storage, the environment of the solution is manipulated against it. This can be done by controlling the water activity, pH or osmolality. Also, more and more natural preservatives are being developed that are effective at controlling microbial growth. These natural preservatives include certain plant extracts or peptides that inhibit microbial growth.

3.6 SUMMARY

Even though the use of enzymes in food industry is well established, the adoption of new technologies in recent years has resulted in considerable progress for the production of such enzymes. Improvements in protein engineering have yielded enzymes which are more efficient, stable and have less side activities. Efforts have been made towards the development of plug-in platforms, that is limiting the number of hosts in which an enzyme can be produced. Baseline processes, including fermentation, downstream processing and formulation, developed for each host, are a starting point for the production of a new enzyme. This standardization allows for a shorter development time.

Most fermentations for food enzyme production require the preparation of a sterile medium. They are usually aerobic, requiring pressure, airflow, power and cooling. Fermentations are most often run in the fed-batch mode, although batch and continuous processes can also be found.

Since most food enzymes are secreted into the fermentation medium, downstream processing simply requires removal of cells by filtration or centrifugation, followed by a concentration step to make the product ready for formulation. In cases where higher purity product is required, a purification step typically followed the concentration step. The choice of unit operations depends on the capabilities of the plant as well as compliance with government food safety regulations and religious dietary restrictions. Where feasible, sustainability of production is achieved by recycling of water and biomass.

Finally, a formulation step is often required, to allow for use in the application and/or to extend shelf life. Most often for food applications, the enzyme is formulated in the solid state, although some liquid formulation examples can also be found. Tablets are a popular form of delivery, as they can allow for homogenous blends of products and a consistent dose delivered. Preservatives are used to control microbial growth in enzyme products. Customer preferences have been evolving towards less preservative use, or at least more natural preservatives.

REFERENCES

1. Birschbach, P., Fish, N., Henderson, W. and Willrett, D. (2004) Enzymes: tools for creating healthier and safer foods. *Food Technology* **58**(4), 20–26.
2. Kragh, K.M., Larsen, B., Rasmussen, P., Duedahl-Olesen, L. and Zimmermann, W. (1999) Non-maltogenic exoamylases and their use in retarding retrogradation of starch. European Patent No. EP 1 068 302 B1, WO 99/50399.
3. Punt, P.J., van Biezen, N., Conesa, A., Albers, A., Mangnus, J. and van den Hondel, C. (2002) Filamentous fungi as cell factories for heterologous protein production. *Trends Biotechnology* **20**, 200–206.
4. Sondergaard, H.A., Grunert, K.G. and Scholderer, J. (2005) Consumer attitudes to enzymes in food production. *Trends in Food Science and Technology* **16**, 466–474.
5. Mule, V.M.R., Mythili, P.K., Gopalakrishna, K., Ramana, Y. and Reddy, D.R.B. (2007) Recombinant calf-chymosin and a process for producing the same. Patent Application US20070166785 A1.
6. Valle, F. and Ferrari, E. (2005) Mutant aprE promoter. Patent US6911322.
7. Widner, B., Thomas, M., Sternberg, D., Lammon, D., Behr, R. and Sloma, A. (2000) Development of marker-free strains of *Bacillus subtilis* capable of secreting high levels of industrial enzymes. *Journal of Industrial Microbiology & Biotechnology* **25**, 204–212.
8. Song, J.Y., Kim, E.S., Kim, D.W., Jensen, S.E. and Lee, K.J. (2008) Functional effects of increased copy number of the gene encoding proclavaminate amidino hydrolase on clavulanic acid production in Streptomyces clavuligerus ATCC 27064. *Journal of Microbiology and Biotechnology* **18**, 417–426.
9. Sharp, J.S. and Bechhofer, D.H. (2003) Effect of translational signals on mRNA decay in *Bacillus subtilis*. *Journal of Bacteriology* **185**, 5372–5379.
10. Condon, C. (2003) RNA processing and degradation in *Bacillus subtilis*. *Microbiology and Molecular Biology Reviews* **67**, 157–174.
11. Jewett, M., Hofmann, G. and Nielsen, J. (2006) Fungal metabolite analysis in genomics and phenomics. *Current Opinion in Biotechnology* **17**, 191–197.
12. Widner, B. (1997) Stable integration of DNA in bacterial genomes. US Patent 5,695,976A.
13. Kitabayashi, M., Tsuji, Y., Kawaminami, H., Kishimoto, T. and Nishiya, Y. (2008) Producing recombinant glucose dehydrogenase (GDH) derived from filamentous fungus by introducing a mutation in a signal peptide sequence present in an N terminal region of GDH, thus increasing an amount of expressed GDH. US Patent Application 20080014611-A1.
14. Perlman, D., Raney, P. and Halvorson, H. (1986) Mutations affecting the signal sequence alter synthesis and secretion of yeast invertase. *Proceedings of the National Academy of Sciences of the United States of America* **83**, 5033–5037.
15. Leisola, M. and Turunen, O. (2007) Protein engineering: opportunities and challenges. *Applied Microbiology and Biotechnology* **75**, 1225–1230.
16. Ferrari, E., Harbison, C., Rashid, H.M. and Weyler, W. (2006) Enhanced protein expression in *Bacillu*s. Patent Application US20060073559 A1.
17. Bodie, E.A. and Kim, S. (2008) New filamentous fungus having a mutation or deletion in a part or all of a gene, useful for producing improved protein, e.g. enzyme, production. WO2008027472-A2.
18. http://www.porex.com/by_function/by_function_filtration/surf_vs_dep.cfm
19. Ingraham, J.L., Maaloe, O. and Neidhardt, F.C. (1983) *Growth of the Bacterial Cell*. Sinauer Associates, Sunderland, MA.
20. Chotani, G.K., Dodge, T.C., Gaertner, A.L. and Arbige, M.V. (2007) Industrial biotechnology: discovery to deliver. In: *Kent and Riegel's Handbook of Industrial Chemistry and Biotechnology*, Vol. **2**, 11th edn (ed. J.A. Kent). Springer Science, New York, pp. 1311–1374.

21. Hatti-Kaul, R. and Mattiasson, B. (2003) *Isolation and Purification of Proteins*. Marcel Dekker, Inc., New York.
22. Lad, R. (2006) *Biotechnology in Personal Care*. Taylor & Francis, New York.
23. Kabara, J. and Orth, D.S. (1997) *Preservative-Free and Self-Preserving Cosmetics and Drugs. Principles and Practice*. Marcel Dekker Inc., New York.
24. Aehle, W. (2007) *Enzymes in Industry: Production and Applications*, 3rd edn. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.
25. Harrison, R.G., Todd, P., Rudge, S.R. and Petrides, D.P. (2003) *Bioseparations: Science and Engineering*. Oxford University Press, Oxford.
26. Ramsden, D.K., Hughes, J. and Weir, S. (1998) Flocculation of cellular material in complex fermentation medium with the flocculant poly(diallyldimethylammonium chloride). *Biotechnology Techniques* **12**, 599–603.
27. Shan J.-G., Xia, J., Guo, Y.-X. and Zhang, X.-Q. (1996) Flocculation of cell, cell debris and soluble protein with methacryloyloxyethyl trimethylammonium chloride – acrylonitrile copolymer. *Journal of Biotechnology* **49**, 173–178.
28. Scopes, R.K. (1994) *Protein Purification: Principles and Practice*. Springer, New York.
29. Becker, T. and Lawlis Jr., V.B. (1991) Subtilisin crystallization process. US Patent 5,041,377.
30. Becker, N.T., Braunstein, E.L., Fewkes, R. and Heng, M. (1997) Crystalline cellulase and method for producing same. PCT WO 97/15660.
31. Birschbach, P. (1987) Method for separating rennet components. US Patent 4,745,063.

4 Asparaginase – an enzyme for acrylamide reduction in food products

Beate A. Kornbrust, Mary Ann Stringer, Niels Erik Krebs Lange and Hanne Vang Hendriksen

4.1 INTRODUCTION

In 2002, it was discovered that relatively high concentrations of acrylamide are found in common carbohydrate rich foods prepared by baking or frying. Acrylamide is classified as 'probably carcinogenic to humans'[1] and its occurrence in food products has therefore caused intensive debate concerning the potential health risk through dietary exposure.[2,3] Extensive international research programmes have been established to investigate and evaluate this risk. Until the results from these studies are available, Joint FAO/WHO Expert Committee on Food Additives (JECFA) has recommended to producers that appropriate efforts be made to reduce the concentration of acrylamide in food.[2] For consumers, the existing recommendation to eat a well-balanced diet high in fruit and vegetables has not been changed.

Figure 4.1 gives an overview of the acrylamide values found in some typical food products with relatively high acrylamide contents. The values shown are from the European Commission's monitoring database[4] and the figure illustrates the median and 25th and 75th percentile values for acrylamide content in each food product category. The classification system used in this database has grouped similar products into broadly inclusive categories. Both within and between categories, acrylamide contents vary widely, reflecting the variety of product recipes, production parameters and raw materials used in the affected foods. Additional public databases can be found in the summary of Stadler and Scholz.[5]

Daily intake of acrylamide varies globally depending upon local eating and cooking habits. In a typical western diet, fried and baked potato products, biscuits, crisp bread and coffee are among the foods accounting for the most significant proportion of dietary exposure, either because of high acrylamide content or because of a high daily intake.[2,6] Average daily intake estimated by JECFA is 1 $\mu g\ kg^{-1}$ body weight/day and by the FDA is 0.4 $\mu g\ kg^{-1}$ body weight/day.[7] For comparison, the WHO reported guideline values for acrylamide in drinking water are 0.1–0.5 $\mu g\ L^{-1}$.[8]

To date no direct legislative regulation for maximum acrylamide content in food products has been made. However, in Germany the so-called signal value and minimization concept has been implemented. In this programme, food products are classified into groups, and signal values are determined for each of these. If acrylamide levels above the signal value are found, food control authorities contact the food producer and initiate a dialogue on minimization.[9]

Several different research groups have established that acrylamide is generated by Maillard reactions between asparagine and reducing sugars, with asparagine contributing the backbone of the acrylamide formed.[4,6,10–14] Maillard reactions, also known as non-enzymatic browning reactions, typically occur at temperatures above 100°C and are responsible for

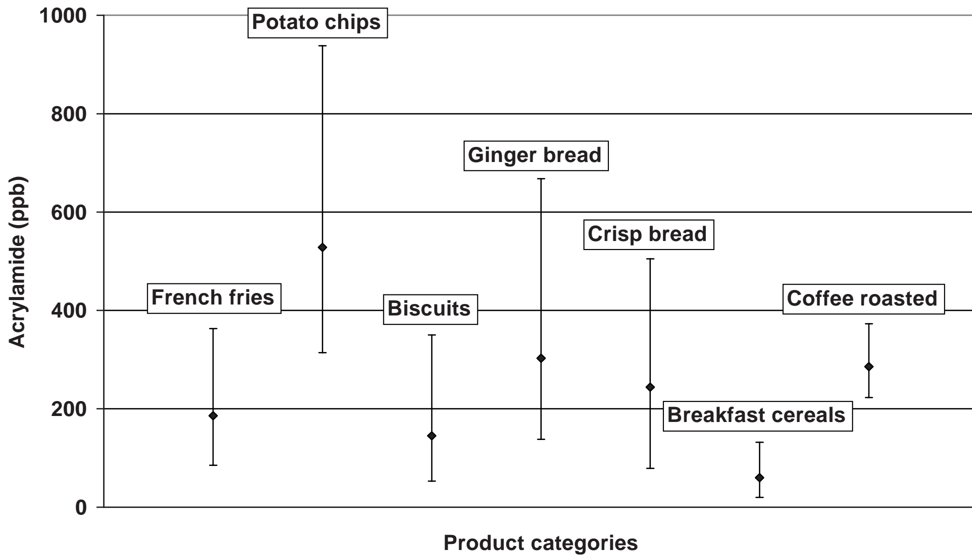


Fig. 4.1 Acrylamide content (ppb) of several typical food categories; median (diamond), 25th and 75th percentile values (bar) are illustrated in Ref. [4].

significant colour and flavour developments in fried and baked starchy products. While the relative importance of different intermediates in acrylamide formation is still discussed, the basic pathway, as generally accepted, is illustrated in Fig. 4.2.[10–13,15–18] Water content, temperature and pH have been identified as critical parameters for acrylamide formation,

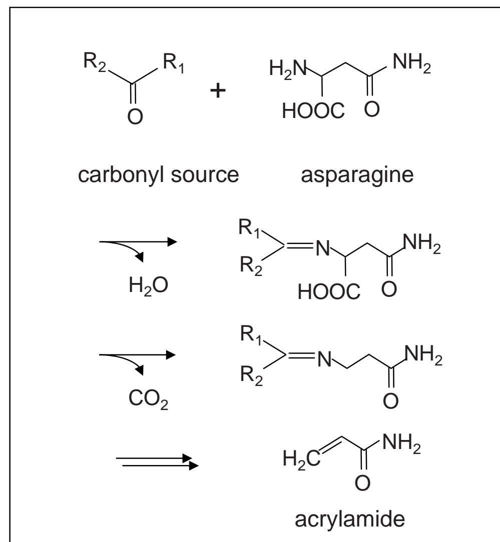


Fig. 4.2 Schematic overview of acrylamide formation from a reducing sugar and the amino acid asparagine.

that is low water content and high temperature favour formation and low pH reduces formation.[15] Several alternative formation pathways have been identified but the general agreement is that these are of far lesser quantitative importance.[15,17,19,20] An overview of studies of acrylamide formation and the major contributing factors has been given by Zhang and Zhang.[21]

As removal of acrylamide from prepared food products is impractical, reduction strategies have focused on limiting the formation of acrylamide. Due to the high number of affected product categories, the differences in acrylamide levels in these products and the broad variety of parameters influencing formation, a range of methods and strategies have been developed.[4,6,22]

For cereal food products, moisture content and thermal input (time/temperature) play a key role in acrylamide formation. The CIAA toolbox 2007[22] suggests the possibility of lowering acrylamide levels by baking products to achieve less colour development but the same final moisture content as is found in traditional products by utilizing lower baking temperatures for longer times. Konings *et al.*[23] and Ahrne *et al.*[24] found 75% reduction of acrylamide in crisp bread when baking temperature and oven speed were reduced. Other technologies for reduction of acrylamide include adding amino acids, which compete with asparagine in Maillard reactions, adding Ca^{++}, lowering pH, optimizing ingredients and carefully selecting raw materials to favour those low in acrylamide precursors.[22] By lowering pH, the rate of formation of key Maillard reaction intermediates is decreased. However, even though significant acrylamide reduction has been reported in literature, addition of acids is probably of limited utility due to the development of acidic off-flavours, and potentially insufficient browning and leavening of biscuits.[23,25,26] An example of ingredient optimization is the replacement of ammonium bicarbonate by other suitable leavening agents, such as sodium bicarbonate. Ammonium bicarbonate enhances acrylamide formation by indirectly catalyzing the formation of sugar fragments, hereby generating more reactive carbonyls that can take part in the Maillard reactions.[4,27,28] Amrein *et al.*[25] reported that the combined use of sodium bicarbonate and sodium acid pyrophosphate (SAPP) in hazelnut biscuits led to a 50% reduction in acrylamide compared to the use of ammonium bicarbonate. Raw material control mainly focuses on low asparagine content, since this is the limiting factor for acrylamide formation in cereal products.[29] Asparagine content in wheat flour differs widely between varieties and harvests and with whole wheat and fibre content. Studies have shown that more asparagine is found in high fibre flours (i.e. flours with higher ash content),[30] resulting in increased acrylamide levels in final products.[31,32]

For potato-based products, several studies have shown that the sugar content of the tuber correlates directly with the acrylamide concentration in the final products.[27,28–36] Controlling sugar levels is therefore currently the primary measure employed by the industry to reduce acrylamide levels in chips and French fries. This is done by selecting potato cultivars with low reducing sugar content and by controlling storage conditions from farm to factory. Optimal storage temperature is between 8°C and 10°C, while storage at lower temperatures leads to significantly increased levels of reducing sugars. Future concepts include breeding new potato varieties with low reducing sugar and asparagine content. An enzymatic solution focusing on removal of the reducing sugars has been suggested but is complicated by the fact that so many different sugars can participate in the reaction. Reported product recipe changes for potato products comprise adding competing amino acids, lowering pH and dipping in Ca^{++}-solutions.[4,37–40] Relevant process changes include decreasing cooking temperatures or cooking duration, where especially vacuum frying has proven very efficient for reducing

acrylamide levels in sliced potato chips.[41] Varying blanching regimes with respect to both time and temperature and introducing longer soaking treatments have been shown to reduce acrylamide by simply washing out the precursors.[22,38,39]

In coffee, no significant mitigation opportunities have yet been identified.[22] Acrylamide formation starts rapidly at the beginning of the coffee roasting, reaching a maximum level approximately halfway through the roasting cycle, at which point acrylamide levels start decreasing due to breakdown or depletion. Final levels in roasted coffee beans are approximately 20–30% of the initial maximum level. Consequently, darker roasting tends to give lower acrylamide levels. Also storing of packed, roasted coffee decreases acrylamide levels significantly. However, both freshness and degree of roasting are important quality parameters, meaning that mitigation based on these parameters is far from optimal. Trials on alternative roasting technologies are still in the exploratory phase.[22]

A drawback to some of the current acrylamide-reducing technologies is that these will not only affect acrylamide levels but also potentially impact final product characteristics, such as colour, texture and flavour, due to a general reduction in Maillard reactions.[23] Additionally, health or nutritional implications should be considered, for example loss of minerals and vitamins during intensive blanching of potatoes, and increased fat uptake upon frying at lower temperatures and longer times. For cereal products, replacement of ammonium bicarbonate with sodium bicarbonate will reduce acrylamide values, but at the same time increase sodium intake. Also, the use of refined flour instead of whole grain flour may reduce acrylamide levels, but will provide a less desirable product due to the lower fibre content.[22,23]

An alternative approach to a general reduction of Maillard reactions is to focus on removal or reduction in the level of the specific precursor, asparagine. This can be done by controlling raw materials and is furthermore the scope of various plant-breeding programmes. Another option is to apply the enzyme asparaginase, which catalyzes the hydrolysis of asparagine to aspartic acid and ammonia. This enzyme reduces the level of free asparagine while leaving other amino acids unaffected.

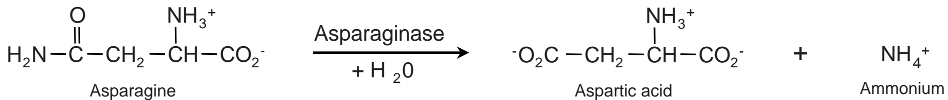
Initially, tests in simple chemical model systems containing only asparagine and glucose or fructose showed that asparaginase could significantly reduce acrylamide formation in these systems.[12,42] Subsequently, asparaginase has been tested in laboratory models of a variety of cereal food and potato-based food products. The resulting acrylamide reductions have ranged from 50% to 90%, suggesting that asparaginases could potentially be used to make a broad impact on dietary acrylamide exposure.[12,42–48] However, since commercial asparaginase products have only very recently become available at pricing and production scales appropriate to the food industry, asparaginase application technology is still very new. Published data are therefore limited and full-scale industrial experience scarce.

In coffee or other roasted products, a few patent publications have included treatments in which using asparaginase leads to a reduction of acrylamide formation during roasting. US patent 7 220 440[49] describes a process where asparagine is extracted from green beans using water. The extract containing solubles and asparagine is treated with an asparaginase and returned to the beans before roasting. In WO 2005/004620,[50] a process where cocoa beans are treated in an asparaginase solution before roasting is described. In both cases, an acrylamide reduction exceeding 10% is obtained.

This chapter will give an introduction to asparaginases, followed by a review of acrylamide reduction achieved by applying asparaginase to cereal and potato-based products and coffee. Advantages and potential limitations of using asparaginase will be illustrated and necessary considerations for enzyme application will be highlighted. Data shown and discussed are derived mainly from Novozymes laboratory testing.

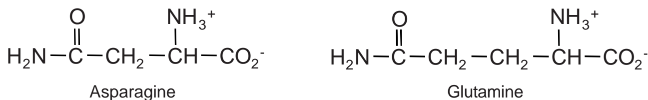
4.2 ASPARAGINASE

Asparaginases (L-asparagine amidohydrolases, EC 3.5.1.1) catalyze the hydrolysis of the amide group of the side chain of asparagine to produce aspartic acid and ammonium. Asparaginases are broadly distributed among living organisms, including animals, plants and microbes.[51,52] Asparaginases participate in basic amino acid catabolism by shuttling asparagine to aspartic acid which, after subsequent conversion to oxaloacetate, can enter the citric acid cycle.[53] In plants, asparagine is used for nitrogen storage and transport; thus asparaginases play a major role in utilization of this essential resource.[54]



Despite their common role in amino acid metabolism, the breadth of asparaginase diversity is not particularly well studied. Borek and Jaskólski[52] compared a handful of protein sequences known to encode asparaginases and concluded that asparaginase activity is found in three distinct and unrelated protein families, which they called Bacterial-type, Plant-type and *Rhizobium etli*-type. These three families correspond to the three asparaginase families currently recognized in the pFam protein family database as Pf00710, Pf01112 and Pf06089.[55] Family Pf00710 includes most of the asparaginases known from bacteria, archaea and fungi and a few animal asparaginases.[56,57] Pf01112 includes plant asparaginases and glycosylasparaginases from a variety of organisms. Pf06089 is a smaller and less-studied family that includes only one characterized enzyme from *R. etli*. The asparaginases described in the rest of this section, where sequences are known, belong to the Pf00710 family.

Many enzymes with asparaginase activity are quite specific for asparagine, but also have a low level of activity towards glutamine, which is very close in structure to asparagine.[58,59] Enzymes that are active on both amino acids but prefer glutamine as a substrate are called glutaminase-asparaginases (EC 3.5.1.38). Crystallographic analyses of microbial asparaginases and glutaminase-asparaginases have shown that they share the same basic structure and catalytic mechanism.[60,61]



Some microbes produce a periplasmic or extracellular asparaginase or glutaminase-asparaginase in addition to the intracellular asparaginase that participates in basic metabolism.[62–65] In the few cases where both enzymes have been studied, the two forms of asparaginase are similar in primary amino acid sequence and protein structure, but differ in other characteristics like pH and temperature activity profiles and, most notably, in substrate affinity. Secreted microbial asparaginases typically have a much higher affinity for asparagine than their cytoplasmic counterparts, probably reflecting the need for a pool of asparagine in the cytoplasm to support protein synthesis. The lower substrate affinities of cytoplasmic asparaginases could function to accommodate asparagine turnover without depleting asparagine supply.[64,66,67] The role of the non-cytoplasmic asparaginases is not known, though there is some evidence that they are involved in obtaining carbon and nitrogen

for the cell.[68] The extracellular asparaginase from *Saccharomyces cerevisiae* accepts a broader range of substrates than the cytoplasmic form, consistent with this suggested role.[64]

Among asparaginases, the periplasmic asparaginase from *Escherichia coli* (known as EcA-II and a variety of other names) is especially well studied because of the discovery in the 1960s that it could be used to treat acute lymphocytic leukaemia. Malignant cells of this leukaemia lack the ability to synthesize adequate amounts of asparagine, and their survival is dependent on utilizing asparagine from the blood serum. Asparaginase is therefore used to deplete the blood serum for asparagine and deprive leukemic cells of this amino acid.[69]

EcA-II can hydrolyze L-asparagine, D-asparagine and L-glutamine, but shows a strong preference for L-asparagine as a substrate. The maximal reaction rate on L-glutamine is only 3% of that on L-asparagine. EcA-II does not show product inhibition, except by ammonium at pH above 8.5.[70] Analysis of a very similar asparaginase from *Erwinia carotovora* has shown that the catalytic mechanism of these enzymes requires a free carboxyl group. Free asparagine and glutamine and their derivatives can be hydrolyzed, as well as carboxyl-terminal asparagine residues in small peptides. Larger peptides and C-terminal glutamines are not accepted as substrates.[51] The crystal structure of EcA-II has been determined, demonstrating that EcA-II functions as a tetramer. The tetramer has been described as a 'dimer of identical intimate dimers' in which two active sites are formed at the intimate dimer interface, and assembled tetramers have four active sites.[71] Structural determination of a number of additional bacterial asparaginases and glutaminase-asparaginases has shown that they function in similarly assembled tetramers.[67] A single asparaginase, from a hyperthermophylic archeaon, has been shown to function as a dimer.[61]

EcA-II is currently used as a component of chemotherapeutic treatment and is commercially available. It has been used in a number of studies testing the effectiveness of asparaginase in the reduction of acrylamide in food products,[12,44–46] but the expense of this enzyme prohibits widespread use.

Two enzymes have been developed and commercialized specifically for the purpose of acrylamide reduction, DSM's PreventASe™[72] and Novozymes' Acrylaway®.[73] Both enzymes are extracellular asparaginases that have been cloned from GRAS (generally recognized as safe) fungi which are commonly used for industrial enzyme production. Acrylaway was found by analyzing gene sequences from *Aspergillus oryzae* and is produced recombinantly in an *A. oryzae* host.[48] PreventASe was developed in a similar fashion from *Aspergillus niger*.[74] The extracellular asparaginases from *A. oryzae* and *A. niger* share sequence similarity with EcA-II from *E. coli*, and belong to the Pf00710 protein family, but differ in pH activity profiles. The enzyme from *A. niger* has an acidic profile, while the enzyme from *A. oryzae* has an optimum closer to neutrality.[48,75] The *A. oryzae* asparaginase, which was used for the examples in this chapter, is quite specific for asparagine and has only trace activity on glutamine. Size exclusion chromatography experiments on native *A. oryzae* asparaginase suggest that it also functions as a tetramer, with a size of approximately 255 kDa.[76]

4.3 ACRYLAMIDE ANALYSIS

Several methods for acrylamide analysis have been established and validated in recent years.[4,77,78] As many different food matrices are involved, sample pretreatment and preparation tend to vary, while the actual detection and quantification of acrylamide is most often done using mass spectrometry (MS). Samples are thoroughly homogenized and

extracted preferably using water, but other solvents have also been tested and implemented. In some set-ups, samples are defatted before extraction. The aqueous extract is cleaned up by combining different solid phase extractions and analyzed directly by liquid chromatography (LC–MS/MS) or gas chromatography (GC–MS). If using GC–MS, derivatization of acrylamide (bromination) can increase sensitivity and selectivity.[4,77–79] Several commercial analytical laboratories have set up analysis for acrylamide quantification in food products and are now routinely offering these. Reproducibility of the applied methods has been assessed by interlaboratory proficiency tests, which revealed relatively large tolerances in the various matrixes, for example 836–1590 $\mu g\ kg^{-1}$ in the same sample of crisp bread, reflecting the differences in methodology.[80]

4.4 APPLICATIONS

Implementing an asparaginase treatment broadly into a range of food products is not a simple undertaking, since for each product the food matrix or components may influence enzyme action and reactivity. Furthermore, the range of food products in which acrylamide formation might be remediated by asparaginase treatment varies greatly from dough-based products such as biscuits, crisp bread, crackers, cereal and potato-based snacks, to intact vegetable or cereal products such as French fries, sliced potato chips, breakfast cereals and coffee. The rate of enzymatic hydrolysis of asparagine is dependant upon physical process parameters, such as temperature, pH, water activity and time, as well as interactions among these parameters. In addition, the content of the precursors, free asparagine and reducing sugars, varies greatly in the affected food products, which will influence acrylamide formation and means that the reaction-limiting factor differs from product to product.

4.4.1 Application testing in cereal food products

Cereal food products are characterized by a wide and heterogeneous range of recipes, raw materials, additives and processing conditions. The influence on acrylamide formation of different processing techniques, such as forming, mixing and baking, or different recipe parameters is not fully understood.[4,23]

A great variety of biscuit types are manufactured today. These are classified based on characteristics such as the method of dough forming, enrichment with fat or sugar, or secondary processing. A major distinction between different types of biscuits is the development of the gluten network which is responsible for dough extensibility and cohesiveness. Short doughs are characterized by an undeveloped gluten network and show no shrinkage after cutting, while hard doughs are characterized by a developed gluten network that is elastic and extensible and tends to shrink. Hard doughs have relatively low fat and low sugar content and high water content which will facilitate the gluten network development. Long shelf life bread substitutes, crisp biscuits and semisweet biscuits (e.g. tea biscuits, cocktail crackers) and snacks (e.g. savoury crackers, pretzels) are included in this category. Hard doughs are widely found in industrial processing and are typically produced by sheeting and cutting. Short doughs usually have higher fat and sugar content and therefore low water content, giving little gluten development. Examples are ginger biscuits and digestives.[81]

In the following section, the results of asparaginase usage in several hard dough and short dough products are shown. The examples were chosen to cover a range of recipes

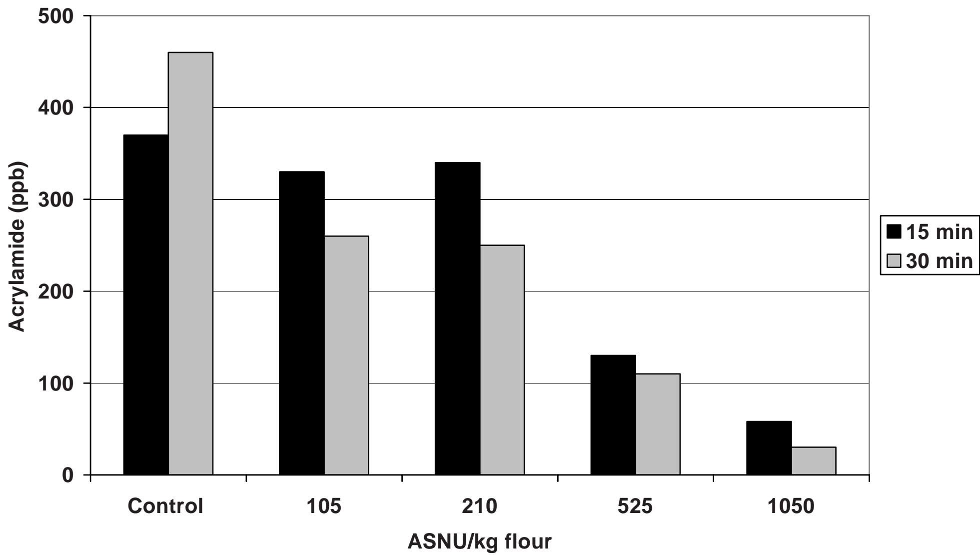


Fig. 4.3 Acrylamide level in semisweet biscuits as a function of asparaginase dosage (105–1050 ASNU kg^{-1} flour) and dough resting times (15 and 30 min at 40°C).

while focusing on the influence of processing conditions such as temperature, holding time, combination with other enzymes and water content.

4.4.1.1 *Semisweet biscuits*

Semisweet biscuits were used as a standard recipe from the hard biscuit range to obtain proof of concept for acrylamide reduction using asparaginase from *A. oryzae*. The biscuits were prepared on laboratory scale with variations in dough holding times and enzyme dosages.[48] Results are shown in Fig. 4.3 and images of the final biscuits in Fig. 4.4.

The differences in the control values are caused by experimental variation during processing and baking and analytical variations rather than the different holding times. It is not likely

Fig. 4.4 (a) Semisweet biscuit prepared in lab bakery: control, no asparaginase addition. (b) Semisweet biscuit prepared in lab bakery: asparaginase treated.

that acrylamide formation will depend on dough resting times except in yeast raised doughs, where the yeast can typically ferment the asparagine. Similar observations were made by Amrein *et al.*[44] who reported that the relative standard deviation of acrylamide content in reference samples was 16% in gingerbread trials.

In the samples treated with asparaginase, a significant reduction of acrylamide in the final biscuits was observed compared to the control. Samples with higher asparaginase dosage had lower acrylamide levels at the same holding times. At 15-min holding time, reductions of approximately 20% for 105 asparaginase units (ASNU) kg^{-1} flour and approximately 85% for 1050 ASNU kg^{-1} flour were seen. Increasing the resting time to 30 min also increased acrylamide reduction to approximately 35% and 90%, respectively. No differences in the texture and appearance were seen for the enzyme-treated sample compared to the control (see Fig. 4.4). Similar trials were carried out on industrial scale and acrylamide reductions up to 90% were achieved (data not shown).

For semisweet biscuits, sodium metabisulphite (SMS) is often used as dough relaxer to avoid shrinkage after cutting. For some recipes, proteases are used instead of SMS or in combination with SMS.[81] To investigate the potential influence of a protease on the effect of the asparaginase, both enzymes were tested in semisweet biscuit dough. The asparaginase and a neutral protease, Neutrase® (Novozymes A/S), were added during mixing. Acrylamide content in the final products is shown in Fig. 4.5. In the control only protease was added. Successful reduction of acrylamide of approximately 80% was achieved, illustrating that the combined use of asparaginase and Neutrase is indeed possible without negative effects of the protease on asparaginase. The 80% reduction corresponds well to the result achieved in the previous trial on semisweet biscuits (Fig. 4.3), even though the acrylamide level in the sample made with protease alone is somewhat lower than the other control (Fig. 4.3).

Additionally, Graham crackers and cheese crackers were tested for enzymatic acrylamide reduction at the American Institute of Baking (Manhattan, Kansas, USA) (data not shown).

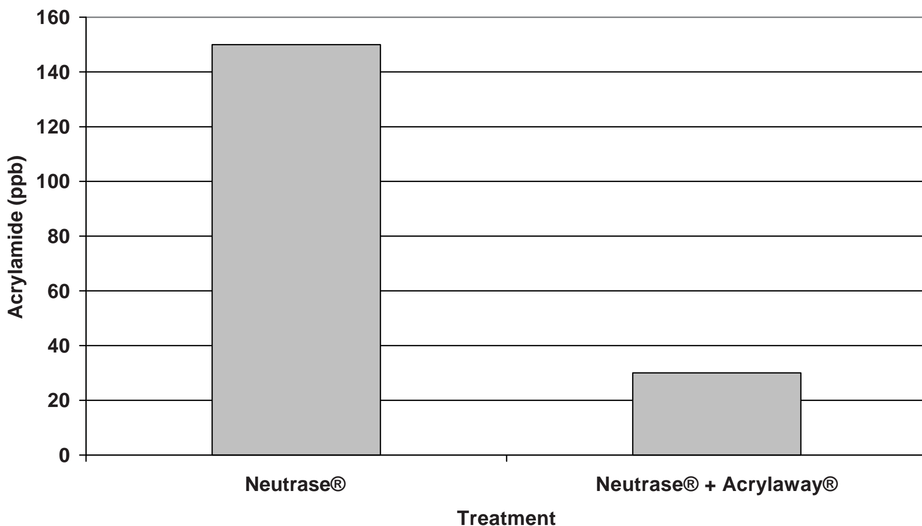


Fig. 4.5 Combination of asparaginase and a neutral protease Neutrase®. Enzyme dosages were 1000 ASNU kg^{-1} flour of asparaginase and 0.225 AU-NH/kg flour of protease in a semisweet biscuit recipe. Dough holding time was 15 min at 40°C and 86% relative humidity.

For Graham crackers, a reduction of 60% at a dosage of 500 ASNU kg^{-1} flour and 87% at 1000 ASNU kg^{-1} flour was seen starting from an acrylamide value of 1040 ppb in the control sample. Further increases in asparaginase dosage up to 5000 ASNU kg^{-1} flour did not improve acrylamide reduction significantly. The observed reduction level is thus comparable to that achieved in semisweet biscuits, even though the acrylamide level in the untreated sample is higher in the Graham crackers, which can, for example, be attributed to higher asparagine content in whole wheat flour compared to refined flour. In cheese crackers, a 75% reduction of acrylamide was found at an asparaginase dosage of 500 ASNU kg^{-1} flour. The control level of 77 ppb acrylamide was low compared to semisweet biscuits and Graham crackers, which again is caused by ingredient variations in the recipe and differences in sheeting thickness or in final baking. An enzyme dosage of 500 ASNU kg^{-1} flour resulted in a maximum effect in these crackers. Higher dosages were tested without improving the effect.

Acrylamide reductions between 75% and 87% for Graham and cheese crackers confirm the findings for semisweet biscuits showing reductions up to 90%. Similar results were reported by Vass *et al.*,[45] who observed acrylamide reductions in wheat crackers of approximately 85% after asparaginase treatment. Amrein *et al.*[25] investigated acrylamide reduction using asparaginase as a function of enzyme dosage and incubation time in hazelnut biscuits. Acrylamide reduction of up to 90% was seen in these trials.

4.4.1.2 *Pretzels*

Asparaginase treatment of cold extruded pretzels was tested at Reading Bakery Systems (Reading, Pennsylvania, USA). After extrusion, the pretzels were passed through a cooker with 1% sodium hydroxide before final baking. Results are shown in Fig. 4.6.

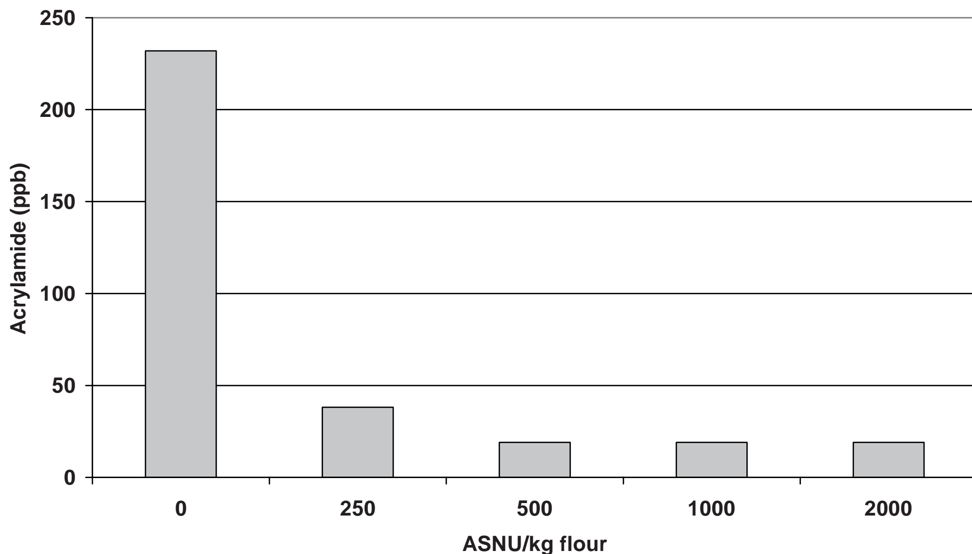


Fig. 4.6 Pretzels produced at Reading Bakery Systems: enzyme dosage was 250–2000 ASNU kg^{-1} flour.

Again a dose response was seen with maximum reduction of more than 90% at 500 ASNU kg^{-1} flour. Further increases in dosage did not result in improved acrylamide reduction in the final pretzels.

4.4.1.3 *Crisp bread*

Crisp bread is a flour-based product made from a dough having a very high water content and held at a low temperature (6°C) during production and handling. Typically rye flour or whole meal flour is used for production.[81] Crisp bread trials were carried out at lab scale as previously described.[48] The water content was limited to 65 bakers% (% w/w flour) as higher water content could not be handled in the laboratory sheeting. The activity of the *A. oryzae* asparaginase is determined at 37°C. Lower temperatures are expected to reduce the activity of the enzyme significantly.[48] To investigate the potential influence of dough temperature, trials were made at 10°C, 15°C and 20°C, keeping a relatively high and constant asparaginase dosage of 2100 ASNU kg^{-1} flour. Compared to semisweet biscuits, where the optimal dosage is 500–1000 ASNU kg^{-1} flour, the high dosage was chosen to compensate for the reduced enzyme activity at low temperatures. Two dough resting times were tested, 30 and 60 min.

Acrylamide values in the control samples were 910 ppb (30 min) and 740 ppb (60 min). Variations in control values were also seen for semisweet biscuits and are related to processing and analytical variations. In asparaginase-treated samples, acrylamide reduction of 80–90% was achieved even at the lowest dough temperature of 10°C, showing that the higher enzyme dosage was adequate at low temperature. No effect of resting time was found. Asparaginase performance was better than expected at such low temperatures, possibly because the high water content in the recipe positively influences the enzyme–substrate interaction. Trials carried out on industrial scale showed a reduction of approximately 50% acrylamide in the final product (results not shown).

4.4.1.4 *Ginger biscuits*

Ginger biscuits are classified as a short dough biscuit with a high sugar and fat content. Trials for ginger biscuits were carried out as previously described.[48] In Fig. 4.7, the acrylamide content in ginger biscuits, made from dough with varying water content and either with or without asparaginase, is illustrated. The water content in the recipe was calculated as the sum of added water and the water content naturally present in the ingredients.

Acrylamide levels measured in the control biscuits with different water contents varied between 530 and 640 ppb, while levels in the enzyme treated ranged from 60 to 530 ppb. The influence of water content in the enzyme-treated samples is clearly visible. By increasing water content from 11% to 19% in the recipe, acrylamide reduction in the final biscuits increased up to 90%. The negative influence of low water content is most likely related to limited enzyme–substrate contact caused both by improper mixing in such dry dough and by low diffusion rates. Amrein *et al.*[44] reported similar results when testing asparaginase from *E. coli* in ginger bread. Here, the acrylamide content was reduced by 55%, while approximately 75% of the free asparagine was hydrolyzed by the enzyme. Low mobility of the substrate and enzyme in the dough was given as an explanation for the incomplete hydrolysis and subsequent partial reduction in acrylamide.

To confirm lab experiments, external trials were carried out at the American Institute of Baking where ginger snaps (estimated to have approximately 18% water content) were

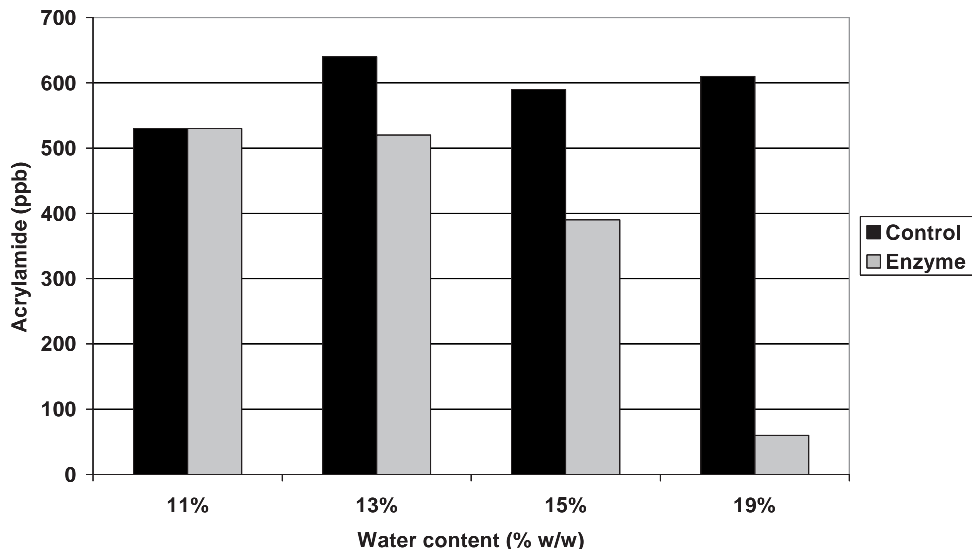


Fig. 4.7 Level of acrylamide in ginger biscuits made from dough of varying total water content (added + content in ingredients) and with or without asparaginase (1000 ASNU kg^{-1} flour). Dough was sheeted for biscuit preparation except for samples with calculated water of 11%, which had to be pressed in a form to make the final biscuit.

produced. Maximum acrylamide reduction achieved was 50% using an enzyme dosage of 2000 ASNU kg^{-1} flour. Increasing dosage had no additional effect.

Asparaginase from *A. niger* has been tested in different foods and similar reductions of up to 80% in crackers and biscuits and up to 90% reduction in Dutch honey cakes have been reported. Process temperature, water content, incubation time, point of enzyme addition, recipe and free asparagine content are reported as the main influencing factors that have to be considered for acrylamide reduction.[47]

4.4.1.5 *Aroma profiles*

For all trials done, no differences in final product characteristics regarding taste and appearance were noted. Crisp bread, crackers and semisweet biscuit samples produced at laboratory scale and pretzels produced at Reading Bakery Systems were analyzed for their volatile aroma components by headspace chromatography (Simec AG, Switzerland). The results showed no significant differences in occurrence and intensity of the volatile aroma peaks identified in the controls and asparaginase-treated samples. This indicated that the enzyme treatment had not influenced aroma and taste of the final product. Amrein *et al*.[44] also found that taste and colour of ginger bread was not affected by asparaginase treatment. These results are consistent with the assumption that by the selective hydrolysis of asparagine, acrylamide formation is reduced, but at the same time other amino acids and reducing sugars are left unaffected and can participate in Maillard reactions.

4.4.1.6 *Conclusions – asparaginase in cereal food products*

To summarize, asparaginase can be successfully applied for acrylamide reduction in a range of cereal-based recipes without changing the taste and appearance of the final product.

Asparaginase has been shown to work in both hard and short doughs, and at temperatures between 10°C and 40°C. Acrylamide reduction of 50–90% was seen for the different recipes and process parameters. For cereal food applications, enzyme performance is dependant upon processing conditions, for example temperature, pH and resting time, and most importantly water availability. Within biscuit types, acrylamide reduction seems to be generally lower in short dough biscuits in comparison to hard dough biscuits, which is probably an effect of the low water content in short dough recipes. Recommended dosage must therefore be determined and optimized for each individual application. Complete acrylamide removal can probably not be achieved due to low enzyme and substrate mobility in recipes with low water content. Furthermore, alternative asparagine-independent formation pathways may contribute small amounts of acrylamide to the final product.

4.4.2 Application testing in potato products

4.4.2.1 *Introducing asparaginase in potato processing*

Potato-based products are among the food products containing the highest levels of acrylamide. This is directly correlated to the very high level of free asparagine naturally present in potatoes compared with cereals and grains, for example 1–7 mg g^{-1} potato fresh weight versus 0.1–0.3 mg g^{-1} flour.[79,82] Levels of acrylamide in commercial potato products vary considerably, reflecting huge differences in both raw materials as well as in processing. Reported levels in French fries range from 5 to 4653 ppb, with a median of 186 ppb, and levels in sliced potato chips range from 5 to 4215 ppb, with a median of 528 ppb.[5] Products reaching the maximum levels are, however, rare as illustrated by the much lower 75th percentile values for the same products: 363 ppb for French fries and 938 ppb for potato chips (see Fig. 4.1).

Compared with dough-based products, where introduction of an exogenous enzyme is relatively simple, French fries and potato chips consist of intact pieces of potato, making contact between enzyme and substrate far from optimal and an enzyme treatment potentially much more complicated. An important initial consideration is whether it is possible for an enzyme like asparaginase to passively penetrate a potato piece, or if asparagine must diffuse out of the cells to an externally applied enzyme. Experiments in apples have shown that when soaking apple cubes in a solution containing pectin methyl esterase, enzyme penetrated only the superficial zone, while enzyme was also found in the core zone when aided by vacuum impregnation.[83] Pore sizes in plant cell walls are estimated to be approximately 3.5 nm meaning that only smaller molecules can penetrate the cell wall. Since the cell membrane will prevent non-specific protein uptake, molecules that have accessed the cell wall will stay in the intercellular spaces irrespective of size.[83] For two known marker proteins, a Stokes radius of 3.55 nm corresponds to a molecular weight of 66 kDa (bovine serum albumin) and of 2.73 nm to 43 kDa (ovalbumin). As the *A. oryzae* asparaginase has an estimated native size of 255 kDa, very little, if any, enzyme will be able to enter a typical plant cell. Therefore, asparagine diffusion out of the cells must instead be facilitated by disruption of the cell wall/cell membrane to improve enzyme–substrate contact in an intact piece of potato. This can be achieved by, for example, heat treatment, as in blanching.

A schematic presentation of the reactions happening in a potato strip during frying is shown in Fig. 4.8. Under typical conditions, the core temperature will not exceed 105°C due to the high water content in the interior, while the surface temperature is much higher and dependant upon oil temperature.[84] Since acrylamide is mainly formed at temperatures above 120°C and at low water content, highest acrylamide levels will be expected to form in the surface region. This was confirmed by Gökmen *et al.*[84] who found up to 17 times

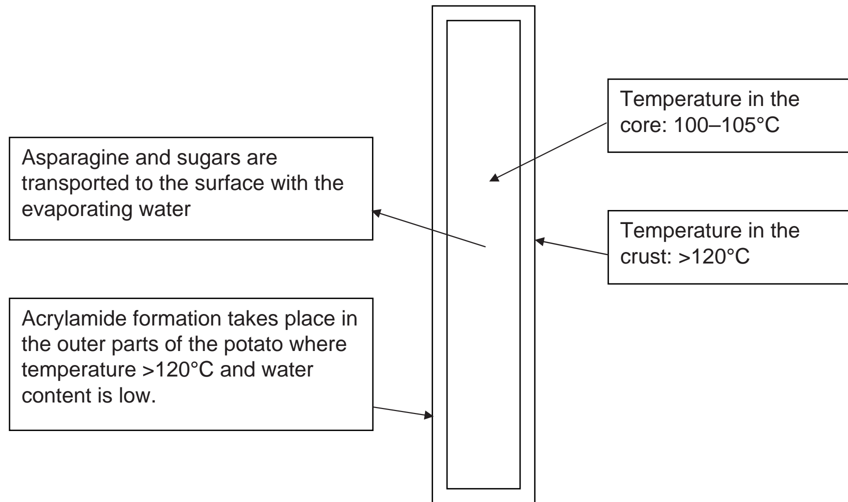


Fig. 4.8 Reactions in a potato strip during frying at oil temperatures >150°C. The surface layer is estimated to be approx. 2 mm (drawing is consistent with results in Gökmen *et al.*[84]).

more acrylamide in the surface than in the core when frying at 190°C. Based on this model, depleting or reducing asparagine in the outer layer would be expected to hinder or at least reduce acrylamide formation quite significantly. However, identifying the action radius of an externally applied enzyme and comparing this to the acrylamide formation zone is not simple and remains speculative. Also, since diffusion of asparagine is a dynamic process, fresh asparagine is very likely transported from the core towards the surface along with evaporating water evolved during drying and frying.

4.4.2.2 *Industrial production of French fries*

In addition to the theoretical considerations concerning enzyme–substrate interaction in the treatment of an intact potato strip, practical considerations have to be made regarding the production process. A typical industrial French fry process is illustrated in Fig. 4.9.

Potatoes are initially washed, sorted, steam peeled and cut. Following cutting, the potato strips are blanched in 2–3 sequential steps typically at 65–85°C for 10–30 min. Blanching is done to inactivate the endogenous enzymes in the potato, to partially cook the potato and to leach out reducing sugars in order to prevent excessive browning of the final product. After blanching, the potato strips are quickly dipped in a warm phosphate salt solution (SAPP) to prevent greying of the final product. The dip is optionally combined with a dip in glucose to control the final colour. The potatoes are dried in a drier with hot circulating air at 75–95°C for 5–20 min giving a weight loss of 5–25%. Finally, the potato strips are par-fried before being quick-frozen and packed. Final frying is done at the restaurant or by consumers.

4.4.2.3 *Lab-scale testing of asparaginase in French fries*

The most simple and direct application point for an enzyme in the French fry production process would be during blanching, where the peeled, cut potato strips are held in hot water for 10–30 min, giving an enzyme a sufficient span of time for action. Unfortunately, typical

(a) Industrial process	(b) Lab set-up
Control and sorting	
Steam peeling	Hand peeling
Cutting	Cutting
Sorting – size, quality	
Blanching, 10–30 min, 60–85°C	Blanching, 4 min, 85°C+10 min, 70°C
SAPP and glucose dip 30–60 s	Dip in water or enzyme, 60 s, 40–55°C
Drying, 10 min, 85°C	Drying, 10 min, 85°C
Par-frying, 45–60 s, 175–195°C	Par-frying, 60 s, 175°C
Freezing	Freezing
	Frying, 2.5–3 min, 175°C
	Acrylamide analysis

Fig. 4.9 (a) Industrial production of French fries.[85] The par-fried frozen product is packed and shipped to the customer where final frying is done. SAPP: sodium acid pyrophosphate. (b) The set-up used for lab-scale production of French fries.

blanching temperatures are too high for standard asparaginase treatment, meaning that an extra process step at a lower temperature has to be introduced in the process. For initial testing in lab scale, the enzyme treatment was therefore established as an extra soak in an enzyme bath at 40–55°C that follows blanching and is just prior to drying (SAPP and glucose dips were not included). A 20-min treatment time was used in the enzyme bath, resulting in acrylamide reductions of up to 85%.[48] However, introducing 20 min of extra processing time in an industrial full-scale operation is far from optimal, so an alternative set-up using a short time dip treatment (1 min, 40–55°C) and exploiting the holding time in the drier (10 min, 85°C) for enzyme action was tested instead. This 1-min additional process step is basically identical to the dip in SAPP and glucose currently used in the industry, except for a lower temperature.

Results from a short dip treatment can be seen in Fig. 4.10. As shown, acrylamide levels are reduced as the enzyme dosage is increased, reaching a maximum effect at 10 500 ASNU L^{-1} of 59% reduction versus the control sample. A short dip in water (sample 0) also reduced the acrylamide level but only by 26%. One sample was soaked in water for 20 min instead of 1 min to see if the longer soaking time could potentially bring down the acrylamide level further. Acrylamide reduction reached 58%, showing a significant effect of the longer time in water. The longer water soak works by washing out more sugar and asparagine from the potatoes. Similar results have been seen in other studies where prolonged blanching or soaking was shown to result in 25–70% reduction, depending upon treatment temperature and time.[35,38,39] By comparing treatment results in Fig. 4.10, it can be seen that a 1-min dip at the highest asparaginase dosage gave an acrylamide reduction (59%) comparable to that achieved with a 20-min soak in water (58%). This illustrates that the time required for a certain level of reduction is decreased significantly in the presence of asparaginase.

During the short dipping treatment, the asparaginase will reduce the asparagine level in the bath directly, which can potentially improve washout of asparagine. In addition, the

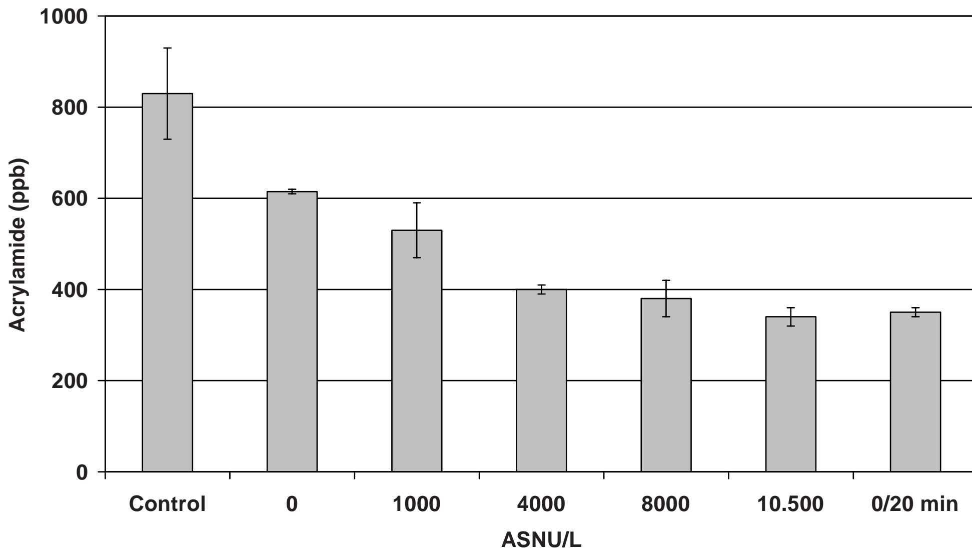


Fig. 4.10 Acrylamide levels in French fries made from blanched potato sticks that were given no treatment (control), a 1-min dip in water (0) or enzyme solution of increasing dosage (1000, 4000, 8000, 10 500 ASNU L^{-1}). 0/20 min: Blanched potato sticks soaked in water for 20 min instead of the 1-min dip. Bars indicate minimum and maximum of duplicate samples. Experimental conditions: enzyme bath: 1 min, 55°C, drying: 10 min, 85°C.

enzyme is also active during the subsequent drying step. To illustrate this, an experiment comparing no holding time during drying and the standard 10-min drying time was made. For the samples having no holding time during drying, the treated potatoes were instead dried using a paper towel. Acrylamide levels after a 1-min dip in water or in enzyme solution followed by paper towel drying and frying were the same (500 ppb and 491 ppb), showing no effect of the enzyme treatment and demonstrating that the 1-min holding time in the bath alone was insufficient for any significant enzyme action. When the enzyme 'coated' potato strips were held for 10 min in the drier (a heating cabinet) following the dip, a clear effect was seen, with a reduction in acrylamide levels of 35% when compared to the similar dip in water (433 vs. 668 ppb acrylamide). In this experiment, the control sample had 927 ppb acrylamide, and the treated sample with standard drying had 433 ppb acrylamide, giving a 53% reduction, while the similar dip in water resulted in 668 ppb acrylamide, equivalent to a 28% reduction. These levels are similar to the results shown in Fig. 4.10.

4.4.2.4 *French fry pilot-scale testing*

Asparaginase treatment of French fries was also tested on larger scale in a pilot potato processing facility at the University of Idaho. Results are shown in Fig. 4.11. Acrylamide levels were decreased significantly by the enzyme treatment, dropping from 960 ppb in the control sample to below 200 ppb. No significant differences in acrylamide levels were found for the different enzyme dosages tested (5000 or 7500 ASNU L^{-1}) or the dip times (0.5, 1 or 3 min). The insignificance of dip time confirmed the importance of the subsequent drying step as the time interval for the actual enzyme action. The reductions achieved ranged from

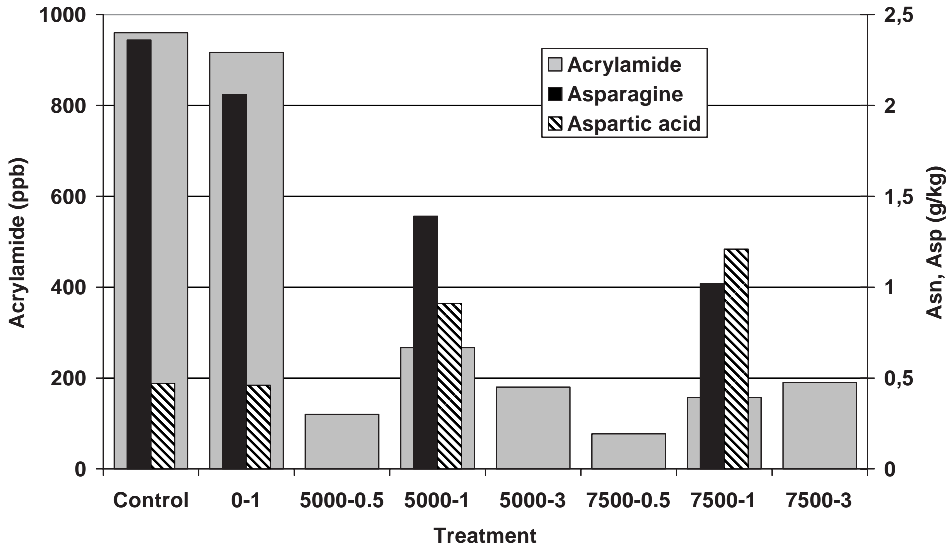


Fig. 4.11 Acrylamide, asparagine (Asn) and aspartic acid (Asp) levels in French fries made from blanched potato sticks dipped in water for 1 min (0-1) or in enzyme solution (5000 or 7500 ASNU L^{-1}) for 0.5, 1 or 3 min (−0.5, −1, −3) at 60°C. Control: no treatment. Results were obtained from a French fry production trial in pilot scale (batch size 10 kg). Blanching was done in two steps: 5 min at 90°C followed by 10–15 min at 70°C. Potatoes were then cooled to 60°C and dipped in the enzyme bath for 0.5, 1 or 3 min. Enzyme dose was 5000 or 7500 ASNU L^{-1}.

72% to 88%, with an average of 83% across dosages and dip times. The 1 min dip in water resulted in only 4% reduction in acrylamide.

Asparagine and aspartic acid were analyzed in selected samples of the final French fries. As seen in Fig. 4.11, asparagine was reduced by around 50% in the 7500 ASNU L^{-1} min sample measured against the control. Aspartic acid increased in parallel, even though final levels of aspartic acid were lower than what would be expected from converted asparagine. The finding that not all asparagine was hydrolyzed possibly reflects the fact that the majority of enzymatic activity was on the surface or in the outer layers of the potato strip, while the asparagine and aspartic acid analyses were done on the entire French fry. The calculated asparagine reduction was therefore an average achieved over the whole potato strip. The actual asparagine reduction in the outer layers, where acrylamide is primarily formed, was probably higher, which is also consistent with the higher acrylamide reduction achieved. Pedreschi *et al.*[43] have tested the asparaginase from *A. oryzae* in lab scale in French fries using a 20-min treatment time in the enzyme bath. Sixty-two per cent acrylamide reduction was observed, while asparagine levels measured directly in the treated potatoes showed a drop of 58%.[43]

4.4.2.5 Sliced potato chips

Compared with the production of French fries, production of sliced potato chips is a very fast process with a total processing time from raw potatoes to finished and packed product of 30–45 min. Major processing steps are illustrated in Fig. 4.12. The blanching step is optional and only included for potatoes that are high in sugar content to control final colour.

(a) Industrial process	(b) Lab set-up
Control and sorting	
Abrasive peeling	Hand peeling
Slicing	Slicing
Wash/rinse in cold water	Wash/rinse in cold water
Optional blanching, 80–85°C, 1–3 min	Blanching, 1 min, 85°C
	Enzyme treatment, 15 min, 40°C
Frying, 2.5–4 min, 175–190°C	Frying, 2.5 min, 180°C
Seasoning	Acrylamide analysis
Packaging	

Fig. 4.12 (a) Industrial production of sliced potato chips.[85] (b) The set-up used for lab-scale production of sliced potato chips.

Asparaginase treatment of sliced potato chips was tested on both un-blanched and blanched potato slices by introducing an extra processing step just before frying, as shown in Fig. 4.12. Holding (15 min) the blanched slices in water without enzyme gave an acrylamide level of 1686 ppb, which was reduced to 659 ppb when asparaginase was included in the bath. If the slices were not blanched prior to the enzyme treatment, no reduction in acrylamide was observed (water, 3500 ppb acrylamide; enzyme treatment, 3752 ppb acrylamide). The effect of a blanching treatment followed by a soak in water was thus significant compared to just a soak in water, resulting in 1686 ppb versus 3500 ppb acrylamide, corresponding to a 52% reduction. Similar results have been reported in several other studies.[38,39] As was observed for French fries, adding an asparaginase to the water increased the reducing effect significantly, achieving a maximum of 81% reduction (659 ppb vs. 3500 ppb acrylamide) in this experiment. The negative results obtained from treating the un-blanched slices confirm that some kind of pretreatment for opening the cell wall or cell membrane structure is required to release asparagine into the surrounding liquid hereby facilitating contact with the enzyme.

4.4.2.6 *Limitations on asparaginase performance*

To further investigate the rate-limiting step in the treatment of potato slices, a test was run with increased enzyme dosage and prolonged treatment time. From a production point of view, a long treatment time of up to 3 h is not an option since the final product completely changes its characteristics and can no longer be considered a chip. However, as a means of studying the system and defining the rate-limiting step, such tests can be valuable. Results are shown in Fig. 4.13. The control sample was blanched and thereafter fried directly, while all other samples were given a treatment in water or enzyme solution for 10 or 180 min after blanching and before frying. The effect of the longer holding time is obvious, especially for the enzyme-treated samples, where acrylamide levels as low as 30 ppb were reached. Very little effect was seen of increasing the enzyme dosage five times for neither the 10 nor the 180 min samples, showing that reaction time more than enzyme dosage is a limiting factor. This further indicates that the system is probably diffusion limited and controlled by

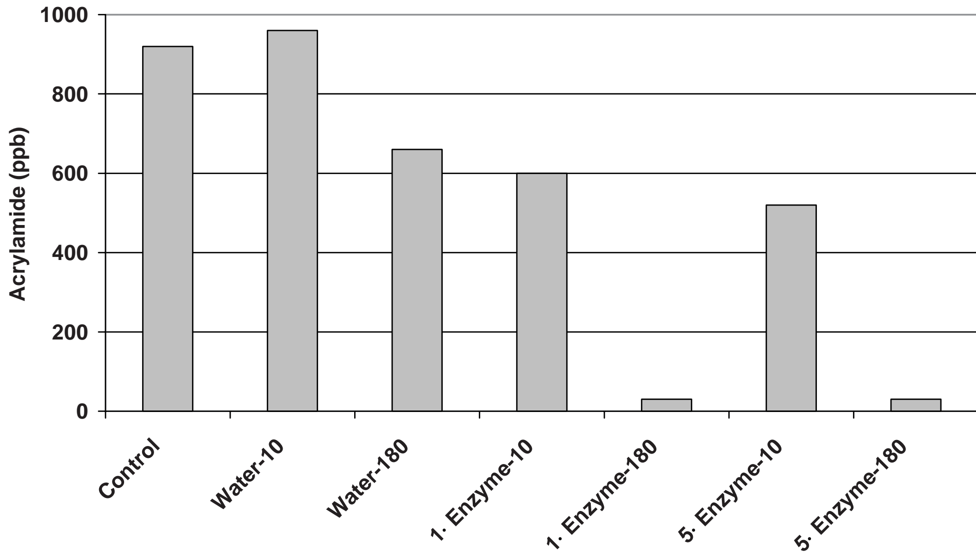


Fig. 4.13 Acrylamide levels in blanched sliced potato chips treated for 10 or 180 min (−10 or −180) at 50°C in water or enzyme solution. Enzyme dosage was 10 500 (= 1×) or 52 500 ASNU L^{-1} (= 5×). Control sample was just blanched. Blanching was done at 80°C for 1 min. Frying was done at 180°C for 3 min.

enzyme–substrate contact. As was also seen for treatment of French fries, the boosting effect of the asparaginase is obvious when comparing the Water-180 sample with the Enzyme-10 sample (Fig. 4.13). These two treatments give similar acrylamide reductions (660 ppb vs. 600 ppb acrylamide), but require very different lengths of time to achieve it.

Ciesarová *et al.*[46] have tested asparaginase from *E. coli* in roasted pancakes made from peeled and shredded potatoes and using both fresh potatoes as well as a dried potato premix. In the fresh potatoes, acrylamide reductions of 45–97% were achieved at a treatment time of 30 min and with increasing enzyme dosage, while in the dry premix reductions ranged from 70% to 97%. Reductions found by Ciesarová *et al.*[46] are thus in the same range or higher than the present results in French fries and sliced potato chips. The higher reduction in treated pancakes probably results from the longer treatment time and, more importantly, the much smaller size of the potato pieces, since these were shredded before being roasted. This will clearly facilitate enzyme–substrate contact.

4.4.2.7 Dough-based potato products

Fabricated potato chips or snacks are typically dough-based products, having dried potato flakes or granules as the major ingredient. Potato flakes and granules are both produced by drying potato mash, but processing and final product characteristics differ. Since dough is a much more homogenous system than intact potatoes, enzyme–substrate contact will be better. However, the low water content characteristic of many snack doughs will present a disadvantage by limiting diffusion.

In Fig. 4.14, results are shown from a test of asparaginase in fabricated potato chips. When chips were made with a dough water content of 40%, a very efficient reduction was achieved

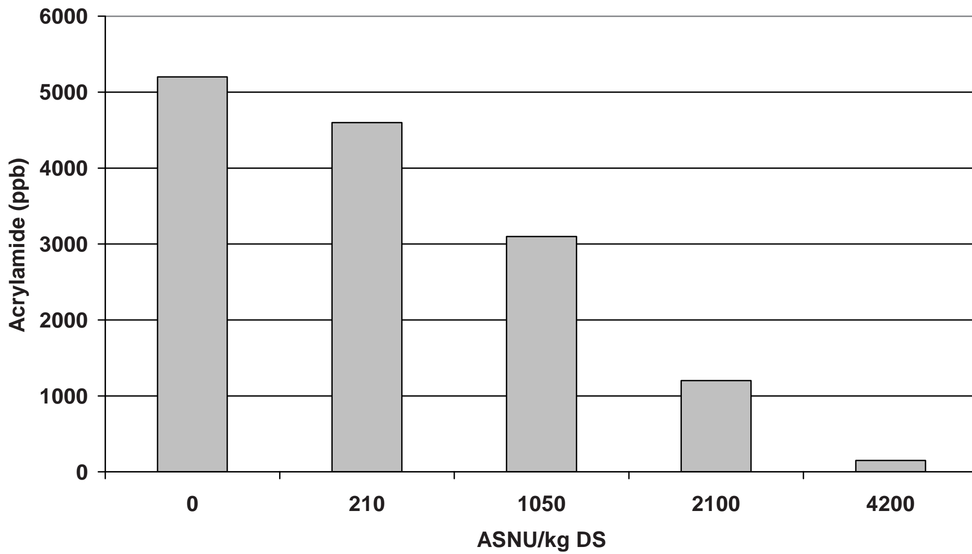


Fig. 4.14 Acrylamide in fabricated potato chips using increasing levels of enzyme in the dough. Dosages were 210–4200 ASNU kg^{-1} dry matter. Dough resting time was 45 min at room temperature.

(up to 95%) and a very clear dosage response was seen (Fig. 4.14). When chips were made at a lower water content of 33%, the maximum reduction achieved was 48% and this response was constant across dosages from 1000 to 5000 ASNU kg^{-1} flour (data not shown). Similar results identifying water content as a very critical parameter were seen in ginger nut biscuits (Fig. 4.7).

Potato-based snack pellets are an intermediate dried, extruded product made from dough using either potato flakes and/or granules. The snack pellets are fried, seasoned and packed at the final producer. Testing in snack pellets was done on industrial pilot scale without any process changes. Asparaginase was added with the water to the dry ingredients during mixing before the dough was extruded cold and dried. The snack pellets were then fried, and the final product analyzed for acrylamide. Results are shown in Fig. 4.15. Acrylamide reduction reached 95% at the highest enzyme dosage. These results show that the asparaginase worked very efficiently in a dough containing 35% water, which illustrates that enzyme performance depends very much on the specific recipe and probably on water activity more than water content.

In a study by Vass *et al.*,[45] addition of asparaginase (from *E. coli*) to a potato cracker dough resulted in 70% reduction in acrylamide in the final product, confirming the potential for such a treatment. Reduction in a similar wheat cracker product was higher (85%), presumably related to the much lower initial asparagine content in wheat compared to potato. Also *A. niger* asparaginase has been tested for acrylamide reduction in potato-based fabricated chips, showing up to 90% reduction.[47]

4.4.2.8 *Conclusion – asparaginase in potato-based products*

In summary, the results reviewed here show that it is clearly possible to significantly reduce final acrylamide levels in fried potato-based products by manipulating the asparagine level

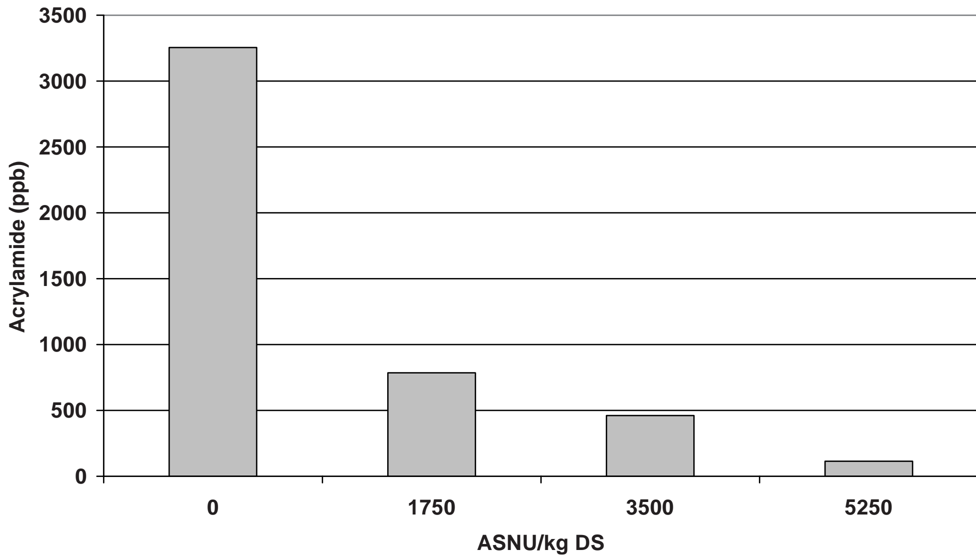


Fig. 4.15 Acrylamide in fried potato snacks after enzyme treatment of the potato snack pellet dough. Dosages were 1750–5250 ASNU kg^{-1} dry matter. Holding time was 20 min at 35°C.

with an asparaginase. This is somewhat unexpected for two reasons. First, most studies on acrylamide formation in potato products have shown that reducing sugars are the limiting factor for acrylamide formation in this type of product, and have proposed focusing on this reactant to reduce acrylamide formation.[27,28,33–36] The results presented and discussed here and in the cited studies show clearly that lowering asparagine levels alone can result in significant reduction of acrylamide formation. Second, for intact potato products like French fries and sliced potato chips, bringing asparaginase in contact with asparagine is an additional challenge, as the potato structure is thought to be impenetrable for the enzyme. Experimental results suggest that cell wall and membrane disruption of the potato cells is essential to facilitate contact between substrate and enzyme. The rate-limiting factor seems to be the diffusion of asparagine to the enzyme, which is mainly located on the outside of the intact potatoes pieces. Asparaginase treatment, therefore, most likely has a primary impact on asparagine found in the outer layers of the potato stick, which is also where acrylamide formation is highest.

Since asparaginase treatment is effective when applied in a short dip process step similar to steps in current industrial French fry production, process modifications required for industrial implementation are minor and potentially worthwhile relative to the reductions achieved. The major issue to be solved for implementation in industrial scale is control of the temperature of the enzyme bath, since this has to be kept within the limits of enzyme activity and stability. Considering current blanching practice, some cooling of the blanched potatoes will therefore be required before entering the enzyme bath. For sliced potato chips, potential industrial implementation will require more significant process changes, including a short heat treatment or blanching step and a holding time to accommodate enzyme activity.

In potato-based dough products, water content or water activity appears to be the most critical factor for enzyme performance, and experiments indicate that at least 35–40% water is needed for maximum performance. At lower water contents, performance can very likely

be improved and optimized based on enzyme dosage, temperature and holding time, or alternatively the asparagine containing ingredient treated in a separate step with maximum water present.

4.4.3 Application testing in coffee

4.4.3.1 *Introducing asparaginase in coffee processing*

Coffee is the roasted kernel of the coffee cherry. In the coffee process, first the fruit flesh is removed, then the kernel is dried, resulting in green coffee beans. These are then roasted at temperatures exceeding 180°C. Green coffee beans contain asparagine as well as sugars, with sucrose being dominant. Since the high roasting temperatures catalyze a degradation of sucrose to glucose and fructose, subsequent formation of acrylamide can take place. Acrylamide forms mainly during the early stages of roasting. During extended roasting, especially at very high temperatures, accumulated acrylamide will break down again.[86,87] Roasted coffee beans have been analyzed to contain 220–375 ppb acrylamide (see Fig. 4.1) resulting in 5–10 $\mu g\ L^{-1}$ in brewed coffee.[88] In coffee-drinking countries, this may constitute 28–36% of the total daily exposure to acrylamide.[88]

Coffee beans contain approximately 0.5 g asparagine and 50 g sucrose per kg.[89] No data are available on localization within the bean, but they are expected to be rather evenly distributed. As described for intact potato pieces (see 4.4.2.1), a major portion of the asparagine is probably present inside the cells and thus not easily accessible.

The green coffee bean has a very tight structure where less than 7% of its pore volume has a diameter of 10 nm or more.[90] The diameter of many enzymes is in the order of 5–10 nm, consequently only a limited part of the bean is accessible to a relatively large enzyme like asparaginase (see also 4.4.2.1). Thus, diffusion of asparagine out of the cells is likely to be the rate-limiting factor for treatment with asparaginase, accentuating the importance of distributing the asparaginase as evenly as possible over the whole bean in order to minimize the distance between enzyme and substrate.

To improve enzyme–substrate contact in the coffee bean, various techniques can be applied to actively aid diffusion. Vacuum infusion is known from fruit and vegetable processing, where it has proven quite effective in getting enzymes into the inner part of plant cells given that these contain a certain minimum amount of occluded air.[83] Applying pressure may have the same beneficial effect, but this technique has not yet been tested. Thermomechanical pretreatments like steam explosion may help open up the bean structure, but will also change roasting kinetics.

4.4.3.2 *Laboratory-scale testing*

Wet green beans typically have a pH of around 5.5, so fungal asparaginases, like the asparaginase from *A. oryzae*, can be applied without pH adjustment; the dosage may range from 2500 to 10 000 ASNU kg^{-1} green beans.

The following three techniques could be applied to enable enzyme–substrate contact in the bean:

1. **Soaking coffee beans in an enzyme bath**: This is an easy process but it will also result in extraction losses of, for example, aroma precursors, thereby lowering both the yield in terms of final product weight and aroma formation. Furthermore, rather large changes in the roasting kinetics are expected.

2. **Vacuum infusion**: Depending on the water absorbtion capacity of the coffee bean, a proper amount of enzyme solution is sprayed evenly onto the beans and vacuum is applied until the enzyme solution is absorbed. This procedure does not result in yield loss, and a loss of aroma precursors is also unlikely, except for the most volatile aroma components.
3. **Pressure infusion**: The procedure is similar to vacuum infusion, but pressure is applied instead of a vacuum. It can be assumed that no volatile aroma precursors are removed in this way.

Following the enzyme application in all three processes, the beans can be incubated at temperatures up to 50°C for a defined amount of time to accommodate enzyme activity. In laboratory trials using the vacuum infusion method, 18 h were found to be sufficient to overcome diffusion hindrances. After incubation the beans are dried, for example in an oven, and roasted.

As a wet process is unavoidable when working with this type of enzyme application, a certain rearrangement of the solutes in the bean will take place and during drying sugars and amino acids will most likely become more concentrated at the surface. Therefore, it must be anticipated that the roasting kinetics will change somewhat, even when using vacuum or pressurized application processes.

Figure 4.16 illustrates results from a trial in which either water or enzyme solution was added to green coffee beans using vacuum infusion. The beans were then roasted for varying times. Acrylamide levels in the untreated control beans were highest at the shortest roasting time, and lower for longer times, consistent with the pattern of degradation of acrylamide

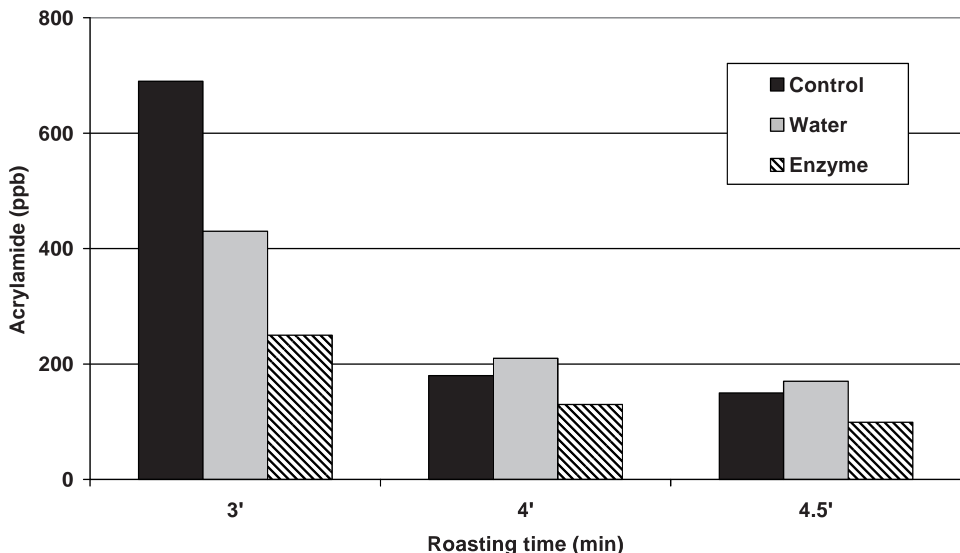


Fig. 4.16 Effect of roasting and asparaginase treatment on acrylamide formation in vacuum-infused green coffee beans. Control sample had no treatment at all, Water sample is beans vacuum-infused with water and Enzyme sample is beans vacumm-infused with an enzyme solution. After vacuum infusion the green beans were oven dried and then roasted. 3 minutes roast corresponds to an insufficently roasted bean. 4–4.5 minutes roast results in a medium to darker roasted bean. Roasting took place in a household roaster.

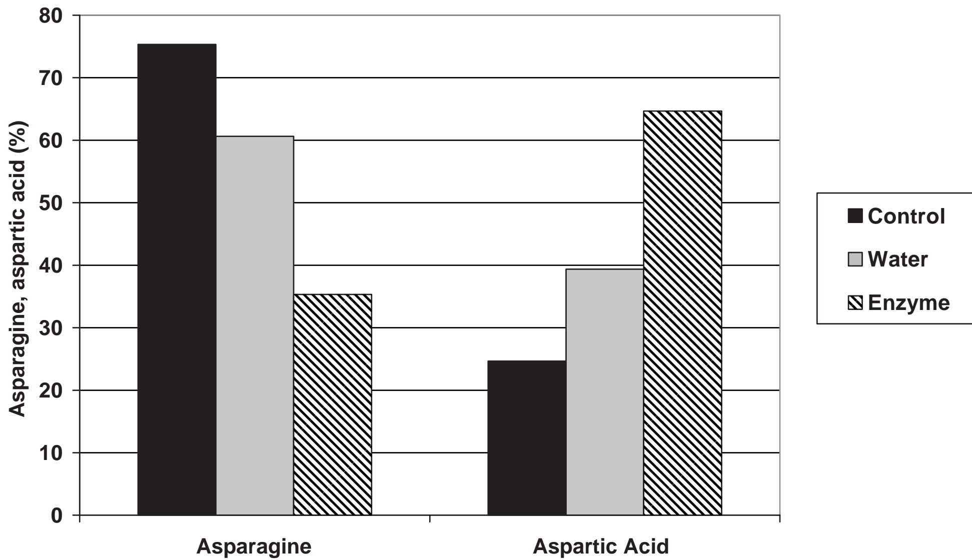


Fig. 4.17 Asparaginase and aspartic acid content of treated and untreated green coffee beans before roasting. The sum of asparagine and aspartic acid is set to 100%. Process conditions: enzyme dosage: 4000 ASNU/kg beans; vacuum: 30 min, incubation for 18 hours at 50°C, subsequent drying of the beans overnight at 70°C.

known to occur during extended roasting. Similar results have been described by Lantz *et al.*[86] and Bagdonate *et al.*[87]

Results illustrated in Fig. 4.16 also show that wetting the green beans caused a lower acrylamide formation in the early stages of roasting, but had very little or no effect when making a medium to dark roast. The addition of asparaginase clearly reduced the amount of acrylamide formed compared with both the untreated (control) and water-treated green beans.

Figures 4.17 and 4.18 illustrate results from an enzyme dosage response trial using vacuum infusion. Figure 4.17 shows the relative amounts of asparagine and aspartic acid in the green beans before and after vacuum treatment using only one enzyme dosage, while Fig. 4.18 shows the acrylamide content of medium roasted beans after treatment with different doses of asparaginase.

The results in Fig. 4.17 show that the amount of asparagine in the green bean was much higher than that of aspartic acid. Wetting the bean resulted in a reduction in asparagine levels by 20% relative to the control, while addition of asparaginase enhanced this effect giving an overall reduction in asparagine level of 40%.

Figure 4.18 shows that with a dosage of 4000 ASNU kg^{-1} green beans or above, the formation of acrylamide was reduced by 40–50% after a medium roast. In this experiment, a trial was also made in which green beans were treated with a mannanase together with the asparaginase, to see if the mannanase could increase enzyme and substrate diffusion. The main polysaccharides in coffee beans are arabinogalactan (approximately 30% w/w bean) and galactomannan (approximately 15% w/w bean). Mannanase degrades galactomannan, and could aid in opening the bean structure. The results in Fig. 4.18 show that mannanase

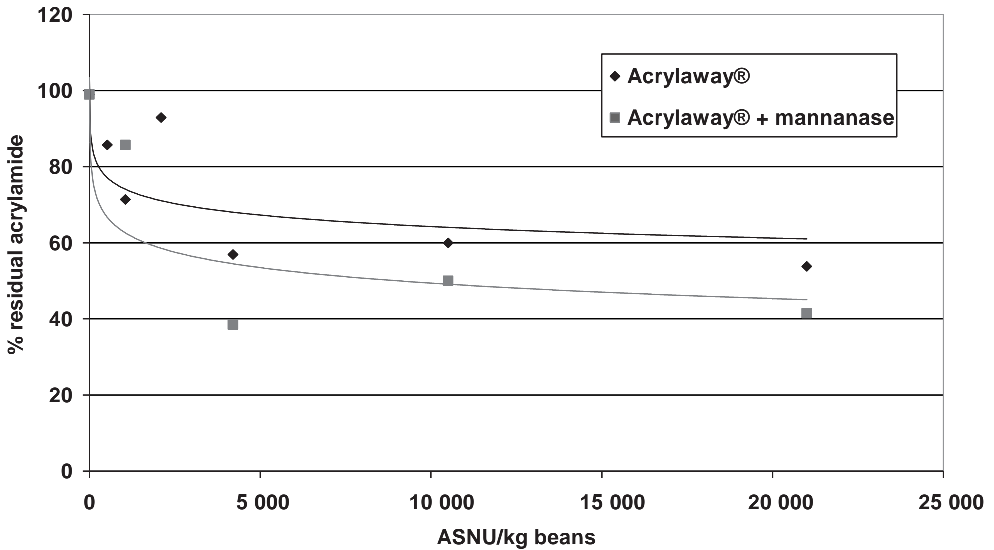


Fig. 4.18 Acrylamide reduction as a function of asparaginase dosage. Hundred per cent acrylamide corresponds to 130 ppb. Process conditions: Acrylaway (or asparaginase) dosages: 525–20 000 ASNU kg^{-1} beans; mannanase dosage: 2000 Units kg^{-1} beans; vacuum treatment with enzyme for 30 min, heat treatment for 18 h at 50°C, drying of the beans overnight at 70°C and roasting in a kitchen coffee roaster for 4 min.

addition did improve acrylamide reduction, possibly because the solubilized galactomannan led to an increase in pore size.

4.4.3.3 *Conclusion – asparaginase in coffee*

Experiments reviewed here show that the asparaginase from *A. oryzae* can effectively remove a sufficient amount of asparagine from green coffee beans and thereby reduce final acrylamide content in the roasted beans by 30–60%, depending on processing conditions and efficiency and degree of roasting. An additional wet processing step is introduced in the coffee process so that solubles and aroma precursors may be redistributed in the bean. Therefore, possible adjustments to the roasting procedure may be needed and should be investigated. Since many other beans and nuts are also roasted, this technology may potentially be applied to products like cocoa, almonds and peanuts, thus opening up for acrylamide reduction in a wider range of different foods.

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REFERENCES

1. IARC (1994) *Monographs on the Evaluation of Carcinogenic Risks to Humans, Some Industrial Chemicals*, Vol. **60**. International Agency for Research on Cancer, Lyon, France, pp. 389–433.
2. JECFA (2005) *Report from the Sixty-Fourth Meeting*. Joint FAO/WHO Expert Committee on Food Additives, Rome, 8–17 February, http://www.who.int/ipcs/food/jecfa/summaries/summary_report_64_final.pdf
3. Wilson, K.M., Rimm, E.B., Thompson, K.M. and Mucci, L.A. (2006) Dietary acrylamide and cancer risk in humans: a review. *Journal für Verbraucherschutz und Lebensmittelsicherheit* **1**, 19–27.
4. http://irmm.jrc.ec.europa.eu/html/activities/acrylamide/database.htm
5. Stadler, R.H. and Scholz, G. (2004) Acrylamide: an update on current knowledge in analysis, levels in food, mechanisms of formation, and potential strategies of control. *Nutrition Reviews* **62**, 449–467.
6. Claus, A., Carle, R. and Schieber, A. (2008) Acrylamide in cereal products: a review. *Journal of Cereal Science* **47**, 118–133.
7. http://www.cfsan.fda.gov/~dms/acryexpo/acryex8.htm
8. http://www.who.int/foodsafety/publications/chem/acrylamide_faqs/en/index3.html
9. http://www.bvl.bund.de/nn_521172/EN/01_Food/04_Acrylamid_en/00_Minimierungskonzept_en/minimierungskonzept_node.html_nnn=true
10. Mottram, D.S., Wedzicha, B.L. and Dodson, A.T. (2002) Acrylamide is formed in the Maillard reaction. *Nature* **419**, 448–449.
11. Stadler, R.H., Blank, I., Varga, N., Robert, F., Hau, J., Guy, P.A., Robert, M.-C. and Riediker, S. (2002) Acrylamide from Maillard reaction products. *Nature* **419**, 449.
12. Zyzak, D.V., Sanders, R.A., Stojanovic, M., Tallmadge, D.H., Eberhart, B.L., Ewald, D.K., Gruber, D.C., Morsch, T.R., Strothers, M.A., Rizzi, G.P. and Villagran, M.D. (2003) Acrylamide formation mechanism in heated foods. *Journal of Agricultural and Food Chemistry* **51**, 4782–4787.
13. Yaylayan, V.A., Wnorowski, A. and Perez Locas, C. (2003) Why asparagine needs carbohydrates to generate acrylamide. *Journal of Agricultural and Food Chemistry* **51**, 1753–1757.
14. Lingnert, H., Grivas, S., Jägerstad, M., Skog, K., Törnquist, M. and Åman, P. (2002) Acrylamide in food: mechanisms of formation and influencing factors during heating of foods. *Scandinavian Journal of Food & Nutrition* **46**, 159–172.
15. Blank, I., Robert, F., Goldmann, T., Pollien, P., Varga, N., Devaud, S., Saucy, F., Huynh-Ba, T. and Stadler, R.H. (2005) Mechanism of acrylamide formation – Maillard-induced transformation of asparagine. In: *Chemistry and Safety of Acrylamide in Food* (eds M. Friedman and D.S. Mottram). Springer Science+Business Media Inc., New York, pp. 171–189.
16. Becalski, A., Lau, B.P.-Y., Lewis, D. and Seaman, S.W. (2003) Acrylamide in foods: occurrence, sources, and modeling. *Journal of Agricultural and Food Chemistry* **51**, 802–808.
17. Yasuhara, A., Tanaka, Y., Hengel, M. and Shibamoto, T. (2003) Gas chromatographic investigation of acrylamide formation in browning model systems. *Journal of Agricultural and Food Chemistry* **51**, 3999–4003.
18. Vattem, D.A. and Shetty, K. (2003) Acrylamide in food: a model for mechanism of formation and its reduction. *Innovative Food Science and Emerging Technologies* **4**, 331–338.
19. Gertz, C. and Klostermann, S. (2002) Analysis of acrylamide and mechanisms of its formation in deep-fried products. *European Journal of Lipid Science and Technology* **104**, 762–771.
20. Stadler, R.H., Verzegnassi, L., Varga, N., Grigorov, M., Studer, A., Riediker, S. and Schilter, B. (2003) Formation of vinylogous compounds in model Maillard reaction systems. *Chemical Research in Toxicology* **16**, 1242–1250.
21. Zhang, Y. and Zhang, Y. (2007) Formation and reduction of acrylamide in Maillard reaction: a review on the current knowledge. *Critical Reviews in Food Science and Nutrition* **47**, 521–542.
22. CIAA (2007) Confederation of the food and drink industries of the EU; Acrylamide 'Toolbox'; Rev. 11, http://ec.europa.eu/food/food/chemicalsafety/contaminants/ciaa_acrylamide_toolbox.pdf
23. Konings, E.J.M., Ashby, P., Hamlet, C.G. and Thompson, G.A.K. (2007) Acrylamide in cereal and cereal products: a review on progress in level reduction. *Food Additives and Contaminants* **24**(S1), 47–59.
24. Ahrne, L., Andersson, C.G., Floberg, P., Rose, J. and Lingert, H. (2007) Effect of crust temperature and water content on acrylamide formation during baking of white bread: steam and falling temperature baking. *LWT – Food Science and Technology* **40**, 1708–1715.

25. Amrein, T.M., Andres, L., Escher, F. and Amado, R. (2007) Occurrence of acrylamide in selected foods and mitigation options. *Food Additives and Contaminants* **24**(S1), 13–25.
26. Amrein, T.M., Schönbächler, B., Escher, F. and Amado R. (2005) Factors influencing acrylamide formation in gingerbread. In: *Chemistry and Safety of Acrylamide in Food* (eds M. Friedman and D.S. Mottram). Springer Science+Business Media Inc., New York.
27. Amrein, T., Bachmann, S., Noti, A., Biedermann, M., Barbosa, M.F., Biedermann-Brem, S., Grob, K., Keiser, A., Realini, P., Escher, F. and Amadó, R. (2003) Potential of acrylamide formation, sugars, and free asparagine in potatoes: a comparison of cultivars and farming systems. *Journal of Agricultural and Food Chemistry* **51**, 5556–5560.
28. Elmore, J.S., Koutsidis, G., Dodson, A.T., Mottram, D.S. and Wedzicha, B.L. (2005) Measurement of acrylamide and its precursors in potato, wheat, and rye model systems. *Journal of Agricultural and Food Chemistry* **53**, 1289–1293.
29. Surdyk, N., Rosén, J., Andersson, R. and Åman, P. (2004) Effects of asparagine, fructose and baking conditions on acrylamide content in yeast-leavened wheat bread. *Journal of Agricultural and Food Chemistry* **52**, 2047–2051.
30. Claus, A., Schreiter, P., Weber, A., Graeff, S., Hermann, W., Claupein, W., Schieber, A. and Carle, R. (2006) Influence of agronomic factors and extraction rate on the acrylamide contents in yeast-leavened breads. *Journal of Agricultural and Food Chemistry* **54**, 8968–8976.
31. Springer, M., Fischer, T., Lehrack, A. and Freud, W. (2003) Development of acrylamide in baked products. *Getreide, Mehl und Brot* **57**, 274–278.
32. Claus, A., Carle, R. and Schieber, A. (2006) Reducing acrylamide in bakery products. *New Food* **2**, 10–14.
33. Becalski, A., Lau, B.P.-Y., Lewis, D., Seaman, S.W., Hayward, S., Sahagian, M., Ramesh, M. and Leclerc, Y. (2004) Acrylamide in French fries: influence of free amino acids and sugars. *Journal of Agricultural and Food Chemistry* **52**, 3801–3806.
34. Biedermann, M., Noti, A., Biedermann-Brem, S., Mozetti, V. and Grob, K. (2002) Experiments on acrylamide formation and possibilities to decrease the potential of acrylamide formation in potatoes. *Mitteilungen Lebensmittel Hygiene* **93**, 668–687.
35. Haase, N.U., Matthäus, B. and Vosmann, K. (2004) Aspects of acrylamide formation in potato crisps. *Journal of Applied Botany and Food Quality* **78**, 144–147.
36. Williams, J.S.E. (2005) Influence of variety and processing conditions on acrylamide levels in fried potato crisps. *Food Chemistry* **90**, 875–881.
37. Bråthen, E., Kita, A., Knutsen, S.H. and Wicklund, T. (2005) Addition of glycine reduces the content of acrylamide in cereal and potato products. *Journal of Agricultural and Food Chemistry* **53**, 3259–3264.
38. Kita, A., Bråthen, E., Knutsen, S.H. and Wicklund, T. (2004) Effective ways of decreasing acrylamide in potato crisps during processing. *Journal of Agricultural and Food Chemistry* **52**, 7011–7016.
39. Pedreschi, F., Kaack, K., Granby, K. and Troncoso, E. (2007) Acrylamide reduction under different pre-treatments in French fries. *Journal of Food Engineering* **79**, 1287–1294.
40. Mestdagh, F., De Wilde, T., Delporte, K., Van Peteghem, C. and De Meulenaer, B. (2008) Impact of chemical pre-treatments on the acrylamide formation and sensorial quality of potato crisps. *Food Chemistry* **106**, 914–922.
41. Granda, C., Moreira, R.G. and Tichy, S.E. (2004) Reduction of acrylamide formation in potato chips by low-temperature vacuum frying. *Journal of Food Science* **69**(8), 405–411.
42. Elder, V.A., Fulcher, J.G. and Leung, H.K.-H. (2004) Method for reducing acrylamide formation in thermally processed foods. US Patent Application 2004, US 2004/0058054.
43. Pedreschi, F., Kaack, K. and Granby, K. (2008) The effect of asparaginase on acrylamide formation in French fries. *Food Chemistry* **109**, 386–392.
44. Amrein, T.M., Schönbächler, B., Escher, F. and Amado, R. (2004) Acrylamide in gingerbread: critical factors for formation and possible ways for reduction. *Journal of Agricultural and Food Chemistry* **51**, 4282–4288.
45. Vass, M., Amrein, T.M., Schönbächler, B., Escher, F. and Amado, R. (2004) Ways to reduce acrylamide formation in cracker products. *Czech Journal of Food Sciences* **22**, 19–21.
46. Ciesarová, Z., Kiss, E. and Boegl, P. (2006) Impact of L-asparaginase on acrylamide content in potato products. *Journal of Food and Nutrition Research* **4**, 141–146.
47. Benschop, C. (2008) PreventASe™ – a practical solution to the acrylamide issue. CCFRA, Chipping Campden, 4 July.

48. Hendriksen, H.V., Kornbrust, B., Oestergaard, P.R. and Stringer, M.A. Evaluating the potential for enzymatic acrylamide reduction in a range of food products using an asparaginase from *Aspergillus oryzae*. Submitted for publication.
49. Dria, G.J., Zyzak, D.V., Gutwein, R.W., Villagran, F.V., Young, H.T., Bunke, P.R., Lin, P.Y.T., Howie, J.K. and Schafermeyer, R.G. (2007) Method for reduction of acrylamide in roasted coffee beans, roasted coffee beans having reduced levels of acrylamide, and article of commerce. US Patent 7,220,440, 2003.
50. Howie, J.K., Lin, P.Y.T. and Zyzak, D.V. (2005) Method for reduction of acrylamide in cocoa products, cocoa products having reduced levels of acrylamide, and article of commerce. International Patent Application 2005, WO 2005/004620.
51. Wriston Jr., J.C. and Yellin, T.O. (1973) L-asparaginase: a review. *Advances in Enzymology and Related Areas of Molecular Biology* **39**, 185–248.
52. Borek, D. and Jaskólski, M. (2001) Sequence analysis of enzymes with asparaginase activity. *Acta Biochimica Polonica* **48**, 893–902.
53. Lehninger, A.L. (1982) *Principles of Biochemistry*. Worth Publishers Inc., New York.
54. Sieciechowicz, K.A., Joy, K.W. and Ireland, R.J. (1988) The metabolism of asparagine in plants. *Phytochemistry* **27**, 663–671.
55. Finn, R.D., Tate, J., Mistry, J., Coggill, P.C., Sammut, J.S., Hotz, H.R., Ceric, G., Forslund, K., Eddy, S.R., Sonnhammer, E.L. and Bateman, A. (2008) The Pfam protein families database. *Nucleic Acids Research* 36 (Database Issue), D281–D288 (Pfam release 23.0. http://pfam.sanger.ac.uk/).
56. Ario, T., Taniai, M., Torigoe, K. and Kurimoto, M. (1996) DNA coding for mammalian L-asparaginase. Patent Application EP726313.
57. Tsuji, N., Morales, T.H., Ozols, V.V., Carmody, A.B. and Chandrashekar, R. (1999) Identification of an asparagine amidohydrolase from the filarial parasite Dirofilaria immitis. *International Journal of Parasitology* **29**, 1451–1455.
58. Wriston Jr., J.C. (1985) Asparaginase. *Methods in Enzymology* **113**, 608–618.
59. Krasotkina, J., Borisova, A.A., Gervaziev, Y.V. and Sokolov, N.N. (2004) One-step purification and kinetic properties of the recombinant L-asparaginase from Erwinia carotovora. *Biotechnology and Applied Biochemistry* **39**, 215–221.
60. Ortlund, E., Lacount, M.W., Lewinski, K. and Lebioda, L. (2000) Reactions of Pseudomonas 7A glutaminase-asparaginase with diazo analogues of glutamine and asparagine result in unexpected covalent inhibitions and suggests an unusual catalytic triad Thr-Tyr-Glu. *Biochemistry* **39**, 1199–1204.
61. Yao, M., Yasutake, Y., Morita, M. and Tanaka, I. (2005) Structure of the type I L-asparaginase from the hyperthermophilic archaeon Pyrococcus horikoshii at 2.16 Å resolution. *Acta Crystallographica: Section D, Biological Crystallography* **D61**, 294–301.
62. Cedar, H. and Schwartz, J.H. (1967) Localization of the two L-asparaginases in anaerobically grown Escherichia coli. *Journal of Biological Chemistry* **242**, 3753–3755.
63. Minton, N.P., Bullman, H.M.S., Scawen, M.D., Atkinson, T. and Gilbert, H.J. (1986) Nucleotide sequence of the Erwinia chrysanthemi NCPPB 1066 L-asparaginase gene. *Gene* **46**, 25–35.
64. Dunlop, P.C., Meyer, G.M., Ban, D. and Roon, R.J. (1978) Characterization of two forms of asparaginase in saccharomyces cerevisiae. *Journal of Biological Chemistry* **253**, 1297–1304.
65. Hüser, A., Klöppner, U. and Röhm, K.-H. (1999) Cloning, sequence analysis, and expression of ansB from Pseudomonas fluorescens, encoding periplasmic glutaminase/asparaginase. *FEMS Microbiology Letters* **178**, 327–335.
66. Sinclair, K., Warner, J.P. and Bonthron, D.T. (1994) The ASP1 gene of Saccharomyces cerevisiae, encoding the intracellular isozyme of L-asparaginase. *Gene* **144**, 37–43.
67. Yun, M.-K., Nourse, A., White, S.W., Rock, C.O. and Heath, R.J. (2007) Crystal structure and allosteric regulation of the cytoplasmic Escherichia coli L-Asparaginase I. *Journal of Molecular Biology* **369**, 794–811.
68. Sonewane, A., Klöppner, U., Derst, C. and Röhn, K.-H. (2003) Utilization of acidic amino acids and their amides by pseudomonades: role of periplasmic glutaminase-asparaginase. *Archives of Microbiology* **179**, 151–159.
69. Kurztberg, J. (2000) Asparaginase. In: *Cancer Medicine*, 5th edn (eds R.C. Blast Jr., D.W. Kufe, R.E. Pollock, R.R. Weichselbaum, J.F. Holland, E. Frei III and T.S. Gansler). B.C. Decker Inc, Hamilton, ON, pp. 699–705.
70. Campbell, H.A. and Mashburn, L.T. (1969) L-asparaginase EC-2 from Escherichia coli. Some substrate specificity characteristics. *Biochemistry* **8**, 3768–3775.

71. Swain, A.L., Jaskólski, M., Housset, D., Rao, J.K.M. and Wlodawer, A. (1993) Crystal structure of Escherichia coli L-asparaginase, an enzyme used in cancer therapy. *Proceedings of the National Academy of Sciences of the United States of America* **90**, 1474–1478.
72. http://www.dsm.com/en_US/html/dfs/preventase_welcome.htm
73. http://www.acrylaway.novozymes.com/
74. Daniells, S. (2007) DSM publishes fungus genome to help R&D into enzymes. *FoodNavigator.com/Europe*.
75. Plomp, P.J.A.M., DeBoer, L., VanRooijen, R.J. and Meima, R.B. (2004) Novel food production process. International Patent Application 2004, WO04030468.
76. Lobedanz, S.S. (2008) Personal Communication.
77. Tareke, E., Rydberg, P., Karlsson, P., Eriksson, S. and Törnqvist, M. (2002) Analysis of acrylamide, a carcinogen formed in heated foodstuffs. *Journal of Agricultural and Food Chemistry* **50**, 4998–5006.
78. Wenzl, T., de la Calle, B.M. and Anklam, E. (2003) Analytical methods for the determination of acrylamide in food products: a review. *Food Additives and Contaminants* **20,** 885–902.
79. Taeymans, D., Wood, J., Ashby, P., Blank, I., Studer, A., Stadler, R.H., Gondé, P., Van Eijck, P., Lalljie, S., Longnert, H., Lindblom, M., Matissek, R., Müller, D., Tallmadge, D., O′Brien, J., Thompson, S., Silvani, D. and Whitmore, T. (2004) A review of acrylamide: an industry perspective on research, analysis, formation, and control. *Critical Reviews in Food Science and Nutrition* **44,** 323–347.
80. Clarke, D.B., Kelly, J. and Wilson, L.A. (2002) Assessment of performance of laboratories in determining acrylamide in crisp bread. *Journal of AOAC International* **85**, 1370–1373.
81. Manley, D. (2001) *Biscuit, Cracker and Cookie Recipes for the Food Industry.* Woodhead Publishing in Food Science and Technology, Cambridge.
82. Claeys, W.L., de Vleeschouwer, K. and Hendrickx, M.E. (2005) Quantifying the formation of carcinogens during food processing: acrylamide. *Trends in Food Science and Technology* **16**, 181–193.
83. Guillemin, A., Degraeve, P., Guillon, F., Lahaye, M. and Saurel, R. (2006) Incorporation of pectinmethylesterase in apple tissue either by soaking or by vacuum-impregnation. *Enzyme and Microbial Technology* **38**, 610–616.
84. Gökmen, V., Palazoğlu, T.K. and Şenyuva, H.Z. (2006) Relation between the acrylamide formation and time-temperature history of surface and core regions of French fries. *Journal of Food Engineering* **77**, 972–976.
85. Lisińska, G. and Leszczyński, W. (1989) *Manufacture of Potato Chips and French Fries in: Potato Science and Technology*. Elsevier Applied Science, London.
86. Lantz, I., Ternit, R., Wilkens, J., Hoenicke, K., Guenther, H. and Van Der Stegen, G.H.D. (2006) Studies on acrylamide levels in roasting, storage and brewing of coffee. *Molecular Nutrition & Food Research* **50**, 1039–1046.
87. Bagdonate, K., Derler, K. and Murkovic, M. (2008) Determination of acrylamide during roasting of coffee. *Journal of Agricultural and Food Chemistry* **56**, 6081–6086.
88. Granby, K. and Fagt, S. (2004) Analysis of acrylamide in coffee and dietary exposure to acrylamide from coffee. *Analytica Chimica Acta* **520**, 177–182.
89. Murkovic, M. and Derler, K. (2006) Analysis of amino acids and carbohydrates in green coffee. *Journal of Biochemical and Biophysical Methods* **69**, 25–32.
90. Schenker, S., Handschin, S., Frey, B., Perren, R. and Eschere, E. (2000) Pore structure of coffee beans affected by roasting conditions. *Journal of Food Science* **65**(3), 452–457.

5 Enzymes in dairy product manufacture

Barry A. Law

5.1 INTRODUCTION

The dairy foods sector of the food manufacturing industry is a traditional user of enzymes. The best-known dairy enzyme preparation is, of course, rennet, a collective name for commercial preparations containing acid proteases extracted from animal tissues.[1] These products clot milk by removing a highly charged peptide fragment from κ-casein on the surface of micellar casein, the majority form of milk protein. Destabilized casein micelles aggregate and form the structure of the milk clot that is then acidified by lactic cultures to make cheese curd.[2] Although this use of enzymes is the single most important in the dairy sector, modern production methods have made possible other applications to meet changing needs and priorities. For example, in the UK and USA the shortage of calves from which to source traditional rennet has led to the development of enzyme production technology with yeasts moulds and fungi as the primary source. Over half of the total milk coagulating enzymes used in these regions are microbial in origin, mostly from genetically modified (GM) food yeast and mould containing copies of the calf gene for the production of chymosin, the main acid proteinase involved in milk clotting. The other main type of microbial rennet (now called 'coagulant') is made from non-GM mould, *Rhizomucor miehei*. This topic of coagulant production and use in the dairy industry will be described and discussed in detail in this chapter.

In addition to the use of milk-clotting enzymes to make cheese, the dairy industry also makes use of enzymes such as lipases, non-coagulant proteases, aminopeptidases, lactases, lysozyme, lactoperoxidase and transglutaminase. Some of these applications are traditional (lipase for flavour enhancement) whilst others are relatively new (lactose hydrolysis, accelerated cheese ripening, control of microbiological spoilage, modification of protein functionality) and all will be discussed in detail below. Table 5.1 summarizes the range of enzymes used in dairy products.

5.2 MILK-CLOTTING ENZYMES

5.2.1 The nature and identity of rennets and coagulants

The first commercial standardized rennet preparation was made and sold by Chr. Hansen A/S, Denmark in 1874, and was probably the first commercial enzyme of any type. It was then, and still is by definition an extract of ruminant abomasum, ideally containing mainly chymosin, the enzyme which is specific for κ-casein hydrolysis and casein destabilization.

Table 5.1 Enzymes used in dairy technology.

Enzymes	Application examples
Acid proteinases	Milk coagulation
Neutral proteinases and peptidases	Accelerated cheese ripening, debittering, enzyme-modified cheese, production of hypoallergenic milk-based foods
Lipases	Accelerated cheese ripening, enzyme-modified cheese, flavour-modified cheese, structurally modified milk fat products
β-Galactosidase	Lactose-reduced whey products
Lactoperoxidase	Cold sterilization of milk, milk replacers for calves
Lysozyme	Nitrate replacer for washed-curd cheeses and cheeses with eyes (e.g. Emmental)

However, dependent on the age of the calves from which it is extracted, rennet can contain more or less pepsin, another acid protease with a wider casein substrate range. Both chymosin and pepsin, and indeed all of the milk coagulating enzymes used in cheese technology, are classified as aspartic proteinases with the Enzyme Commission (EC) number 3.4.23. Because there are now several different types and sources of milk-clotting enzymes on the market, the International Dairy Federation (IDF/FIL) official definitions decree that the name 'rennet' be reserved for enzyme preparations from ruminant stomachs, and other milk-clotting enzymes (mainly the microbial ones) should be named 'coagulants'.[1]

As far as the cheese technologist is concerned, the rennets and coagulants are most usefully categorized by their source, not only to distinguish animal, vegetable, microbial and GMO-sourced preparations, but also to select the most suitable enzyme for any particular cheese variety. This is a very important aspect of cheese manufacturing technology, affecting the yield of cheese, its storage life and its final quality of flavour/texture after maturation. These interrelationships are discussed in detail in Section 5.4.3. For now, we will consider the range of rennets and coagulants available in Table 5.2.

5.2.2 Main characteristics of rennets and coagulants from different sources

Of the animal rennets, calf rennet is widely regarded as the ideal milk-clotting enzyme for cheese making. This is partly through traditional familiarity with the product through long use, but the preference also has a sound scientific basis in that calf rennet is typically 80–90% chymosin (EC 3.4.23.4). This means that most of the casein breakdown in the cheese vat is directed very specifically at κ-casein to clot the milk, and not at the other caseins. Non-specific proteolysis of α- and β-casein during curd formation can result in the loss of casein nitrogen in the whey, reducing the yield of the cheese process. The second enzyme in calf rennet, pepsin (EC 3.4.23.1) is thought by traditional cheese makers to add to the ripening qualities of rennet in maturing cheese, but there is no hard evidence for this. Indeed, cheese makers now make excellent quality long-hold cheeses (especially Cheddar) using pure chymosin from GM yeast and fungi expressing cloned copies of the calf (pro)chymosin gene.

Sheep, goats and pigs can provide rennet preparations that are enzymatically similar to calf rennet, but not ideally suited to clotting cows' milk.[3] Rennet 'paste' is a crude form of rennet made from the macerated stomachs of suckling calf, lamb or kid and contains pregastric lipase to add piquancy to the flavour of the cheese. It is mainly used in traditional Italian cheeses.

Table 5.2 Main types and sources of milk-clotting enzymes (rennets and coagulants) used in cheese making.

Type	Source	Component enzyme(s)	Technological characteristics
Animal			
Calf rennet; bovine rennet	Bovine stomachs	Chymosin A and B, pepsin and gastricin	High ratio of κ-casein: general casein hydrolysis; maxim yield; traditional texture and flavour in hard and semi-hard cheeses
Rennet paste	Calf, kid or sheep stomachs	As calf rennet + pregastric lipase	Optimum coagulation and yield, with piquant flavour production
Sheep, pig and kid rennet	Corresponding animal stomach	Chymosin, pepsin and gastricin	Not widely used; best used with milk from same animal
Vegetable	*Cynara cardunculus*	Cyprosin, cardosin	Produced locally on small scale for artisanal cheeses
Microbial			
Miehei coagulant type L, TL and XL	*Rhizomucor miehei*	*R. mucor* aspartic proteinase	Native L-type enzyme is too heat stable and proteolytic for hard cheese manufacture; destabilized TL and XL chemical variants are used for cheeses in this category (see Section 5.4.3 for details)
Pusillus coagulant	*Rhizomucor pusillus*	*R. mucor* aspartic proteinase	Similar properties to L-type miehei enzyme, but more pH-sensitive
Parasitica coagulant	*Cryphonectria parasitica*	*C. parasitica* aspartic proteinase	Very heat resistant – use confined to high-cook cheeses like Emmental
Fermentation-produced chymosin (Chymax™; Maxiren™)	*Kluyveromyces lactis*;[a] *Aspergillus niger*[a]	Calf chymosin	Identical to calf chymosin in all respects

[a] Genetically modified to express copies of the calf prochymosin gene.

Many plants produce proteinases that clot milk. However, plant coagulants are not produced on a commercial scale, but made locally (mainly in Portugal) for artisanal cheese making.[1] The principle plant coagulant in use for these speciality cheeses is extracted from the cardoon flowers of *Cynara cardunculus*.[4] Recent cheese making trials with goat's milk cheese[5,6] suggest that this type of coagulant may be applicable for accelerated cheese ripening. However, there is no reliable commercial source of cardoon rennet at the time of writing. Local users make their own preparations 'in house'.[4]

The best-known and most widely used microbial coagulant is that produced from *R. miehei* (Table 5.2). Its usage volume is now greater than that of extracted natural calf rennet. The commercial product is a mixture of aspartyl proteinases (EC 3.4.23.23) and is commercially available in three types. The native, unmodified enzyme ('type L') is very heat stable and hydrolyzes all of the caseins, not just κ-casein. Although this has been used successfully

to make soft, short-hold cheese varieties, its non-specific proteolytic action reduces yields of cheeses whose curd spends a long time in the whey (hard and semi-hard cheese), and caused bitterness in long-hold cheeses. The heat resistance of the enzyme is also a potential drawback in cheese plant from which the whey is processed as a food ingredient. The heat treatment and processing does not eliminate the activity of the coagulant and it can cause protein breakdown in other food products in which whey protein is a supplementary ingredient (sausages, meat pies, soups, etc.).

To overcome these problems, dairy enzyme producers have developed heat-labile versions of the *R. miehei* coagulant ('TL' and 'XL') using chemical oxidation to modify methionine side chains. These enzymes can be denatured by pasteurizing the whey and they are also less specific than the native proteinase. These coagulants are a good alternative to microbial chymosin (see below) in the manufacturing of 'vegetarian' cheeses, but the texture of hard cheese made with them becomes crumbly more quickly than that of cheese made with chymosin. Also the flavour profile of hard cheese made with fungal rennet is not the same as chymosin-made cheese.

The most widely used alternative to calf rennet in the cheese industry worldwide is fermentation-produced chymosin (FPC), which currently accounts for half of the world production of enzyme-coagulated cheese.[7]

It is produced by large-scale fermentation of GM *Kluyveromyces lactis* or *Aspergillus niger*. In both cases, the microorganism has been modified using gene technology by the incorporation of the calf prochymosin gene into the host organism with a suitable promoter to ensure its efficient secretion into the growth medium. The enzyme is relatively easy to harvest and purify from the culture, unlike the earlier production system using *Escherichia coli* to produce chymosin in inclusion bodies.[1,8]

Recently, a new FPC from camels (*Camelus dromedarius*) has become available,[7] and its suitability for Cheddar cheese is documented by[9] showing that the enzyme's high C/P ratio resulted in yield improvements even over calf-derived FPC.

The details of cheese making with alternative rennets and coagulants are beyond the scope of this chapter and the reader should study *Technology of Cheesemaking*[10] for full coverage of the topic.

5.2.3 Production of rennets and coagulants

Animal rennets are secreted from the stomach mucosa as inactive proenzymes that can easily be extracted by maceration with water, weak brine or a buffer solution. A preservative (usually sodium benzoate) is normally added at this stage to prevent microbial growth during the next stages of production, involving filtration and acidification to activate the proenzymes. After neutralization to pH 5.5 and a second filtration to clarify the extract, the preparation is standardized to the 'advertised' milk-clotting activity, sterile filtered and packaged as a liquid enzyme product to be transported and stored refrigerated. Animal rennets are not purified products, but contain whatever enzymes were secreted by the mucosal tissue at the time of slaughter. However, the enzymes in good-quality calf abomasal tissue are mainly chymosin and pepsin and only standardization is necessary.

Microbial coagulants are produced by submerged fed-batch fermentation of the production organisms *Rhizomucor* or *Cryphonectria*. The fermentation is usually for several days, after which the enzyme is recovered as a crude filtrate, concentrated by ultrafiltration and standardized. No attempt is made by the manufacturer to purify the product by removing other co-produced enzymes such as lipases and starch hydrolases, though the production strains of

the mould are selected to minimize these contaminants. Nevertheless, it is important to note that *Rhizomucor* coagulants contain significant amounts of starch-degrading enzymes which pass through to the cheese whey and are not removed by whey processing. Whey protein concentrates (WPCs) from cheese plants using microbial coagulants should not therefore be used in the formulation of sauces and other food products containing starch as a thickener.

FPC is mainly sourced from either GM yeast (*K. lactis*) or the GM filamentous fungus (*A. niger*). Both organisms have a long history of safe use in food fermentations and in food enzyme production. The genetic modifications employed are fully approved as safe in Europe and North America. Law and Goodenough[11] summarized the GM techniques used to enable these organisms to secrete calf chymosin. Harboe and Budtz[1] described the generic production scheme used for the fermentation and isolation of the coagulant product, which is similar to that used in the production of microbial rennets. Special emphasis is put on an acidification step in the FPC production process to ensure that the source organism is killed and that the DNA is broken down before the enzyme is isolated from the fermentate. The FPC sourced from *Aspergillus* by Chr. Hansen A/S is purified by chromatography to minimize the activity of non-coagulant enzymes and to provide added product purity assurance to cheese makers and consumers.[1]

5.2.4 Formulation and standardization of rennets and coagulants

The most common type of rennet/coagulant product is the liquid form, inexpensive to produce, easy to measure out for addition to the cheese milk and easy to mix. All products are formulated in a similar way irrespective of their source and the same types of stabilizers (sodium chloride, buffer, sorbitol, glycerol) are used. The only permitted preservative is sodium benzoate, but some manufacturers also sterilize their products by filtration to prevent microbial growth in the stored liquid products. Some rennets and coagulants are sold as powders or tablets, especially for shipment to hot countries. Whether dry or liquid, the products are all standardized so that a particular volume or weight always has the same milk-clotting activity, or 'strength'. Rennet/coagulant strength is determined by an international standard method, IDF standard 157,[12] developed jointly by IDF, International Standards Organisation (ISO) and the Association of Official Analytical Chemists (AOAC). This standard uses International Milk Clotting Units (IMCU) and measures clotting at the pH of most milk (6.5). Standard 157 uses a calf rennet preparation as the reference standard and is used to standardize rennets and FPC. A similar standard method is also available for microbial coagulants (IDF Standard 176[13]). The standard method for determining rennet composition (% chymosin and pepsin) is IDF 110B.[14] The application technology and enzymology of rennets and coagulants is a major subject that is too big to cover in this chapter, but is described and discussed in great detail in *Technology of Cheesemaking*.[10]

5.3 LACTOPEROXIDASE

Lactoperoxidase (LP) (EC 1.11.1.7) occurs naturally in raw milk, colostrum and saliva; it is thought to be part of the protective system for suckling animals against enteric infections. Lactoperoxidase is bactericidal to gram-negative bacteria and bacteriostatic to gram positives. It is a peroxidase that uses hydrogen peroxide to oxidize the thiocyanate ion to hypothiocyanate the active bactericidal molecule. Law and John[15] demonstrated that the LP

'system' (LPS) (LP + thiocyanate + hydrogen peroxide) irreversibly inhibits the membrane-energizing D-lactate dehydrogenase in gram-negative bacteria, leading to cell death. In gram-positive bacteria, the membrane ATPase is reversibly inhibited and this may be the basis of bacteriostasis, rather than death.

Although all raw milk contains LP and thiocyanate, there is not sufficient natural hydrogen peroxide to activate the enzyme system (LPS). Several methods have been devised to increase hydrogen peroxide levels in commercial raw milk supplies to provide a 'cold sterilization' system for countries with insufficient energy resources for heat treatment to preserve raw milk before consumption. Its efficacy in eliminating psychrotrophic gram-negative spoilage bacteria from raw milk stored at 4°C was demonstrated by Reiter and Marshall.[16] Although hydrogen peroxide alone can be used as a preservative in these countries, it must be used at 300–500 mg L^{-1} to be effective, and at this concentration it destroys some vitamins and impairs the functionality of the milk proteins. With the LPS, hydrogen peroxide can be generated in situ using glucose oxidase and free hydrogen peroxide levels are too low to damage the milk. Even if chemical peroxide is used instead of glucose oxidase, it needs to be added at only 10 ppm to activate the LPS. Law and Goodenough[11] have summarized these options.

5.4 CHEESE-RIPENING ENZYMES

5.4.1 Types of enzyme available commercially

The enzymes and enzyme 'packages' used to modify, enhance or accelerate the maturation of cheese are generally composed of more than one class of enzyme, and for the sake of clarity they are discussed here as a technological group, rather than as individual classes. The classes used in commercial ripening technology include many hydrolases represented by proteinases, peptidases and lipases, and if current research is successful, this list may soon extend to metabolic enzymes such as acetyl-CoA synthases and amino acid-catabolizing enzymes to generate volatile esters and sulphur compounds.

Considering the very extensive worldwide research effort and literature on the enzymology of cheese ripening,[17] it is remarkable that only a few enzyme companies have successfully developed commercial enzyme packages for cheese technology, other than the ageing enzyme-modified cheese (EMC) production methods used to make flavour ingredients for processed cheese and cheese-like foods.[18,19]

This state of affairs is partly due to the poor availability of commercial enzyme preparations that are both dedicated to cheese ripening and of proven efficacy. The research literature contains hundreds of reports of small-scale and pilot-scale enzyme trials with well-known cheese varieties but few reach the market. Thus, in contrast with the widespread use of animal (bovine, porcine, caprine) and fungal (*Aspergillus*, *Candida*, *Rhizomucor*) lipases and proteolytic enzymes in the manufacture of EMC, only one commercial system, Accelase[R] has been widely trialled in the manufacture of the established cheese varieties and their reduced fat variants.

This product was developed by IBT Ltd., now part of Danisco, from the author's basic and applied research into the role of starter enzymes and cell lysis in flavour development.[20,21] Smith[22] has described the efficacy of the commercial system. It is made up of a food-grade microbial endopeptidase (proteinase) active against all of the casein components in cheese, together with general and specific lactic acid bacteria (LAB) aminopeptidases, and undefined

esterases and flavour enzymes present in LAB cell homogenates. Extensive trial data from commercial cheese manufacture suggest that, when the product is added to cheese curd, the cheese reaches the equivalent of 9 months' maturity in only 5 months. In addition, this enzyme treatment is claimed to reduce bitterness due to certain cultures and to enhance flavour notes such as 'sulphur', acid and Cheddar. The precise mechanism of flavour enhancement is not defined but, like its research prototype, this product increases the amino acid pool in cheese, provides taste enhancement directly and increases the supply of flavour and aroma precursors in the presence of added LAB biomass. The emerging research on amino acid-catabolizing enzymes in LAB also suggest that Accelase may not only increase the cheese amino acid pool, but also increase its enzymatic turnover to flavour and aroma compounds.

Enzymes such as 'Rulactine' (Rhone-Poulenc) and 'Flavorage' (Chr. Hansen, US, Inc.) have been marketed as cheese-ripening enzymes, but little information is available as to their efficacy and market uptake. 'Rulactine' is a proteinase from *Micrococcus* sp. and 'Flavorage' contains a lipase from *Aspergillus* sp. together with proteolytic enzymes.

The research literature suggests that one day cheese makers will *knowingly* benefit from enzymes that convert amino acids into sulphur volatiles, esters, aldehydes, amines, ammonia and fatty acids. However, many obstacles stand in the way of commercialization, not least the instability and low production levels of these enzymes in their natural host microorganisms. Also, some of these conversions require enzyme/cofactor complexes that are only sustainable in whole cell environments, and GM variants of the wild-type cheese bacteria with selectively enhanced activities may be the only route to the technology.

Before leaving the topic of enzyme availability, it is worth mentioning the very interesting approach[23] using urokinase added to cheese milk to activate plasminogen to plasmin in the cheese. The increased plasmin activity accelerates proteolysis during maturation, and would presumably accelerate texture development in hard and semi-hard cheese as a consequence. If this enzyme technology could be economically viable, it would link up well with various forms of peptidase enrichment ('Accelase' and GM peptidase mutants). The activation of plasmin as an indigenous milk proteinase component of a ripening system after whey separation and curd formation would also overcome one of the practical difficulties of incorporating enzymes intimately into the cheese matrix and is discussed next.

5.4.2 Enzyme addition technology

As is the case with most good scientific ideas, technology transfer to the real world of process innovation and product manufacture presents unique and often unforeseen challenges. Thus was clearly the case when proteolytic enzymes were introduced into cheese. Figure 5.1 illustrates the stages in the manufacture of hard and semi-hard cheese that could be the addition points for ripening enzymes. This plan applies to any enzyme, but the process implications are more serious in the case of proteinases, hence the emphasis.

Proteinases added to break down casein in cheese are needed only in very small amounts because, like all enzymes, they are catalysts and a small quantity will convert a large amount of substrate. This is fine from the point of view of cost and conversion efficiency, but it means mixing grams of the active enzyme with tonnes of cheese. Putting enzymes evenly into the complex cheese matrix is difficult enough in itself, but the problem of distributing such *small* amounts is far from trivial. Enzyme addition to the cheese milk at point A (Fig. 5.1) would be ideal logistically, because the starter and rennet are also added here and thoroughly mixed in. However, unlike these traditional parts of the recipe, ripening proteinases quickly begin removing soluble peptides from caseins. These peptides are lost into the whey when

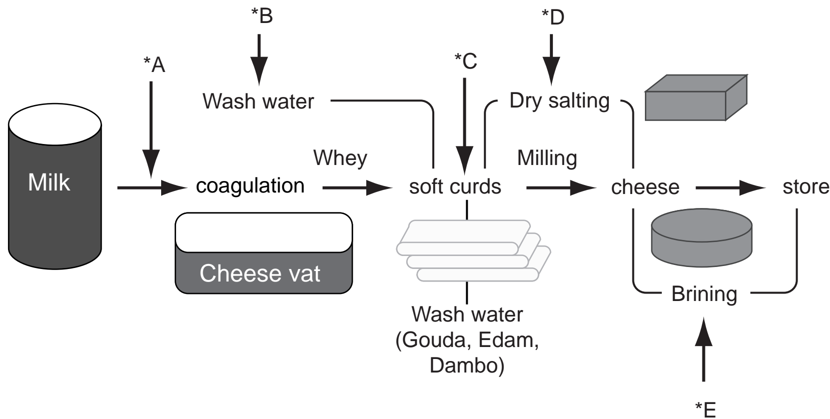


Fig. 5.1 Possible enzyme addition points (*) during the manufacture of hard and semi-hard cheese.

the cheese curd is separated, causing unacceptable losses to cheese yield. Also, the early breakdown of caseins disrupts their orderly structure, prevents proper gel formation and renders the curds too soft and unworkable in the later stages of curd acidification, prior to salting and pressing into cheese. Add to these problems the loss of added enzymes into the whey (at a rate of about 95%) and it is clear that addition of proteinases directly to the milk is not an option. If peptidase preparations were very inexpensive, they could be added by this route, but most large cheese plants sell their whey as concentrates to be added to foods for their functionality. Any carry-over of ripening enzymes would have to be removed or destroyed before the whey was processed and sold.

Enzyme microencapsulation is the obvious solution to the above problem, to protect caseins in the milk and ensure physical entrapment of the enzymes in the curd gel matrix. Options include fat, starch or gelatine capsules, but none of these has a satisfactory 'release' mechanism in cheese. The author's research group developed a special type of phospholipid liposomes[24,25] as an effective technology to overcome this problem. Proteinases and peptidases were entrapped in the liposomes and added to cheese milk. Most of the ripening enzymes were entrapped in the water spaces of the curd particles and very little was lost in the whey. The liposomes were degraded naturally in the cheese matrix after whey separation and this allowed full contact between the protein matrix and the ripening enzymes. However, although the technique has since been adopted in numerous experimental cheese trials,[26] the high cost of the pure phospholipids necessary to make stable, high-capacity liposomes rules this out as a large-scale commercial technology. More recently, liposomes have been available commercially in the form of proliposome mixture (Prolipo-Duo™, Lucas Meyer, France) and used to put enzymes into Cheddar cheese.[27] However, an economical assessment of this technology compared with the earlier work with 'home-made' liposomes has not been made yet. Other approaches to proteinase encapsulation have been summarized by Kailasapathy and Lam,[28] including the use of food-grade gums, and this seems to offer new opportunities for cheese-like products. However, the cheese regulations in the major cheese-producing countries would currently not permit gums as ingredients in natural cheese.

The semi-hard cheeses typified by Gouda and Edam are made by a process which includes a curd 'washing' stage to replace some of the whey with water to reduce acidity. Although this stage (point B in Fig. 5.1) and the soft curd stage (point C) offer a further opportunity

to introduce enzymes uniformly into the cheese matrix, they both create problems through premature curd softening, yield reduction and loss of 'unincluded' enzyme from the curds. Washed-curd cheeses are brine-salted, rather than dry-salted, and this may appear to offer an enzyme addition point (E in Fig. 5.1), but researchers can predict that enzyme penetration from brine into the closed texture of pressed cheese would be very low, ruling out this route, and making washed-curd cheese very difficult to treat with ripening enzymes.

In dry-salted cheese varieties, such as Cheddar, the addition of enzymes to milled curd with the salt (point D, immediately before pressing to form cheese blocks) was originally proposed[29] for laboratory-scale cheese making and this was successfully adapted to a 180 L vat scale.[30] However, this technique is difficult to adapt and scale up to automated salting equipment which is used in large throughput cheese plants. Although enzymes can be granulated with dry salt, this is an expensive process for cheap ingredients and it is not widely used. An alternative method for enzyme addition was patented recently, involving mixing curds and enzyme in a vessel to which 'negative pressure' is applied, so that the enzyme is 'sucked in' to the curd matrix.[31] However, it is not clear how the problems of even distribution and alteration of curd moisture and structure are solved by this invention. Although Wilkinson and Kilcawley[32] suggested in their review that mechanical injection of enzymes may offer new solutions to addition at the finished cheese stage, there are no such efficacious technologies on the market at this stage.

Whatever physical method is employed to place enzymes into cheese curd, some kind of vehicle is needed to disperse them and this is either water or some other natural constituents of cheese (salt, fat, etc.).

Thus, this whole area of enzyme addition technology is in urgent need of radical new ideas from the research base, but researchers also need feedback from cheese technologists and business economists. For example, there is sufficient know-how in molecular and applied enzymology to devise matrices and support materials to create microparticulate enzyme complexes which could both liberate and metabolize amino acids, fatty acids and sugars to known flavour and aroma compounds. Such expertise has been generated in the fields of low-water enzymology, immobilized enzyme science, cellular enzymology and membranology, but as yet there has been no incentive to apply this to cheese-ripening research. This is understandable in current circumstances, because the logical route to the use of complex enzyme systems that are easy to put into cheese is *via* whole cell technology – nature has done the work for us, so why undo it? The simple answer is that nature designed microbial cells for efficient life processes, not for efficient cheese-ripening technology, and the natural microbial cell chemistry and architecture need modification to put the (technologically) right combinations together. Gene technology can do this for us within the whole cell technology concept but however safe this is made, the tide of media and consumer opinion is firmly against developments along this route. Nevertheless, whole cell options are available, some without GM and some with and they are currently favoured by companies involved in developing cheese-ripening systems.[17]

5.4.3 Enzyme-modified cheese technology

EMC is not really cheese from the consumer food point of view. It is a highly flavoured ingredient for processed cheese, cheese-flavoured snack foods and sauces, made by incubating emulsified cheese homogenates with animal or GRAS (generally recognized as safe) microbial lipases and proteinases. This technology was first approved in the USA in 1969 and its products quickly came into large-scale use in processed cheese in 1970. All of the major dairy ingredient suppliers now have extensive and relatively sophisticated EMC-based

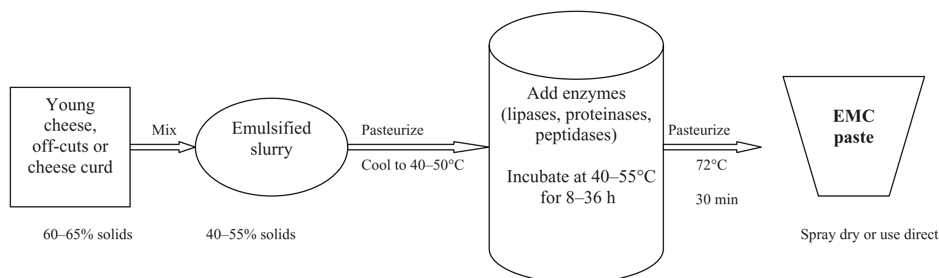


Fig. 5.2 Production flow diagram for enzyme-modified cheese (EMC).

flavour product lines; their production methods are based on patents and a large body of proprietary knowledge,[19] but the general flow of the process is universal (Fig. 5.2).

The raw material for EMC manufacture is young bland hard or semi-hard cheese, cheese off-cuts and/or fresh salted Cheddar cheese curd. This is blended to homogeneity with emulsifying salts as a semi-liquid slurry (40–45% solids), pasteurized for 10 min at 72°C, then cooled ready for enzyme treatment. The incubation temperature for enzyme treatment depends on the flavour reactions to be used to make the product. For example, 25–27°C is suitable for making a blue cheese flavour product using a *Penicillium roqueforti* mould culture to grow and metabolize milk fatty acids to the characteristic methyl ketones. However, the slurry would need a pretreatment at 40–45°C with a lipase to generate sufficient short and medium chain free fatty acids for rapid development of blue cheese flavour.

The most common emulsifiers and stabilizers used in EMC production include mono- and diglycerides, phosphates, citric acid and xanthan gum. Antioxidants are usually added in the form of plant oils and fat-soluble vitamins (e.g. tocopherols). The basic flavours generated enzymatically can be 'topped up' using food-grade nature-identical aroma compounds such as aldehydes, lactones and alcohols, or refined by fermentation with dairy cultures of LAB and moulds.

Cheddar, Parmesan, Romano, Swiss-type and Gouda-flavoured EMCs require a composite enzyme treatment, using lipases, proteinases and peptidases to develop the characteristic savoury, pungent and lipolyzed notes in balance. The choice of incubation temperature (or indeed temperature*s*) is critical for the balance of these flavour notes, but is generally in the range of 40–55°C. This range is also governed by a compromise between the need for a short incubation time at high temperature for process efficiency and reduction of microbiological spoilage on one hand; and the need to avoid temperature denaturation of the enzymes. EMC producers would like to have more robust microbial enzymes that could produce the required flavour biochemicals from the raw material in a few hours at temperatures up to 70°C. However, this might create new shelf life and users' problems from residual enzyme activity in the product. Currently, EMC products are pasteurized at 72°C for 30 min after enzyme incubation to destroy residual activity and eliminate spoilage microorganisms.

EMCs are spray dried or packaged as pastes of different water content, dependent on customer preference and intended food use.

5.5 LYSOZYME

Lysozyme (EC 3.2.1.17) is a hydrolase widely distributed in nature; it is bactericidal to many gram-positive species because it breaks down their cell walls. The enzyme is a mucopeptide

N-acetyl muramoylhydrolase, available commercially from hen egg white or *Micrococcus lysodeikticus*. The food-grade preparations are from egg albumin.

Lysozyme is sold by the major dairy enzyme suppliers as an alternative control agent for 'late blowing', the textural defect of slits and irregular holes caused by the butyric fermentation in Gouda, Danbo, Grana Padano, Emmental and other important hard and semi-hard cheese varieties. Traditionally the defect, caused by *Clostridium tyrobutyricum* in raw milk, has been controlled by the addition of potassium nitrate to the cheese milk. However, this practice will be phased out because it is associated with the production of carcinogens, and lysozyme has become the preferred control agent.[11,33] *C. tyrobutyricum* is a spore former and as such cannot be killed by pasteurization, hence the need to treat the milk by alternative methods. Lysozyme kills vegetative cells and also inhibits outgrowth of spores in cheese; it is stable for long periods in the cheese matrix and because it binds to the cheese curd, little of the enzyme is lost on whey separation. Although lysozyme also inhibits the LAB used as starters in cheese making, they are less sensitive than the Clostridia and a typical enzyme dose rate of 500 units mL^{-1} is sufficiently selective (commercial lysozyme preparations contain about 20 000 units mg^{-1}). Nevertheless, some thermophilic lactobacilli used in Grana cheese making are very sensitive, but can be 'conditioned' by unknown mechanisms by repeated growth on lysozyme-containing media.[33]

Lysozyme also inhibits the growth of *Listeria monocytogenes* in yoghurt and fresh cheese with high acidity ($<$pH 5.0), but the effect is not consistent enough to rely on in commercial-fermented milk products, and in any case high acidity is usually sufficient in itself to inhibit these pathogens.

5.6 TRANSGLUTAMINASE

With the recent availability of commercial microbially derived transglutaminase (protein-glutamine γ-glutamyltransferase; EC 2.3.2.13) preparations, there has been considerable interest in their application to the gelation of caseins and whey proteins, though this is not a widespread technology yet. However, transglutaminase is effective in reducing syneresis in acid milk gels and has been investigated as a method of improving the texture and shelf life of yoghurt.[34] Specifically, transglutaminase has been shown to improve the emulsifying properties of milk proteins[35] and increases the viscosity and water-holding capacity of yoghurt.[36]

5.7 LIPASE

Although lipases are used in cheese flavour technology as components of the ripening systems already discussed in this chapter, they are also used to produce modified milk fat products for other food applications.[37]

5.7.1 Lipolyzed milk fat (LMF)

LMF has a creamy, buttery and cheesy aroma derived from short to medium chain fatty acids and fatty acid chemical derivatives released from milk fat by lipases. The raw material substrate for manufacturing LMF is either condensed milk or butter oil emulsified to maximize

the fat surface area to activate the lipase. Lipases are added and left in contact with the substrate at the optimum temperature for the enzyme(s) used, until the required flavour/aroma is achieved, or until a predetermined acid degree value (ADV) is reached, corresponding to a measurable release of fatty acids by the lipase.

The product is pasteurized, spray dried or otherwise adjusted to a standard solids content and packaged. LMF products include chocolate coatings and syrups, butter flavours for margarine, artificial creams and sauces, flavourings for coffee whiteners and cheese flavour additives.[38]

The type of lipase used to make LMF products depends on the intended food application. Generally, good LMF products for use in baked products can be made using pancreatic lipase preparations, lamb and kid pregastric esterase and fungal/mould lipases from *A. niger*, *Geotrichum candidum* and *P. roqueforti*. Some bacterial lipases are also suitable (*Achromobacter lipolyticum* and *Pseudomonas* lipase) but the LMFs for bread baking should not be prepared with *Achromobacter*, *Penicillium* or *Geotrichum* lipase to avoid soapy and musty flavours. Furthermore, the pregastric esterases are also known to produce too high a proportion of butyric acid for bread making, in which they tend to produce rancid, sweaty flavour notes.[37]

This technology and its food applications have been comprehensively reviewed recently, and the interested reader can find detailed information therein.[39]

5.7.2 Lipase-catalyzed intra- and intermolecular modification of milk fat

Chemical interesterification, acidolysis, alcoholysis and transesterification have been used for many years to modify the physical/functional properties of milk fat, but more recently lipase technology has replaced this chemical technology to give more precise and 'cleaner' processing.[39,40, 41] In particular, milk fat substitutes have been prepared as a partial replacement for the milk fat in baby foods.[42] However, fat fractionation by physical methods is the commercially preferred option for milk fat modification in dairy product applications. Coverage of lipase-catalyzed molecular modification will be confined in this chapter to reference to the reviews cited above.

5.8 LACTASE

Lactase (β-galactosidase; EC 3.2.1.23) hydrolyzes lactose to its constituent monosaccharide sugars, galactose and glucose. The enzyme is widespread in animals, but it has only become important technologically since microbial sources have become readily available.[43] The principal commercial preparations are sourced from *A. niger, Aspergillus oryzae, Candida pseudotropicalis* and *K. lactis.* Lactase applications are in batch an immobilized enzyme technology favouring the *Aspergillus* and *Kluyveromyces* sources, respectively.

5.8.1 Commercial dairy products of lactase technology

Lactase in tablet form (e.g. Lactaid^{R}) is sold as an in vitro remedy for lactose intolerance, a widespread condition (affecting 30–50 million people in the USA, for example) caused by a deficiency of lactase in the digestive tract. Sufferers experience stomach cramps, bloating

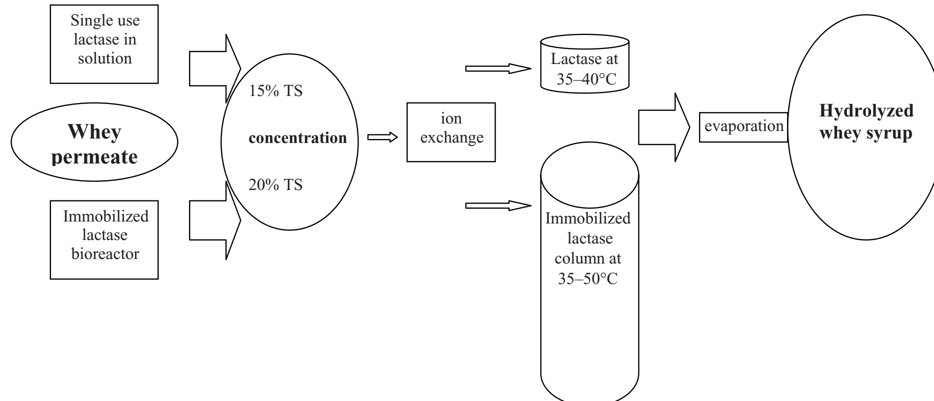


Fig. 5.3 Flow diagram of lactase-based lactose hydrolysis process using batch solution method and immobilized enzyme method.

and diarrhoea due to the accumulation of lactose in the gut lumen. Lactaid^{R} tablets deliver active lactase to the gut to break down the ingested lactose and alleviate the intolerance symptoms.

Hydrolyzed whey syrup is produced from whey, the by-product of cheese making and casein production. The hydrolysis step can be on the whey itself, or on the permeate from ultrafiltration (UF) plant used to make WPC. The UF permeate still contains some whey protein but is enriched in lactose. It is concentrated to 15–20% total solids (TS), demineralized, usually by ion exchange, though electrodialysis or nanofiltration can be used for this, then heated according to the type of lactase treatment to be used. The hydrolysis step can be by batch treatment with yeast lactase, though the use of immobilized enzyme reactor columns is more efficient, using *Aspergillus* lactase. Immobilized lactase reactors can achieve up to 90% lactose hydrolysis in whey permeate, whereas the batch process converts only about 70%. However, both processes produce sufficient free glucose and galactose to make the product sweet, and this property is enhanced by evaporation of the hydrolyate to 60% TS to make the final syrup. A typical flow diagram for the process is shown in Fig. 5.3.

The whey syrup is made sticky by the high concentration of glucose and galactose, so it is not dried, but sold and used in that form. It is used in food manufacture to replace sweetened condensed milk, sugar and skim milk in many products such as ice cream, milk desserts and sauces. The syrup is also an excellent caramel ingredient and as a sweetener/binder in cereal bars.

REFERENCES

1. Harboe, M. and Budtz, P. (1999) The production, action and application of rennet and coagulants. In: *Technology of Cheesemaking* (ed. B.A. Law). Sheffield Academic Press, Sheffield, pp. 33–65.
2. Lomholt, S.B. and Qvist, K.B. (1999) The formation of cheese curd. In: *Technology of Cheesemaking* (ed. B.A. Law). Sheffield Academic Press, Sheffield, pp. 66–98.
3. Foltmann, B.F. (1992) Chymosin: a short review on foetal and neonatal gastric proteases. *Scandinavian Journal of Clinical and Laboratory Investigation* **52**, 65–79.
4. Fernandez-Salguero, J., Tejada, L. and Gomez, R. (2002) Use of powdered vegetable coagulant in the manufacture of ewe's milk cheese. *Journal of the Science of Food and Agriculture* **82**, 464–468.

5. Galan, E., Prados, F., Pino, A., Tejada, L. and Fernandez-Salguero, J. (2008) Influence of different amounts of vegetable coagulant from cardoon *Cynara cardunculus* and calf rennet on the proteolysis and sensory characteristics of cheese made with sheep milk. *International Dairy Journal* **18**, 93–98.
6. Tejeda, L., Abellan, A., Cayuela, J.M., Martinez-Cacha, A. and Fernandez-Salguero, J. (2008) Proteolysis in goats' milk cheese made with calf rennet and plant coagulant. *International Dairy Journal* **18**, 139–146.
7. Harboe, M., Broe, M.L. and Qvist, K.B. (2009) The production, action and application of rennets and coagulants. In: *Technology of Cheesemaking* (eds B.A. Law and A. Tamime). Wiley Blackwell, Oxford (in press).
8. Goodenough, P.W. (1995) Food enzymes and the new technology. In: *Enzymes in Food Technology*, 2nd edn (eds G.A. Tucker and L.F.J. Woods). Blackie Academic & Professional, Glasgow, pp. 41–113.
9. Bansal, N., Drake, M.A., Pirainoc, P., Broe, M.L., Harboe, M, Fox, P.F. and Mc Sweeney, P.H.L. (2008) Suitability of recombinant camel (*Camelus dromedarius*) chymosin as a coagulant for Cheddar cheese. *International Dairy Journal* **18** (in press).
10. Law, B.A. (ed.) (1999) *Technology of Cheesemaking*. Sheffield Academic Press, Sheffield.
11. Law, B.A. and Goodenough, P.W. (1995) Enzymes in milk and cheese production. In: *Enzymes in Food Technology*, 2nd edn (eds G.A. Tucker and L.F.G. Woods). Blackie Academic & Professional, Glasgow, pp. 114–143.
12. International Dairy Federation (2007) *Standard 157 Determination of Total Milk-Clotting Activity of Bovine Rennets*. International Dairy Federation, Brussels.
13. International Dairy Federation (1996) *Provisional Standard 176. Determination of Total Milk Clotting Activity of Microbial Coagulants*. International Dairy Federation, Brussels.
14. International Dairy Federation (1997) Standard 110 B. Calf Rennet and Adult Bovine Rennet: Determination of Chymosin and Bovine Pepsin Contents (Chromatographic Method). International Dairy Federation, Brussels.
15. Law, B.A. and John, P. (1981) Effect of LP bactericidal system on the formation of the electrochemical proton gradient in *E. coli*. *FEMS Microbiology Letters* **10**, 67–70.
16. Reiter, B. and Marshall, V.M. (1979) Bactericidal activity of the lactoperoxidase system against psychrotrophic *Pseudomonas* spp. in raw milk. In: *Cold Tolerant Microbes in Spoilage and the Environment* (eds A.D. Russel and R. Fuller). Academic Press, London, pp. 153–164.
17. Law, B.A. (2001) Controlled and accelerated cheese ripening: the research base for new technologies. *International Dairy Journal* **11**, 383–398.
18. West, S. (1996) Flavour production with enzymes. In: *Industrial Enzymology*, 2nd edn (eds T. Godfrey and S. West). Macmillan Press/Stockton Press, Basingstoke/New York, pp. 209–224.
19. Kilcawley, K.N., Wilkinson, M.G. and Fox, P.F. (1998) Enzyme-modified cheese. *International Dairy Journal* **8**, 1–10.
20. Law, B.A., Sharpe, M.E. and Reiter, B. (1974) The release of intracellular dipeptidase from starter streptococci during Cheddar cheese ripening. *Journal of Dairy Research* **41**, 137–146.
21. Law, B.A. and Wigmore, A.S. (1983) Accelerated ripening of Cheddar cheese with commercial proteinase and intracellular enzymes from starter streptococci. *Journal of Dairy Research* **50**, 519–525.
22. Smith, M. (1997) Mature cheese in four months. *Dairy Industries International* **62**, 23–25.
23. Barrett, F.M., Kelly, A.L., McSweeny, P.L.H. and Fox, P.F. (1999) Use of exogenous urokinase to accelerate proteolysis in Cheddar cheese during ripening. *International Dairy Journal* **8**, 421–427.
24. Law, B.A. and King, J.C. (1985) The use of liposomes for the addition of enzymes to cheese. *Journal of Dairy Research* **52**, 183–188.
25. Kirby, C.J., Brooker, B.E. and Law, B.A. (1987) Accelerated ripening of cheese using liposome-encapsulated enzyme. *International Journal of Food Science and Technology* **22**, 355–375.
26. Skie, S. (1994) Development in microencapsulation science application to cheese research and development: a review. *International Dairy Journal* **4**, 573–595.
27. Laloy, E., Vuillemard, J.C. and Simard, R.E. (1998) Characterisation of liposomes and their effect on Cheddar cheese properties during ripening. *Le Lait* **78**, 401–412.
28. Kailasapathy, K. and Lam, S.H. (2005) Application of encapsulated enzymes to accelerated cheese ripening. *International Dairy Journal* **15**, 929–939.
29. Kosikowski, F.V. (1976) Flavour development by enzyme preparations in natural and processed cheese. US Patent 3,975,544.
30. Law, B.A. and Wigmore, A.S. (1982) Accelerated cheese ripening with food grade proteinases. *Journal of Dairy Research* **49**, 137–146.

31. Rhodes, K. (1999) System and method of making enhanced cheese. World Patent, 09921430A1.
32. Wilkinson, M.G. and Kilcawley, K.N. (2005) Mechanisms of incorporation and release of enzymes into cheese during ripening. *International Dairy Journal* **15**, 817–830.
33. International Dairy Federation (1990) Use of enzymes in cheesemaking. *IDF Bulletin* **247**, 24–38.
34. Motoki, M. and Seguro, K. (1998) Transglutaminase and its use in food processing. *Trends in Food Science and Technology* **8**, 204–210.
35. Hinz, K., Huppertz, T., Kulozic, U. and Kelly, A.L. (2007) Influence of enzymatic cross-linking on milk fat globules and emulsifying properties of milk proteins. *International Dairy Journal* **17**, 288–293.
36. Ozer, B., Kirmaci, H.A., Oztekin, S., Hayaloglu, A. and Atamer, M. (2007) Incorporation of microbial transglutaminase into non-fat yogurt production. *International Dairy Journal* **17**, 199–207.
37. Kilara, A. (1985) Enzyme-modified lipid food ingredients. *Process Biochemistry* **20**, 33–45.
38. Dziezak, J.D. (1986) Enzyme modification of dairy products. *Food Technology* **40**, 114–120.
39. Balcao, V.M. and Malcata, F.X. (1998) Lipase catalysed modification of milk fat. *Biotechnology Advances* **16**, 309–341.
40. Macrae, A.R. and Hammond, R.C. (1985) Present and future applications of lipases. *Biotechnology and Genetic Engineering Reviews* **3**, 193–217.
41. Pandey, A., Benjamin, S., Soccol, C.R., Nigam, P., Krieger, N. and Soccol, V.T. (1999) The realm of microbial lipases in biotechnology. *Biotechnology and Applied Biochemistry* **29**, 119–131.
42. King, D.M. and Padley, F.B. (1990) Fat composition. European Patent 0209327.
43. Wigley, R.C. (1996) Cheese and whey. In: *Industrial Enzymology*, 2nd edn (eds T. Godfrey and S. West). Macmillan Press/Stockton Press, Basingstoke/New York, pp. 133–154.

6 Enzymes in bread making

Maarten van Oort

6.1 INTRODUCTION

Baking is a common name for the production of baked goods, such as bread, cake, pastries, biscuits, crackers, cookies, pies and tortillas, where wheat flour is both the most essential ingredient and key source of enzyme substrates for the product. Wheat's popularity is also due to the ease at which it grows under a variety of moderate temperature conditions, soils, regions and seasons.

All wheats belong to the genus Triticum, of the Gramineae family. Common wheat (Triticum aestivum) and durum wheat (Triticum durum) are the two major wheat groups which are used for food applications. Bread is the product of baking a mixture of flour, water, salt, yeast and other ingredients. The basic process involves mixing of ingredients until the flour is converted into a dough, followed by baking the dough into bread.

The aims of the bread-making processes are to produce dough that will rise easily and have properties required to make good bread for the consumer. To make good bread, dough made by any process must be extensible enough to expand during proofing. Bread dough must also be elastic. Elastic dough has the strength to hold the gases produced while rising and stable enough to hold its shape and cell structure.

6.1.1 Wheat

Wheat is one of the major crops in the world with an annual production of around 550 million ton. Depending on the region, either corn, wheat or rice is the dominant food grain.

Compared with corn and rice, wheat contains the unique gluten-forming proteins, which are intrinsically linked to baking.

6.1.2 Wheat flour constituents

Endosperm is the interior of a wheat kernel and makes up about 83% of the whole grain of wheat and once ground down to a powder, is flour.

Flour contains mostly starch. However, other components in the flour also clearly affect its properties. The main components are starch (70–75%), protein (9–14%), lipids (1–3%), non-starch carbohydrates (1–2%), ash (around 0.5%), lipids (1–2.5%) and moisture (13–14%).

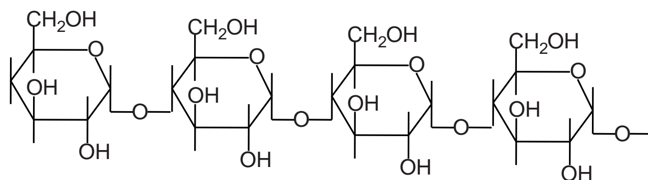


Fig. 6.1 Schematic representation of amylose.

6.1.3 Starch

Starch is the most abundant component and the most relevant reserve constituent of cereals. Starch consists primarily of D-glucopyranose polymers linked together by α-1,4 and α-1,6 glycosidic bonds. The glycosidic linkages in starch are in the alpha (α) configuration. This is determined by the orientation of the hydroxyl (—OH) group on C1 of the pyranose ring. The α-linkage allows starch polymers to form helical structures. The significance of the helical geometry becomes clear when starch is compared with cellulose. Cellulose is a glucose polymer with β-1,4 linkages between the glucopyranose subunits. Because of this configuration, cellulose forms sheeted structures whereas starch polymers are usually helical. This has a strong effect on physicochemical properties and enzyme susceptibility.

In starch there are two types of polymers, amylose and amylopectin.

Amylose (Fig. 6.1) is essentially a linear polymer, although it is known that some branches are present.[1] From the shape of the amylose chain, it becomes clear that this molecule forms helices due to the α-linkages.

Amylopectin (Figs 6.2(a) and (b)) is a branched molecule and is on average a much larger polymer than the amylose polymers.

The structural differences between the two polymers determine the differences in starch properties and starch functionality. Some of the functional differences are listed in Table 6.1.

Although amylose is usually illustrated as a linear chain, the molecule is often helical. The interior of the helix contains hydrogen atoms and can be considered as hydrophobic. This in turn allows amylose to form complexes with free fatty acids, fatty acid components of certain lipids, some alcohols and also iodine.

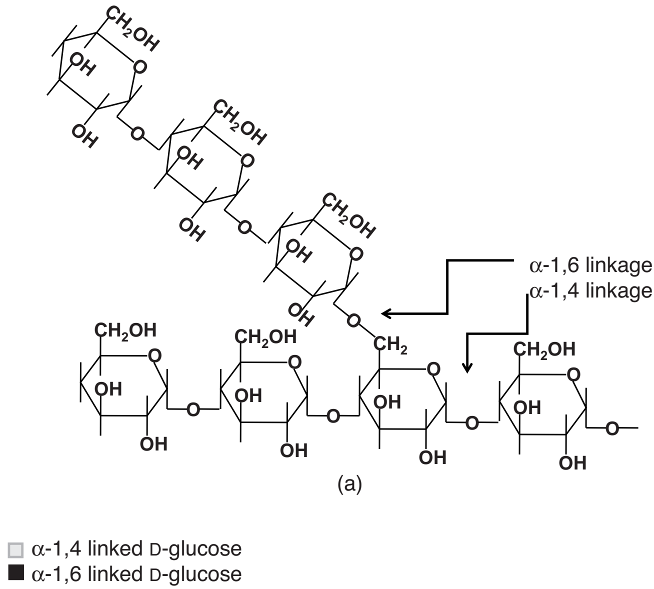
Complexation with lipids is an especially well-known property of the amylose helix and can alter the properties of starch, such as gelatinization temperature, viscosity and retrogradation, considerably.

Native starch granules are insoluble in cold water. However, upon heated with water, granules undergo a substantial change resulting in a complete change of properties and behaviour. This irreversible process is named gelatinization, which in wheat occurs between 52°C and 85°C.

During heating in water, linear amylose molecules start leaking out of the starch granule, and with continued heating additional amylose and also amylopectin leach out.

After heating, solubilized starch polymers and remaining insoluble granular fragments start to reassemble into an organized structure again. Ultimately, a crystalline structure is formed. This process is known as retrogradation. Linear amylose molecules have a greater tendency to reassociate and form crystalline and gel structures than the larger amylopectin molecules.[2]

The process of retrogradation is closely linked to the problem of staling in baked products. This will be discussed further in Section 6.2.



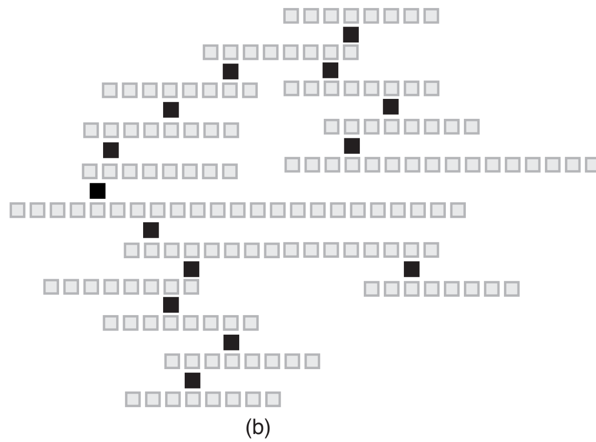


Fig. 6.2 Schematic representations of amylopectin, showing the 1,4 and 1,6 glucosidic linkages (a) and what that means for the whole structure (b).

Table 6.1 Different characteristics of amylose and amylopectin.

Characteristic	Amylose	Amylopectin
Shape	Essentially linear	Branched
Linkage	α-1,4 ($\pm$1/1000 α-1,6)	α-1,4 and α-1,6 ($\pm$1/25)
Molecular weight	Typically 10^5–10^6	10^7–10^9
Films	Strong	Weak
Gel formation	Firm	Non-gelling/soft
Colour with iodine	Blue	Reddish brown

6.1.4 Gluten

Wheat protein is seen as the most important factor governing bread-making quality.[3–5] A high protein content is related to good bread-making quality. There are also a number of other factors, together described as protein 'quality' that determine the bread-making potential of a wheat flour.[6]

In wheat kernels, 80% of the protein is found in the endosperm. Wheat flour proteins have been classified into four types based on their solubility[7]: albumins, soluble in water; globulins, soluble in salt solutions; gliadins, soluble in 70% ethanol; and glutenins (partly) soluble in dilute acid or alkali. The bulk of the protein is formed by the latter two: gliadins and glutenins. All these fractionations lead to broad overlapping classes of proteins, indicating the complexity and variability of the various classes. There is also only a limited relationship between the amounts of each of the Osborne fractions and the final bread-making quality.[8]

When flour is mixed with water, a viscoelastic mass is formed. From this mass, starch can be washed out and then gluten remains. On a dry basis, gluten contains around 70–85% protein, 5–15% carbohydrates (starch and non-starch polysaccharides (NSP)), 3–10% lipids and 1–2% ash.[5,9]

Gluten proteins contain relatively high amounts of glutamic acid, which exists as glutamine in the wheat kernel, proline, hydrophobic amino acids and the sulphur-containing cysteine. Glutamine and also the hydrophobic amino acids ensure sufficient hydrogen bonding during mixing. This helps in film formation. Proline, because of its ring structure, disrupts the α-helix formation that normally occurs in protein polymers. The result is a higher percentage of β-sheet structure, which helps in giving gluten the necessary elasticity. The sulphur-containing amino acids ensure the formation of disulphide bridges between protein chains and also within protein chains, giving the protein network sufficient strength.

The effect of all these amino acids together is a rapid continuous film formation which gives wheat dough its unique viscoelastic properties and gas holding capacity.[10]

The viscoelastic behaviour of wheat gluten sets it apart from other grains or other vegetable protein sources.[11] The formation of gluten is the result of interaction between the two major classes of wheat proteins, the gliadins and glutenins. When flour is mixed with water, a viscoelastic mass is formed, to a large extent due to this interaction.

The sulphur-rich glutenins are able to form polymeric networks. The sulphur-poor gliadins are present mainly as monomers. Glutenins consist of a high molecular weight (HMW) group and a low molecular weight (LMW) group of proteins. Both groups are able to form disulphide polymeric networks. Gliadins behave as a viscous liquid, whereas glutenins behave as a cohesive elastic solid.[12] In the gluten network, these properties are combined into a protein network with viscoelastic properties, which enables gas cells to be retained in a dough during the bread-making process. This highly specific property allows wheat flour to be used for production of a variety of yeast leavened or chemically leavened products.

The composition and strength (quality) of the gluten is partly determined by the presence and abundance of individual glutenin subunits. The relative amounts of each subunit are determined by genetic factors, growing conditions and fertilization. Glutenin polymers are very heterogeneous in composition and size. Certain fractions of the HMW glutenins are highly correlated with loaf volume, whereas others are not.[13,14] A glutenin fraction that is insoluble in sodium dodecyl sulphate (SDS) solution, has been called glutenin macropolymer (GMP).[15] This fraction has been studied in great detail and evidence has been presented showing that this GMP can be seen as a wheat quality parameter.[16]

A large number of HMW glutenin subunits have been sequenced, relations between various proteins and protein fractions have been established; the influence of glutenin subunits and combinations of subunits on bread-making quality has been investigated.[17] Nevertheless, there is still a great deal unknown, mainly due to the complexity of the gluten network and the large amount of various subunits in the network.

Oxidizing and reducing agents have shown to affect the gluten structure. Ascorbic acid, potassium bromate and cysteine have been used for decades as dough improvers. Modification of the redox status of disulphide bonds or thiol groups significantly changes the polymerization of glutenin subunits of wheat gluten. This in turn affects the mechanical and viscoelastic properties of the dough.

6.1.5 Non-starch polysaccharides

NSP originate from the cell wall of the aleurone and the endosperm of the wheat kernel. NSP represents different polysaccharides. One group of polysaccharides is built up by pentose sugars and some hexose sugars. These are called pentosans and main components are pentose sugars arabinose (A) and xylose (X). The structure of arabinoxylans (AX) is a linear backbone of β-1,4 linked xylose residues carrying single arabinose residues on their C-3 position or both on C-2 and C-3 positions. The molecular weight of AX ranges from 20 000 to 5 000 000 D.[18,19] AX together with cellulose, β-glucans, arabinogalactan-peptide and other minor constituents like galactomannan, glucomannan and xyloglucan are referred to as NSP. Some arabinose residues are esterified with ferulic acid (FA). FA (4-hydroxy-3-methoxycinnamic acid) is the natural component of water extractable AX (WE-AX) and water unextractable AX (WU-AX), although free, soluble bound FA and insoluble bound FA all have been found in flour and in gluten.[20]

Despite their low content in flour (2–3% at normal extraction rates), pentosans are very important in determining dough properties, gluten quality and final bread quality.[21,22] Due to their high water binding capacity, AX play a regulatory role with respect to the water economy in bread making.[23] Furthermore, gluten properties and also dough properties are influenced by interactions between proteins and pentosans.[24]

WE-AX have some unique physical properties, such as binding up to 10 times its own weight of water,[18,25] formation of highly viscous solutions[26] and gels due to covalent cross-linking.[27,28] All these properties have direct functional implications in gluten formation and dough properties. In general, it is believed that WE-AX have a positive effect on bread making,[29] whereas WU-AX are reported to have a strong negative effect on bread-making quality.[30–32] However, Wang[24] has shown that both WE-AX and WU-AX have similar effects on gluten yield, GMP yield, on the composition of gluten and GMP, and on the properties of gluten and GMP.

The negative effects of WU-AX (and WE-AX) on bread-making quality is the main reason why arabinoxylan-modifying enzymes are successfully implemented in almost all bread-making procedures all over the world.[33]

Thanks to the feruloyl adducts (Fig. 6.3), pentosans are subject to oxidative gelation.[26,27] Many studies have been carried out on the oxidative gelation of both WE-AX and also WU-AX.[22,28,34–36] Among various oxidative systems, peroxidases and laccases have been successfully applied as gelling agents of AX solutions.[28,37,38]

This oxidation is generally assumed to occur through cross-linking of free FA residues.

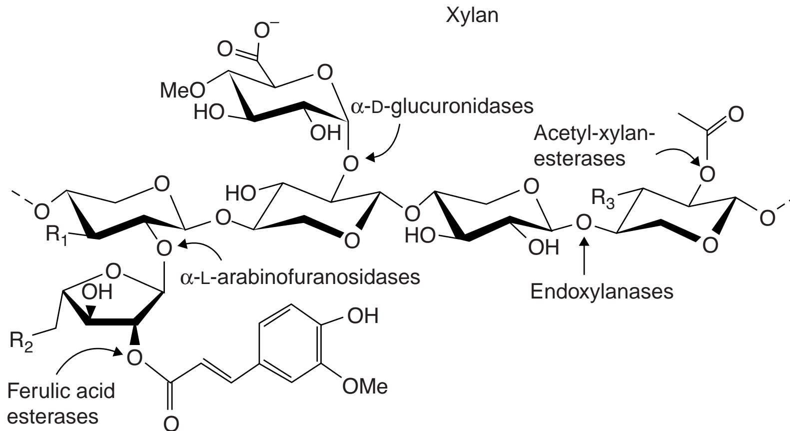


Fig. 6.3 Plant xylan structure with bound ferulic acid.[26]

One possible mechanism is through dimerization of FA residues on adjacent arabinoxylan chains. Proteins also participate in the gelation process, since the gel fraction contains about 25% protein and is dissolved by proteases.[27,35] This indicates that FA is also coupled to the protein, most likely to tyrosine and/or cysteine residues. In total three different possible mechanisms were suggested as explanation for this phenomenon[35,39]: Cross-linking of two FA moieties through their aromatic rings; cross-linking of a FA reside with the ring structure of tyrosine amino acid moieties; or cross-linking of two tyrosine rings.

In feruloylated AX, laccase catalyzes gelation by dimerization of feruloyl esters. Although there was no evidence for a coupling of thiol compounds to phenoxyl radicals, it has been proposed that these phenoxyl radicals, oxidized by laccase from that FA, are involved in the conversion of the thiol compounds into disulphide bonds and thus influence the overall dough firming which is seen upon use of laccase.[36,40]

6.1.6 Lipids

Wheat flour lipids form a highly heterogeneous group of molecules with different chemical structures and compositions. The lipids can be divided into free lipids and bound lipids.[41] Both fractions contain polar and non-polar components. Polar lipids can be divided into glycolipids and phospholipids. The glycolipids are predominantly composed of monogalactosyl diglycerides (MGDG) and digalactosyl diglycerides (DGDG), whereas lysophosphatidylcholines (LPC) and phosphatidylcholines (PC) are major components of the phospholipid class of lipids. Both classes are also highly heterogeneous with respect to the position and the structure of the esterified fatty acids. The non-polar lipid class is mainly composed of triglycerides.[42] The fatty acids in the various lipids are dominated by linoleic acid, whereas other fatty acids, such as palmitic and oleic acid are found in lower levels.[43]

Bound lipids are mainly bound to starch and to a lesser extent to proteins. Starch lipids (around one third of total) comprise mostly LPC. These lipids form inclusion complexes with amylose during gelatinization, whereby the LPC fatty acid chains form complexes with the hydrophobic interior of the amylose α-helices.[44] Such complexes may already exist in

the native starch as well. These lipids are therefore effectively unavailable before most of the starch is gelatinized.

The non-starch lipids consist of similar levels of polar and non-polar lipids. Part of this fraction is most likely bound to proteins.[45]

Polar lipids are known to play a role in dough stability and processing tolerance of yeast-leavened baked products. The ability of the polar flour lipids to form lipid monolayers at the gas/liquid interface is believed to positively influence the gas retention in the dough. Furthermore, polar flour lipids and gluten proteins interact as well. This interaction is also believed to positively affect gas retention.[46,47]

6.2 ENZYMES IN BREAD MAKING

Bakery products have undergone radical improvements in quality over the past 10 years in terms of flavour, texture and shelf life. The usage of enzymes is the biggest contributor to these improvements. Among the enzymes used in food applications, those used in bakery industry constitute nearly one-third of the market.

Baking enzymes are used as flour additives and they are used in dough conditioners to replace chemical ingredients and to perform other functions in a label-friendly way.

The baking industry predominantly makes use of five types of enzymes (Table 6.2). Amylases are used to convert starch to sugar and to produce dextrins. For strengthening and bleaching of the dough, oxidases are used. Hemicellulases and proteases are the enzymes which have an effect on wheat gluten. While hemicellulases improve gluten strength, proteases reduce gluten elasticity. All these enzymes together play an important role in maintaining bread volume, crumb softness, crust crispiness, crust colouring or browning and in maintaining freshness.

6.2.1 Amylases

α-amylases (**EC 3.2.1.1**) are the enzymes which are most frequently used in bakeries.[48] The reasons for this are their positive influence on bread volume, crumb grain, crust and crumb colour, flavour development and anti-staling effect.[49,50] There is also evidence that amylases have an effect on dough development.[51]

6.2.2 Classification

Amylases belong to the family of glycohydrolases (GH), based on structural and amino acid similarities.[52] Various amylases are found in GH families 13, 14 and 15. Besides α-amylases

Table 6.2 General effect of enzymes in bread making.

	Improved gluten network	Gas retention/ increased volume	Improved colour and flavour	Improved crumb structure	Improved shelf life properties
Amylase		X	X	X	X
Protease	X				
Xylanase	X	X		X	
Oxidase	X	X		X	
Lipase	X	X	X	X	

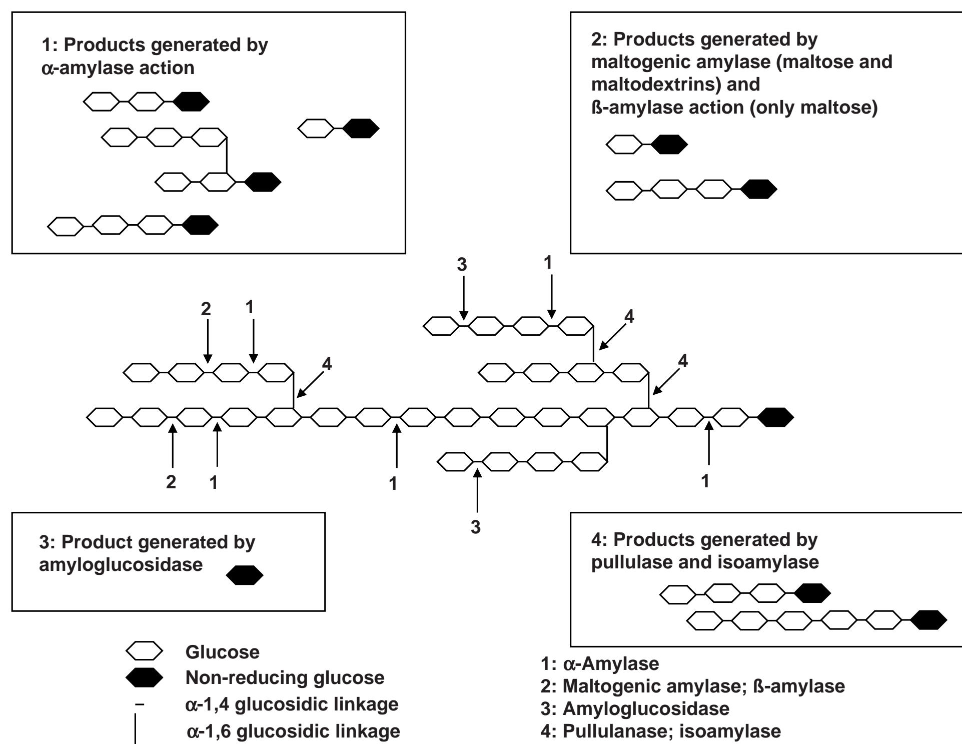


Fig. 6.4 Attack sites and breakdown products for various starch degrading enzymes.

(1,4-α-D-glucan glucanohydrolase **EC 3.2.1.1**), also maltogenic amylase (**EC 3.2.1.33**), β-amylase (**EC 3.2.1.2**), amyloglucosidase (also named glucoamylase, **EC 3.2.1.3**), pullulanase (**EC 3.2.1.41**) and isoamylase (**EC 3.2.1.68**) belong to these families. In the above schematic overview (Fig. 6.4) the action of these enzymes on a starch molecule is shown.

α-Amylase is an endo-acting enzyme that randomly hydrolyzes the α-1,4 glucosidic linkages in polysaccharides, resulting in short chain dextrins. The α-amylases degrade damaged starch in wheat flour into small dextrins of DP2-DP12,[9,53] thus allowing yeast to work continuously during dough fermentation, proofing and the early stage of baking. This results in improved bread volume and crumb texture. In addition, the small oligosaccharides and sugars such as glucose and maltose produced by amylases enhance the reactions for the browning of the crust and baked flavour. If the amylase content is low, this leads to low dextrin production and poor gas production. This in turn results in inferior quality bread with reduced size and poor crust colour.

However, this is not the only effect of α-amylases. As demonstrated by Pritchard,[54] one of the main effects is the reduction of dough viscosity during starch gelatinization. Gelatinization of non-damaged starch granules starts at 55°C. This leads to amylose leaking out of the granules and initial melting of amylopectin crystallites. These events lead to a sharp increase in dough viscosity, which terminates oven spring. When α-amylases attack gelatinized starch, this will result in a prolonged oven spring and thus larger volume.[48]

Wheat and wheat flour contain endogenous enzymes, of which amylases take an important part. However, the level of α-amylase in some flour is sometimes very low[55] and thus there is

a need for wheat flours to be supplemented with α-amylase. These can be added in the form of malt flour or fungal amylases. Since the 1960s, bakers have supplemented the naturally occurring enzymes in wheat flour to minimize natural differences caused by, for example, weather conditions.

6.2.3 Amylases in bread making

α-Amylases are endoglucanases. This means that they hydrolyze random α-1,4 and α-1,6 linkages. Amylases can act only on damaged or gelatinized starch, since these are susceptible to enzymatic attack. The amount of damaged starch is dependent on wheat variety and especially on milling conditions. Standard UK flour has a higher percentage of damaged starch in order to increase water binding in the dough. Suitable dosages of fungal amylase lead to the desired improvement of dough and the final product. However, extensive degradation of damaged starch due to too high levels of α-amylase leads to sticky dough.

In Figs 6.5(a) and (b) the effect of increasing levels of a fungal α-amylase on volume, crumb structure and stickiness is shown[67] using two different flour qualities.

The volume and crumb structure (manual scores) improve with increasing levels of amylase added to the flour. This effect is seen with different flour types, although the extent of the effect is flour dependent. Even though the positive effects increase with the amylase dose rate, there is an optimum dose level, since the stickiness of the dough also increases, leading, in this case, with flour 1 to an unworkable dough at higher amylase dose levels.

6.2.4 Other amylases

Pullulanase and isoamylase are the two best-known debranching enzymes. Both enzymes are capable of hydrolyzing α-1,6 glucosidic linkages, thereby releasing side chains from the branched amylopectin molecule.

β-Amylase and amyloglucosidase are typical exo-acting enzymes, cleaving α-1,4 glucosidic linkages at the non-reducing end of linear chains in the starch molecule, thereby catalyzing successive removal of β-maltose and β-glucose, respectively.[9,56] β-Amylase is stopped by α-1,6 linkages, whereas amyloglucosidase can bypass the side chains and thus, in theory, can completely degrade starch to β-glucose.[57] These latter four classes of amylases all have a limited effect on dough properties and bread quality.

6.2.5 Anti-staling enzymes

Bread rapidly looses its freshness and is subject to microbial spoilage. Changes in flavour and texture, other than due to microbial spoilage, taking place during storage are commonly called staling. This phenomenon, which makes bread hard and dry, is often attributed to starch retrogradation. Changes that are observed are crumb firming, increased crumb-texture harshness, increase in opacity of the crumb, loss of crust crispness, disappearance of fresh bread flavour and appearance of stale bread flavour.[58] All these factors result in a loss of consumer acceptance of the product.

About 85 million ton of wheat flour is used every year to bake bread. By adding specific agents, such as emulsifiers or enzymes, bread stays fresh longer. It is assumed that 10–15% of bread is thrown away because it not longer fulfils the consumer demands for quality, crumb

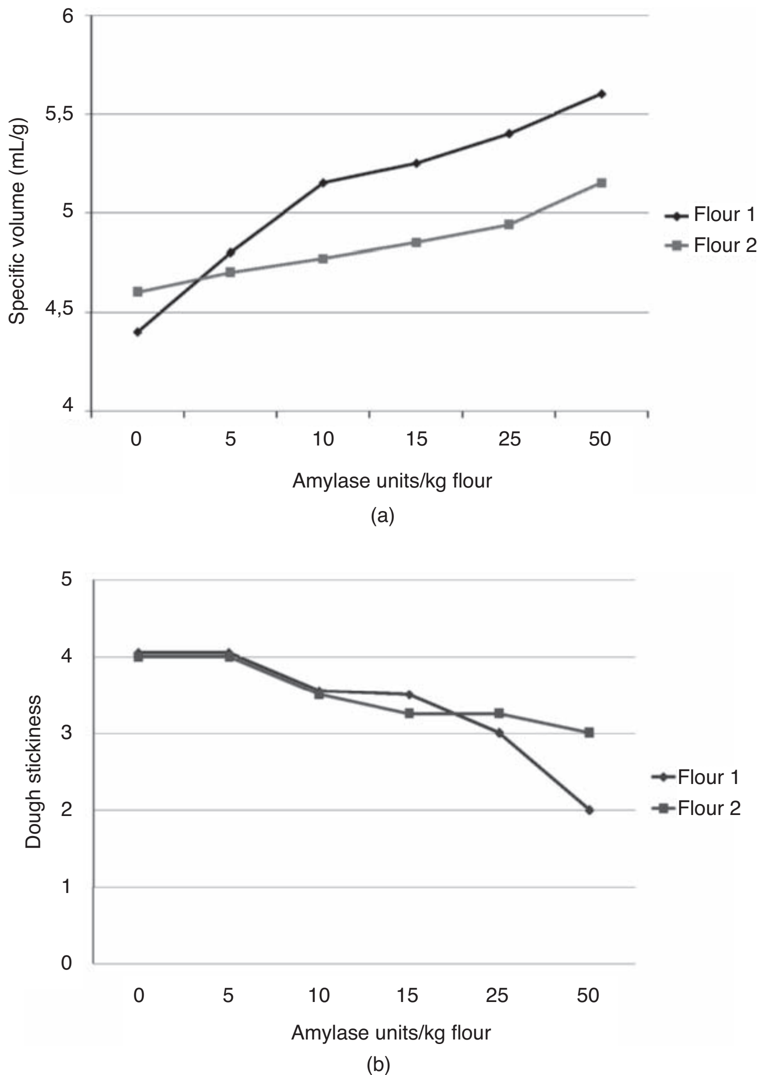


Fig. 6.5 (a) Effects of increasing levels of a fungal α-amylase on specific volume (left) and crumb structure (right). (b) Effects of increasing levels of α-amylase on dough stickiness (score of 5 is not sticky; 0 is most sticky).

softness, taste, etc.; the possibility of keeping it a few days longer fresh could save 2 million ton of flour per year. This is 40% of the annual bread consumption in the US.

Starch retrogradation is seen as the main factor responsible for the observed changes.[32,59,60] However, several authors state that in addition to starch retrogradation gluten, lipids and/or specific dextrins also play important roles in bread staling.[61–66] Furthermore, there are several other factors also having an effect on crumb softness, without necessarily involving starch retrogradation.[67]

- The quality of wheat flour in terms of endogenous enzymes and percentage starch damage has an influence on the total amylase efficiency and thus on the bread quality.

- It is well known that bread volume has a clear relationship with crumb softness. Higher specific volume leads to a softer bread crumb. Fine crumb structure with thin cell walls gives a softer crumb than a coarse structure with thick cell walls.
- Formulation also has a clear influence on staling since any ingredient, like shortening, having an effect on volume will also have an effect on softness.
- Processing has a certain influence on staling as well. Sponge and dough processing gives a different structure and softness compared with straight dough processing. In the same way a twisting step, resulting in a finer and more uniform crumb, will also positively affect softness.
- Finally, storage conditions play a role in staling since storage at lower temperature enhances starch retrogradation and thus will have a clear influence on crumb softness.

In the beginning of this century, the extended shelf life (ESL) concept was introduced in the US. This concept used already existing enzyme technology in order to achieve dramatic improvements in industrial bread making. These improvements were significantly ESLs for industrial bread up till 11 days, strongly reduced stale bread returns and also strongly reduced logistic complexity and costs due to a reduced number of transport routes.[68]

The most visible applications of ESL have been in bread and, to a lesser extent, in snack cakes. However, important improvements are also feasible in other grain-based food products, ranging from cookies via cakes to frozen dough products.

Following these improvements, the demand for even longer shelf lives became obvious. The objectives for further ESL developments can be found within four major areas[69]: texture, flavour, microbial stability and crumb moistness.

With regards to texture, with the use of specific enzymes, like bacterial amylases or intermediate stable maltogenic amylases (see section on amylases) sufficient softness can be obtained. However, sufficient crumb elasticity (or crumb resilience or crumb springiness) is more difficult to achieve. Since it is not fully understood which structures determine resilience, that is gluten, starch, amylopectin, modified amylopectin, etc., it is extremely difficult to find enzymes and/or ingredients which positively influence resilience.

When bread is kept for a longer time, a stale bread flavour develops and the well-known fresh bread flavour and aroma disappear.[70] Removal or masking of this stale flavour is a prerequisite for ESL. Keeping bread soft for a longer period may be feasible by using proper enzymes, but the microbial deterioration has to be suppressed. Increasing the level of propionate in the dough is not an option, due to problems with taste and yeast growth. Longer storage times will result in enhanced evaporation of water from the crumb, via the crust to the outside, thus leaving an unacceptable dry crumb. Specific precautions are needed in order to prevent this.

Further, ESL developments will lead to highly interesting opportunities for industrial baking. Merchandizing, for example showing bread on displays in groceries and supermarkets, baking to inventory – just as is done for cookies and biscuits – and further optimization of distribution and transportation are currently being discussed.

Fungal amylases have limited effect on staling. These enzymes act predominantly on damaged starch, but at the temperature at which starch starts to gelatinize, fungal amylases are already inactivated and thus cannot act on starch when it has become accessible.

Bacterial amylases are much more heat stable and these enzymes have a significant action on gelatinized amorphous starch. Modification of gelatinized starch results in a clear anti-staling effect. However, since bacterial enzymes are extremely heat stable, these

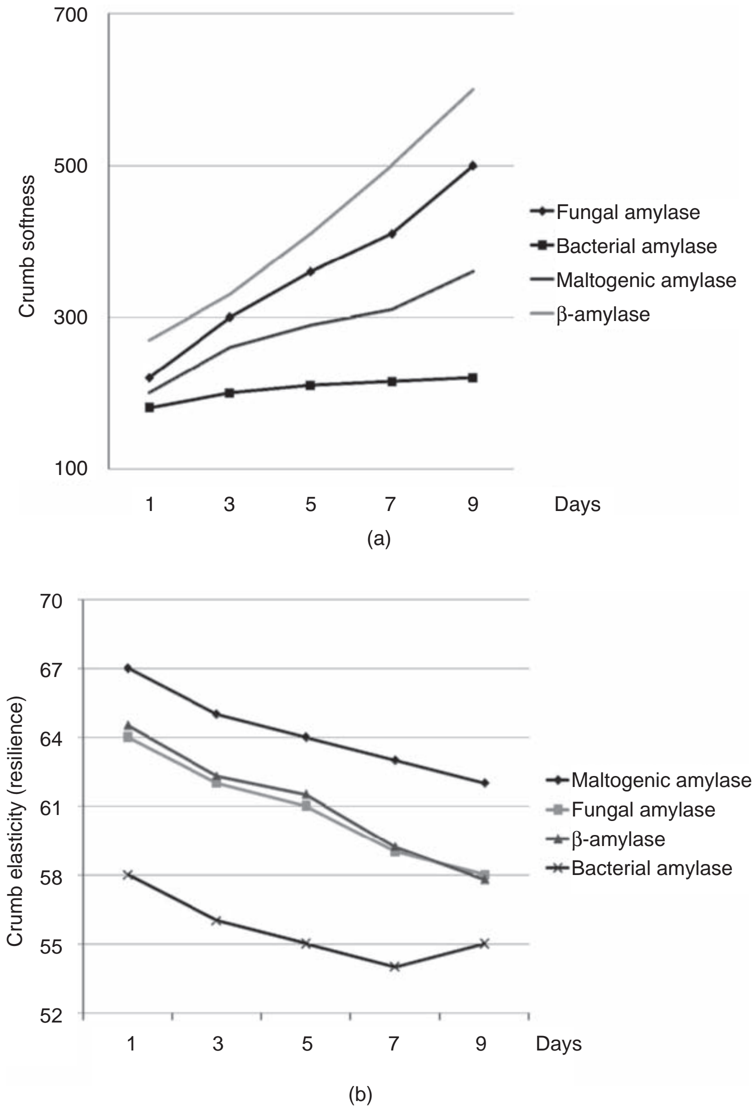


Fig. 6.6 (a) Effect of various starch degrading enzymes on crumb softness as a function of time. (b) Effect of various starch degrading enzymes on crumb elasticity (springiness or resilience).

enzymes exhibit residual enzymatic activity after baking, which can lead to excessive starch degradation, causing a collapse of the bread upon storage after baking. Only at very low dose rates, bacterial amylases can safely be used, but the risk of overdosing remains significant.[71]

Maltogenic amylase (glucans, 1,4-α-maltohydrolase, **EC 3.2.1.33**) produces maltose (and some longer maltodextrines) in the α-configuration. The enzyme is most active between 60°C and 70°C and is able to degrade amylopectin to a greater extent than fungal amylases or β-amylase.

In the above graph (Fig. 6.6(a)), the effects of several different amylases on crumb freshness are shown.[72]

Table 6.3 The effects of various amylases on staling.

Enzyme	Mechanism	Thermostability	Softening	Springiness
α-Amylase (A. oryzae)	Mainly endo	Low	+	Very limited
α-Amylase (A. niger)	Mainly endo	Intermediate	+	Little
α-Amylase (B. amyloliquefaciens)	Endo	High	++++	Negative
α-Amylase (Maltogenic)	Exo and endo	Intermediate	++++	Positive
β-Amylase (e.g. from wheat)	Exo	Low	+	Little

As can be seen in Fig. 6.6, maltogenic intermediate stable amylase has a significant effect on crumb softness as function of storage time. A specific bacterial amylase even has a greater effect on softness, but this same enzyme completely ruins the crumb elasticity (Fig. 6.6(b)), whereas the maltogenic enzyme gives the crumb a relatively good springiness, even after prolonged storage.

Fungal amylase and β-amylase have a very limited effect on crumb softness and on crumb resilience. The effects of these enzymes are comparable with effect of distilled monoglyceride.

The maltogenic amylase has a thermostability which is in between those of fungal amylase and bacterial amylase.[73] Therefore the enzyme is able to reduce retrogradation of amylopectin. It can hydrolyze glucosidic linkages in gelatinized starch before it is inactivated during the baking process. Since the enzyme is inactivated at the end of the baking process, it does not excessively hydrolyze starch. Apart from the optimal thermostability of this enzyme, it has other benefits compared with fungal or bacterial enzymes.[74]

The enzyme can degrade amylase and amylopectin into maltose and longer maltodextrines, and in doing so it does not need an unblocked non-reducing end. This indicates that the enzyme is also capable of attacking starch through an endo-type mechanism. Besides that, these maltodextrines are also assumed to have an anti-staling effect by specifically blocking interactions between starch and gluten.[62,63] The effects of various amylases on staling are summarized in Table 6.3.

6.3 XYLANASES

Numerous studies have been performed to demonstrate the positive effects of pentosans-modifying enzymes, which are presented by industry as pentosanases, xylanases, arabinoxylanases and/or hemicellulases, here further referred to as xylanases.[29,75–78] The way these enzymes are considered to have their effect is by reducing the water binding of WU-AX and by solubilizing WU-AX and later also WE-AX into smaller molecules.[79,80] Another effect ascribed to xylanases is to offset reduced gluten coagulation caused by pentosans by hydrolyzing the pentosans to an extent whereby this effect is not longer occurring.[81] Hamer[79] reported that the use of xylanases in a batter significantly improved gluten coagulation. The resulting gluten also was shown to have a much better bread-making quality. This effect was explained by the absence of any detectable pentosans in the remaining gluten, whereas normally 2–3% pentosans were attached to gluten. These gluten-linked pentosans were considered to have a steric hindrance effect on gluten coagulation.[82] Currently, industrial xylanases are sold to the starch industry for processes whereby gluten and starch are separated, which are based on this principle.

6.3.1 Classification

Glycoside hydrolases have been classified into 93 families[52] based on sequence homologies, which reflect structural and mechanistic features.

Xylanases (endo-1,4-β-D-xylanase, **EC 3.2.1.8**) can be classified in at least three ways. The first is based on molecular weight and pI.[83] They either have HMW or LMW and have either a high (basic) or low (acidic) pI. The second is based on crystal structure. This can be derived indirectly by a determination of DNA sequence. Endo-β-1,4-xylanases are generally classified in families 5, 8, 10 (formerly family F), 11 (formerly family G), 16, 26 and 43.[52] However, the majority belong to families 10 and 11 and are found to frequently have an inverse relationship between their pI and molecular weight. The family 10 xylanases are generally larger and more complex than the family 11 xylanases. The third classification is based on kinetic properties, substrate specificity or product profiles. Virtually all xylanases are 'endo' acting, as readily determined by chromatography, but the more detailed determination of kinetic properties, measuring the relative reaction rates on various substrates and determining the kinetics of intermediate product formation, is much less common.[84]

Unfortunately, only very few studies have been performed in order to relate sequence or structural family classification to action patterns, substrate specificity or functionality (e.g. in bread making).

Family 10 xylanases occasionally exhibit endocellulase activity; they generally have a higher molecular weight, and they occasionally will possess a cellulose-binding domain. Also in general these enzymes are considered to be less specific.[85]

Members of family 10 (including all plant xylanases, such as xylanase from cereals) will act on both PNP-xylobiose and PNP-cellobiose (artificial chromogenic substrates); however, the overall catalytic efficiency on PNP-xylobiose is about 50 times higher. This suggests that family 10 enzymes act mainly on xylan. Family 10 xylanases are capable of attacking the glycosidic linkages immediately next to branching points and the endoxylanases require two unsubstituted xylopyranosyl residues between the branches.[85]

Even though all xylanases are endo acting, they show variations in their product profiles. Some enzymes generate predominantly xylose and xylobiose and others predominantly (or exclusively) form xylotriose or a range of other/higher oligosaccharide products. Family 10 xylanases have relatively HMWs, and they tend to form oligosaccharides with a low degree of polymerization (DP). These xylo-oligosaccharides can be further degraded by β-xylosidases (**EC 3.2.1.37**), which remove xylose from the non-reducing end of the polymeric xylan residue.

Family 11 xylanases are true xylanases. They do not have cellulase activity; they consistently exhibit a LMW, and they can have either a high or low pI. They are formed by both bacteria and fungi. The positions of many amino acids are essentially identical in the family 11 xylanases from bacterial (*Bacillus circulans*) and fungal (*Trichoderma harzianum*) origins. Thus, there has been a tremendous conservation of the basic structure of the catalytic site of family 11 xylanases during evolution.[86,87] This is remarkable when considering the differences in functionality between the two classes of enzyme in bread making. Family 11 endoxylanases require three consecutive unsubstituted xylopyranosyl residues and thus also in this way can be distinguished from family 10 xylanases.[88]

Some xylanases belonging to GH families 5, 8 and 43 have been identified[89–91] (see Ref. [52] with the EC code for xylanase 3.2.1.8. for an overview). These have not been studied in any detail and their bread-making potential has not been elucidated in any detail. Among these, one example is the xylanase from Pseudoalteromonas haloplanktis TAH3a belonging

to glycoside hydrolase family 8.[92–94] This enzyme is a typical psychrophilic enzyme and presents a high catalytic activity at low temperatures. It is not homologous to family 10 or 11 xylanases, but has 20–30% identity with glycoside hydrolase family 8 members (formerly family D), a family that comprises mainly endoglucanases, but also lichenases and chitosanases.

6.3.2 Mechanism

The mechanism of action of xylanases in bread preparation is still not clearly elucidated. Many types of hemicellulase preparations have been used for the applications mentioned above, and are commercially available. They are produced by microbial fermentation using various microorganisms as enzyme sources. Many of these enzymes are produced by genetically modified microorganisms. All documented commercial uses of xylanases relate to enzymes belonging to either glycoside hydrolase family 10 or family 11, as defined previously. Examples of commercial xylanases are the xylanases from *Bacillus* sp., *Trichoderma* sp., *Humicola* sp. and *Aspergillus* sp.

It has been assumed for a long time that, depending on the application, preferential attack of WE-AX or WU-AX is needed, whereas activity towards the other fraction is not desired.[29] In bread making, endoxylanases that have a preference towards WU-AX have been considered beneficial.[29,95] The difference in substrate selectivity is therefore an important parameter in developing and selecting proper xylanases.[26] Comparison of selectivities and activities of a Bacillus xylanase and an Aspergillus xylanase showed that the Bacillus enzyme has a clear preference for WU-AX, whereas the Aspergillus xylanase more readily hydrolyzed WE-AX.[96] Nevertheless, both enzymes have a certain (although not the same) positive effect on bread making, confirming the findings of Wang[24] that both WE-AX and WU-AX affect gluten network formation in a similar negative manner. This means that hydrolyzing either one of these components can have a positive effect.

Figure 6.7 shows that the positive effect of xylanases is more pronounced when bread is made with low protein flour.[97]

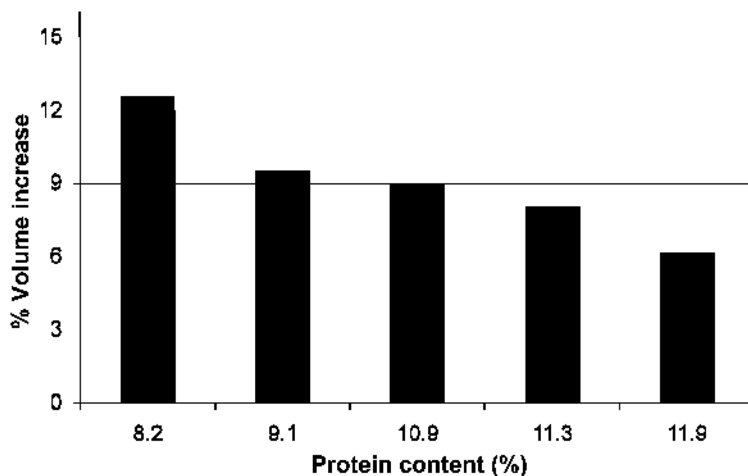


Fig. 6.7 Effect of a xylanase, in terms of per cent volume increase, on flour types with varying protein contents.

Again this is in line with the results from Wang.[24] A negative influence on gluten formation will be more difficult to handle in low protein flour than in high protein flour. Offsetting the negative effect will therefore be more effective in low protein flour.

6.3.3 Xylanases in bread making

Xylanases are broadly used in bread making, and depending on the application there is generally an appropriate xylanase or a mix of different xylanases that gives the desired effects in terms of dough-handling properties;[29] stability and oven spring[98] and volume.[95] This immediately indicates that there is not one single xylanase giving all desired effects in any application, but that the xylanase type(s), usage and dose rates need to be optimized in each case.

In spite of all research that has been done on xylanases and in spite of the wide acceptability of this type of enzymes, there is still no complete understanding of the mechanism and effects of different xylanases. This becomes obvious from a range of experiments (results not shown) in which four different xylanases (a monocomponent genetically modified organism (GMO)) – Aspergillus xylanases, an Aspergillus xylanase produced by solid state fermentation (thus having a wide range of side activities), a bacterial (Bacillus) xylanase and a fungal (Trichoderma) xylanase – are compared for different applications. Each of these four enzymes, in spite of similar dose rates, has a different effect on dough and bread properties when tested in different applications, varying in mixing time, water addition, mixing type and bread type. This indicates that there is not one single xylanase which performs equally well under all circumstances. Bakeries and bread improver companies need to establish optimal dose rates and optimal xylanase blends for each application and they can do this by trial and error only. There is no way to predict the performance of a xylanase.

6.4 LIPASES

Since a few years lipases (glycerol ester hydrolases, **EC 3.1.1.3**) and phospholipases (A2 and A1 type; **EC 3.1.1.4** and **EC 3.1.1.32**, respectively) are recognized as an additional tool for improving bread-making properties and in particular for their strong, positive effects on dough conditioning and dough characteristics.

Lipases hydrolyze ester bonds of acylglycerols, yielding mono- and diacylglycerols, free fatty acids and, in some cases, also glycerol. Lipases preferably hydrolyze ester bonds at the sn-1 and sn-3 position of the glycerol molecule.[99] Lipases usually function at lipid-air or lipid-water interfaces and their activity is sharply increased by the presence of organized lipid structures, which are normally found at such interfaces.[100,101]

Lipases can generally be divided into four groups, according to their specificity: substrate specific lipases, regioselective lipases, fatty acid specific lipases and stereospecific lipases.

6.4.1 Mechanism

The structure of a bread dough can be seen as a foam structure. The individual gas cells are separated by a continuous gluten film in which the starch granules are also embedded.

Bread-making quality is largely determined by the gas cell stability.[102] The distribution of the gas bubbles in the dough and also their size are largely determined by the flour quality, the ingredients and the mixing conditions. Some studies have focused on the role

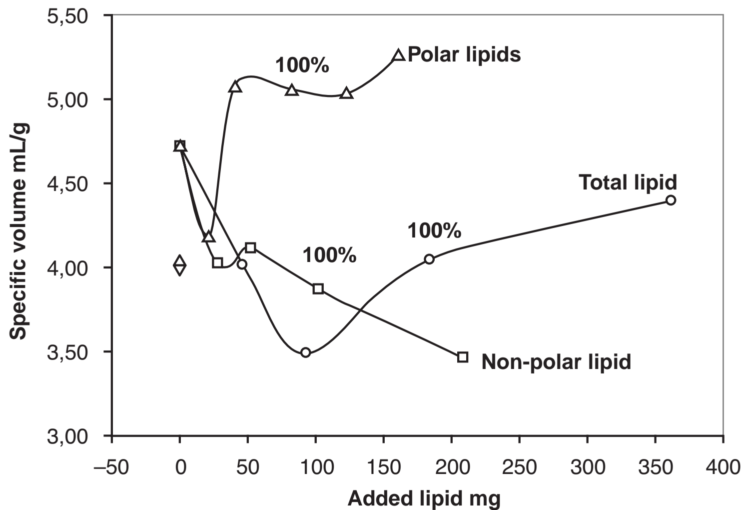


Fig. 6.8 Effect of addition of various wheat lipids on bread volume (adapted from Ref. [97]). Guy, R.C.E.; Sahi, S.S. (2002) Comparison of effects of xylanases with fungal amylases in five flour types. In: Recent advances in enzymes in grain processing. Courtin, Veraverbeke and Delcour eds. Lab. Food Chem., Catholic University Leuven, Belgium.

of gluten proteins on the gas holding capacity,[103] but there is also evidence that a lipid film surrounding the gas cell is also contributing to the gas cell stability.[47,104] Surface-active materials (emulsifiers) are able to counteract instability of gas cells. In fact these ingredients prevent coalescence and disproportionalization of gas cells by stabilizing the interface.

Initially, positive effects of lipase action were explained by assuming that lipases break down non-polar lipids, such as triglycerides, thereby removing a negative component from the dough. As can be seen in Fig. 6.8,[105] non-polar lipids generally have a negative effect on bread-making properties. It is well-known fact that triglycerides do not form stable monolayers at the lipid-air interface, and following this logic it is understandable that breakdown of triglycerides was considered to be positive. The 1,3 specific lipases were also assumed not to attack the polar lipids, which seemingly have a positive effect on bread-making quality.[106]

The current generation of lipases hydrolyze both polar lipids (see Fig. 6.9) as well as non-polar lipids. The resulting products show clear structural resemblance to well-known emulsifiers, such as DATEM and SSL. In that way, lipase action results in the stabilization of gas cells. However, there is a clear difference in effects of specific 1,3 specific lipases and lipases having other specificity, that is towards polar lipids.

Figure 6.9 shows the effect of lipase and phospholipase action on the structure of specific lipids. DGDG (digalactosyl-diglyceride) is converted into DGMG (the monoglyceride variant), and phosphatidylcholine (lecithin) is converted into lysophosphatidylcholine.

6.4.2 Lipases in bread making

1,3 Specific lipases are claimed to improve dough-handling properties, to increase dough strength and stability, to improve dough machinability and to increase oven spring. Besides this, such lipases also improve crumb structure and crumb whiteness.[107] The first generation of lipases in baking were almost exclusively of this type and were claimed to be alternatives to chemical dough strengtheners and emulsifiers. However, the technical and commercial

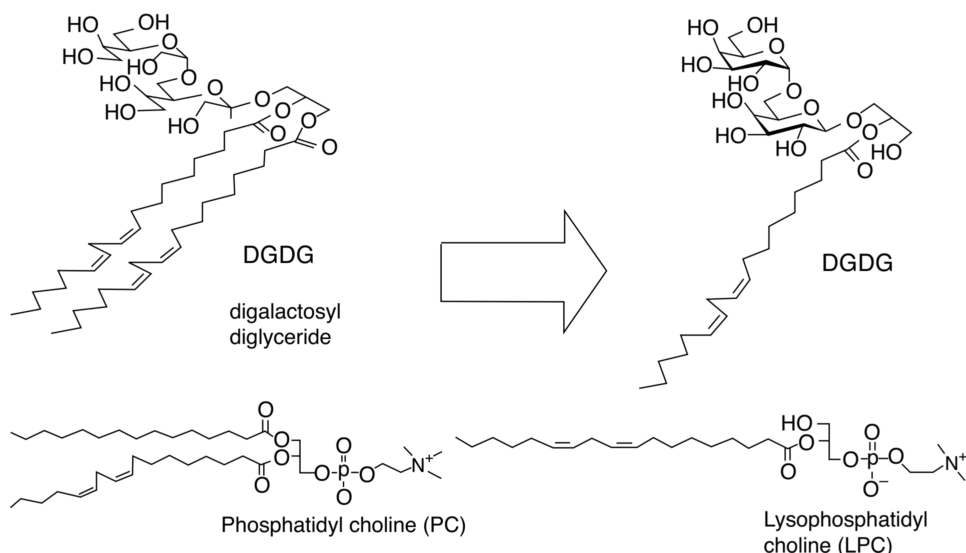


Fig. 6.9 Molecular structure of various wheat lipids before and after lipase hydrolysis (adapted from Ref. [105]).

benefits were limited. The second generation were enzymes with much broader specificity, acting also on polar lipids. These enzymes exhibited phospholipase activity as well as lipase activity. Phospholipases act much more like emulsifier replacers.[108] Both types of lipases were shown to give an increase in surface pressure of gas cells, although the effect of phospholipases was much more pronounced.[108] A similar increase in surface pressure is also obtained by addition of DATEM. Increased surface pressure leads to a better distribution of more stable, smaller gas cells. This results in finer, more silky crumb structure with optically whiter colour, better dough-handling properties and, to a certain extent, a larger loaf volume.[109] However, the surface pressure alone cannot account for the positive effects of the enzymes or of the emulsifier.[108] Further research is needed in order to elucidate the reaction mechanism of the various lipases, their breakdown products and the effects of these products in the bread-making process.

A third generation lipase is currently entering the baking market. These enzymes are protein engineered in order to give a better effect in high speed mixing and no-time dough processes. Especially in these processes, the first and second generation lipases were not very successful. Furthermore, the third generation lipases that become available now have a lower affinity for short chain fatty acids, leading to lower release of such fatty acids, which result in lower risk for off-flavour formation upon prolonged storage of the baked goods and upon use of butter or milk fat in baked products.

Lipases are also claimed to have a direct effect on the gluten network.[110,111] This can partly be explained by the generation of free fatty acids, which can be oxidized by endogenous lipoxygenase, leading to an increased oxidation potential which in turn may positively affect gluten network formation. Furthermore, lipase may influence the interaction between gluten proteins and lipids and the interactions between starch and lipids. Especially, the anti-staling and crumb softening effects of lipases are said to be the results of amylose–lipid complex formation.[112,113] These effects could not be obtained by simply adding monoglycerides to

the dough, indicating that the amount of emulsifier-like structures formed due to the lipase action is not fully explaining positive lipase effects.[108]

Concluding, it will be clear that there is still a lot of uncertainty about the mechanism of lipase action in dough and in bread making. More research with even more specific lipases is needed in order to reach well-defined conclusions.

6.5 OXIDOREDUCTASES

Oxidoreductases are widely distributed among microbial, plant and animal organisms. These enzymes catalyze the exchange of electrons or redox equivalents between donor and acceptor molecules. This occurs in reactions involving electron transfer, proton abstraction, hydrogen extraction, hydride transfer, oxygen insertion or other key steps.[114] In general two half reactions, one oxidative and one reductive, take place and at least two substrates (one reducing and one oxidizing) are activated or transformed.

To accomplish this physiological function, oxidoreductases have various redox-active centres.[115] Common redox centres include amino acids, metal ions, metal complexes (e.g. Fe-S clusters; heme cluster) or coenzymes (e.g. FAD, NAD, pterin, PQQ).

Many oxidoreductase substrates, such as carbohydrates, unsaturated fatty acids, phenolics and thiol-containing proteins, are important components of wheat flour. Their modification by oxidoreductases may lead to new functionalities, quality improvements and/or cost reduction.

6.5.1 Classification

Oxidoreductases can be classified according to their amino-acid sequence, their three-dimensional structure or their application, that is type of catalysis and/or cofactor dependence.[114] In the latter classification according to application, four groups can be distinguished:

- Oxidases
- Peroxidases
- Oxygenases
- Dehydrogenases/reductases

Within each of these groups, various subtypes exist, mainly dependent on differences in active centres. Within the baking industry, several oxidases have been investigated and some have been commercialized.

6.5.2 Oxidases in baking

In bread making, bread improvers and dough conditioners are widely used and accepted. The main action of these agents is to help rebuild the gluten network and the GMP, in order to improve texture, volume, freshness and also dough machinability and stability. Dough conditioners are specifically meant for gluten strengthening. Gluten strengthening results in improved rheological and handling properties of the dough. Such conditioners also have a long history in bread making and are well known. Non-specific agents, such as iodates, peroxides, ascorbic acids, potassium bromate and azodicarbonamide, all have a

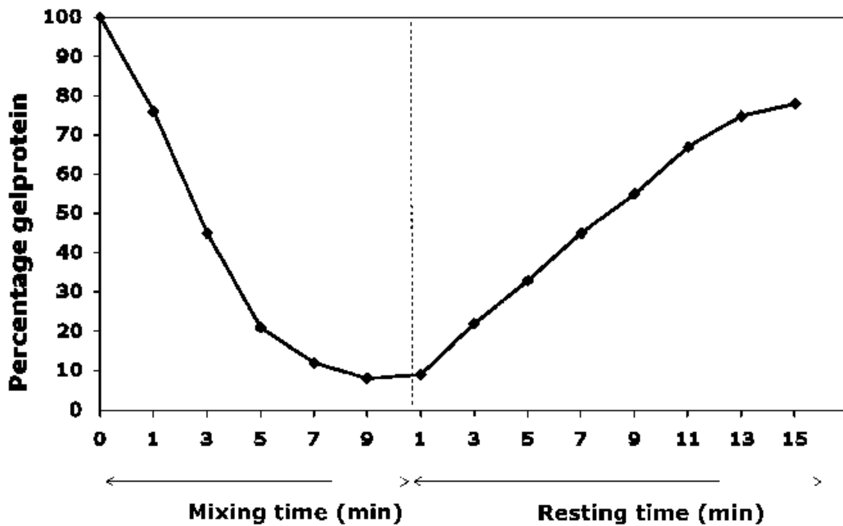


Fig. 6.10 Breakdown and rebuilding of gel protein during dough mixing and dough rest.[16] Weegels, P.L.; Hamer, R.J.; Schofield, J.D. (1997), Depolymerization and repolymerization of wheat glutenin during dough processing II. Changes in composition. J. Cereal Science. 25: 155–263.

gluten strengthening effect by inducing the formation of protein–protein bonds that strengthen the protein network and thereby strengthen and stabilize the dough.[116]

The glutenin sub fraction, which is unextractable in SDS solution (gel protein or GMP) and which is highly correlated to various quality parameters of bread, is changing during bread making. During dough mixing, GMP partly depolymerizes, which leads to more SDS-soluble glutenin. During resting, these extractable proteins repolymerize, thus increasing GMP again (see Fig. 6.10).[16] This process of disaggregation and rebuilding can be influenced by longer or shorter mixing; but the whole process is catalyzed by oxidoreduction reactions.

It is generally accepted that the properties of dough and its three-dimensional protein network are dependent on the arrangement and number of disulphide bonds and sulfhydryl groups of the protein. The vital contribution of disulphide bonds to dough stability has been shown by rheological studies,[117] and it is specifically this group of reactive groups that are targeted by oxidative agents or oxidases.

After mechanical development of the gluten network, the three-dimensional protein structure needs to be stabilized by oxidants. Small amounts of oxidizing reagents, such as potassium bromate or dehydroascorbic acid, improve the dough handling and baking characteristics of wheat flour; loaf volume increases and bread crumb improves as well.[118] Bromate is assumed to oxidize LMW, SH-containing peptides (glutathione) into disulphide bonds.[119] On the other hand, a small amount of cysteine or reduced glutathione sharply increases the extensibility of dough. Both the viscous and elastic component of dough deformation are increased by addition of these reducing agents.[117]

In general, due to the great number and also the complexity of oxidoreduction reactions occurring during bread making, the effects of oxidative reagents are only poorly understood.[120–122]

Oxidizing effects can also be obtained by using enzymes instead of chemical dough conditioners. In Table 6.4, a number of oxidases are listed which are currently used in commercial bread making or which have been investigated specifically for this purpose. As

Table 6.4 Oxidases investigated and/or commercialized for bread-making applications.

Enzyme	EC number
Glucose oxidase	1.1.3.4
Hexose oxidase	1.1.3.5
Pyranose oxidase	1.1.3.10
Sulfhydryl oxidase	1.8.3.2
Glutathion oxidase	1.8.3.3
Glutathione dehydrogenase (DHA reductase)	1.8.5.1
Diphenyl oxidase (catechol oxidase)	1.10.3.1
Laccase	1.10.3.2
Ascorbic acid oxidase	1.10.3.3
Peroxidase	1.11.1.7
Glutathion peroxidase	1.11.1.19
Lipoxygenase	1.13.11.12
Tyrosinase (polyphenol oxidase)	1.14.18.1

can be seen, there is quite some variety in enzymes from different subclasses, which all are claimed to give a beneficial effect in bread making. The reactions of most of these enzymes in wheat dough are schematically shown in Fig. 6.11.

Glucose oxidase and hexose oxidase most likely catalyze their reactions following a similar reaction mechanism. Glucose, preferably in the β form, is oxidized by glucose oxidase to form gluconolacton, which is immediately converted into glucuronic acid. For

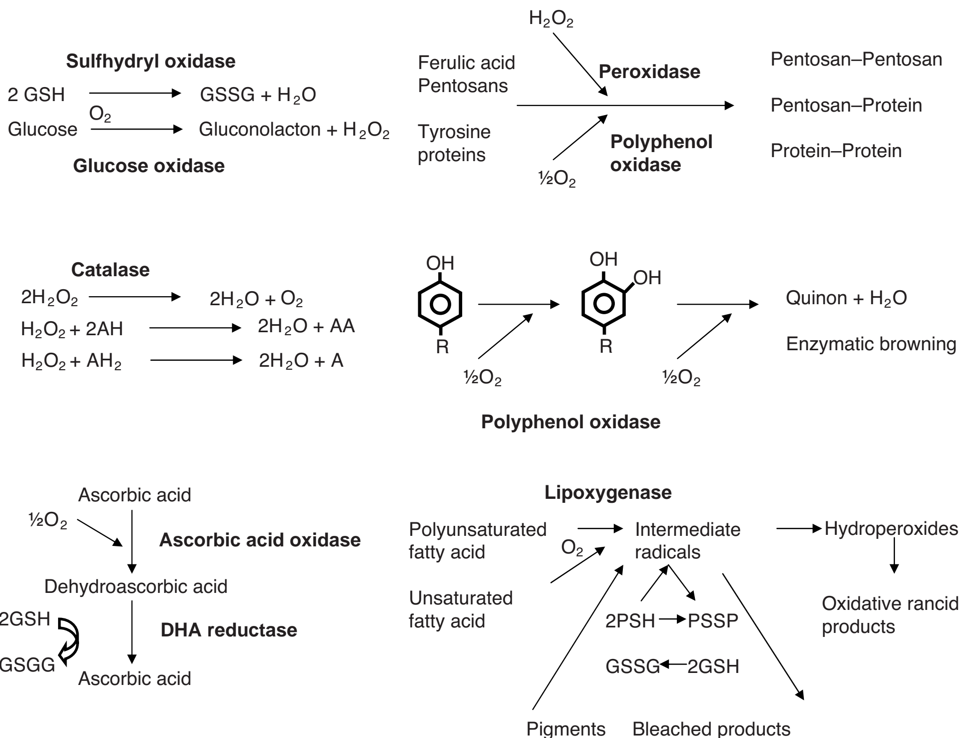


Fig. 6.11 Oxidation reduction systems in wheat dough.

the reaction the presence of oxygen is required. Oxygen serves as an electron acceptor. In this reaction hydrogen peroxide is formed. One explanation of the reaction mechanism is that hydrogen peroxide, in the presence of endogenous peroxidase, naturally occurring in flour, promotes the oxidation of sulfhydryl (—SH) groups to disulphide (S—S) bridges in the gluten network, as well as the formation of a gel from the water-soluble pentosans.[123,124] Another explanation is that hydrogen peroxide reduces the level of reduced glutathione, which normally has a weakening effect on the gluten network formation.[125]

The increase in gluten network results in increased strength of the gluten structure in the dough. This leads to improved dough stability, reduced dough stickiness and improved dough machinability. These improvements in turn lead to increased volume, improved crumb structure and better softness of the baked product. However, there are also studies which do not support these theories, since no evidence could be found for increased or changed gluten structures.[126] The main difference between glucose oxidase and hexose oxidase is that the latter can use various monosaccharides and even oligosaccharides as a substrate.

Lipoxygenase converts polyunsaturated fatty acids, more specifically those containing a *cis*, *cis*-1–4-pentadiene moiety, to fatty acid peroxy radicals. This reaction also requires the presence of oxygen. The free radicals react further to yield monohydroperoxides with conjugated double bonds and these compounds can react further with a wide variety of products.[121,127]

In bread making, lipoxygenase from soya bean flour has been used for decades not only for its bleaching effect, resulting in a whiter crumb,[128] but also for its improving effect on dough rheology (viscoelasticity), on mixing tolerance, loaf volume and on the gluten stability.[129–132] The hydroperoxides react with the naturally occurring yellow carotenoid pigment in wheat flour, leading to a reduction of the yellow colour. Furthermore, it has been claimed that lipoxygenase has a direct oxidizing effect on gluten formation.[121,133,134] This effect cannot be ascribed to hydroperoxides, since addition of lipid hydroperoxides did not show any effect.[135,136] Most of the oxygen uptake by wheat dough during mixing is due to the oxidation of free and esterified polyunsaturated fatty acids (PUFAs).[137–141] The improving effect of lipoxygenase may thus be due to the oxidation of gluten proteins through the co-oxidation of accessible thiol groups of the gluten protein by the enzymatically oxidized lipids.[142–146]

Wheat flour itself also contains lipoxygenase, but the activity of this enzyme is confined to free linoleic acid, linolenic acid and monoglycerides containing these fatty acids.[147]

Currently there are no other commercial sources of lipoxygenases other than enzyme-active soya bean flour and, to a lesser extent, flour from other beans (e.g. faba beans). With the current trend towards liquid bread improvers, either water based or oil based, the usage of soya bean flour is gradually reducing as a consequence of the limited solubility of soya bean flour. This increases the necessity for a microbial lipoxygenase. However, it seems extremely difficult to find a lipoxygenase with exactly the right specificity. Also in soya beans there are three distinct isoforms of lipoxygenase (LOX1, LOX2 and LOX3), of which only LOX1 and LOX3 have a positive effect on loaf volume. LOX2 is mainly responsible for undesirable aroma formation in bread dough.[148]

Polyphenol oxidases (PPO) are normally involved in enzymatic browning reactions. Enzymatic browning is the enzymatic oxidation of phenols leading to the formation of pigments. The colour of those pigments varies widely in colour and intensity.[149]

Several names are in use for PPO, including phenolase, creolase, tyrosinase, diphenolase, catecholase, laccase, etc. According to the official nomenclature, two kinds of enzymes are classified under the trivial PPO name. The first group, catechol oxidases (or diphenol oxidase)

(**EC 1.10.3.1**) catalyzes two distinct reactions in the presence of oxygen: the hydrolxylation of monophenols into *o*-diphenols and the oxidation of the *o*-diphenols into *o*-quinones. Both reactions require oxygen. The second group, laccases (**EC 1.10.3.2**) oxidizes both *o*-diphenols and *p*-diphenols, thereby forming the corresponding quinines. However, laccases can also act on monophenols. A third group of enzymes exists, which is contributing to the confusion about nomenclature of those enzymes: tyrosinase (or polyphenol oxidase, **EC 1.14.18.1**). The latter also catalyzes phenol oxidation, but by a different mechanism, involving two electron transfers.[150] Oxidation of tyrosine moieties in proteins can also lead to the formation of new covalent bonds, for example with cysteine groups, which in turn leads to changed rheological properties.[151–153] In spite of positive findings,[154,155] there are currently no commercial tyrosinases available for baking.

Laccases (**EC 1.10.3.2**) are copper containing enzymes that have been extensively tested for baking applications but this has not resulted in a successful commercial product. Laccases are claimed to increase dough stability and dough strength and to reduce dough stickiness.[65] Both laccases and peroxidases (cf. below) catalyze the oxidative gelation of arabinoxylans in model systems.[36] In bread systems, it has been demonstrated that laccase reduces the extractability of arabinoxylans due to cross-linking of AX chains as a result of dimerization of FA residues.[23] It has also been suggested that these enzymes only catalyze the formation of a sugar network and not the formation of a gluten network.[156] It is more likely, though, that both cysteine and tyrosine residues are also involved in oxidative cross-linking reactions.[33] In this reaction, laccase is assumed to catalyze the formation of thiol radicals as a result of the formation of phenoxyl radicals. Finally, proteins may also be coupled to arabinoxylan chains by FA moieties and tyrosine or cysteine residues.[27,157]

Currently, several industrial laccases are for sale (for use in textile, in juice, in brewing), but none of them has been commercialized directly for the baking industry and thus there are little or no laccase sales for baking applications.

Peroxidases (**EC 1.11.1.7**) are also able to catalyze the oxidation of phenolic groups. Peroxidases use hydrogen peroxide as electron acceptors and can use a wide variety of substrates, leading to the formation of radicals which can react further, non-enzymatically, with other substrates.[158] In this way, peroxidase shows a dough strengthening effect, leading to improved volume and crumb characteristics.[158] Peroxidase causes oxidative gelation of soluble flour arabinoxylans (pentosans). This oxidative gelation of pentosans by hydrogen peroxide is ascribed to FA residues. One possible mechanism is through dimerization of FA residues on adjacent arabinoxylan chains.[159] Proteins also participate in this gelation, since the gel fraction contains around 25% protein. In this case, the mechanism is by coupling FA residues to tyrosine or cysteine residues in protein chains.

Peroxidases are commercially available, although the only food grade version is derived from plant material (soya bean hulls), whereas microbial-derived peroxidases are commercialized for non-food applications, such as the production of textile materials.

Sulfhydryl oxidase (**EC 1.8.3.2**) catalyzes the formation of disulphide bonds from a variety of thiol groups. This enzyme was originally used for removal of cooked flavours in UHT milk.[160] It was speculated that sulfhydryl oxidase would give a similar effect as that of chemical oxidants[161] and that chemical oxidants would therefore lead to formation of disulphide bonds between protein chains. For this reason the enzyme has been extensively studied for its effect in bread making. However, it was found that the enzyme had no effects on loaf volume, dough strength, dough stability and mixing tolerance.[161] The conclusions were that sulfhydryl oxidase had no or very limited affinity for thiol groups in protein chains, but only for small thiol containing molecules.

Nevertheless, the enzyme has been commercialized since it was claimed that there was a positive synergism with glucose oxidase[162] and it has been available for a number of years. Nowadays it has been withdrawn from the market for baking enzymes.

*Amino acid oxidase (**EC 1.10.3.3**) and Dehydroascorbic acid dehydrogenase (**EC 1.8.5.1**)* are both mentioned in relation to the oxidation and reduction of ascorbic acid (AA) in wheat dough and the concomitant oxidation of glutathion. The improving effect of AA is mediated through its oxidation in dehydroascorbic acid (DHA). The latter compound is able to oxidize two thiol groups into a disulphide bridge.[163] Although AA oxidase has been characterized in wheat flour by many researchers,[164–166] the possible oxidation of AA by other enzymes[167] or by non-enzymatic reactions[165,168] cannot be excluded.

Reduction of DHA into AA with the concomitant formation of disulphide bridges from thiol groups is an enzymatic reaction, especially when the thiol groups are from glutathion. [134,169] Glutathion DHA reductases are present in wheat flour and wheat bran.[170] This enzyme is specific for glutathion and inactive against cysteine and cysteine-containing peptides.

In spite of the clear effects these enzymes have on the oxidation reduction systems in wheat dough, none of them has been commercialized.

Concluding remarks on oxidases may be that in spite of extensive research done globally on oxidases, the commercial success is so far rather limited. One of the reasons that has frequently been given is the requirement of oxidases for molecular oxygen. Since the amount of oxygen in a dough is limited and also taken up by yeast, this could explain the limited success of oxidases. However, incidentally, very positive results have been obtained with oxidases.[171] This, in combination with the lack of complete mechanistic understanding of the oxidation processes occurring in dough, leads to the conclusion that positive oxidase effects must be possible. Most likely the right oxidases still need to be discovered and commercialized.

6.6 PROTEASES

Proteolytic enzymes, also referred to as proteases, proteinases and peptidases, catalyze the hydrolysis of peptide bonds in proteins. A wide variety of proteases exist in nature; in plant material, animal tissue and in many different microorganisms. Commercially, proteases are by far the largest group of enzymes sold for a wide variety of applications.

Commercial proteases can be of cereal (or other plant materials), animal, fungal or bacterial origin. In contrast to, for example, amylases, proteases do not differ much in terms of heat stability. They differ widely, however, in terms of pH dependence and even more in terms of catalytic specificity.

6.6.1 Classification

Proteases can be divided into two subclasses: endoproteases and exoproteases. The first group hydrolyzes peptide bonds of proteins in the interior of the polypeptide chain, thereby generating smaller peptides and sometimes even free amino acids. Endoproteases can be further subdivided into four groups:

Serine proteases (**EC 3.4.21**. . .)
Cysteine proteases (**EC 3.4.22**. . .)
Aspartic proteases (**EC 3.4.23**. . .)

Metalloproteases (**EC 3.4.24**. . .)
(. . . indicates that various sub-types exist.)

This classification is based on the catalytic mechanism of the enzymes and specific functional groups or molecules involved in the catalytic action.

Exoproteases or peptidases cleave the protein chain at the end, thereby generating free amino acids and sometimes even small peptides. Peptidases are usually subdivided into two classes, each with various subclasses:

Carboxypeptidases (**EC 3.4.16**. . ., **EC 3.4.17**. . ., **EC 3.4.18**. . .)
Aminopeptidases (**EC 3.4.11**. . .)

This subdivision is based on the specificity of the enzymes, that is from which side of an amino acid the peptide bond is hydrolyzed.

6.6.2 Proteases in baking

The most important functional component in wheat flour is gluten. Therefore, anything that influences or modifies the gluten network or the individual gluten proteins, and thus the ability to form a network, will have a strong influence on the dough and final bread quality. Degradation of gluten proteins has an immediate effect on the covalent interactions in the gluten network.

Proteases have a long history in bread making and were traditionally used to treat ‘bucky’ dough resulting from overly strong and too elastic flours.[172] Originally the aim of protease addition was to improve softness, dough-handling properties and dough machinability.[172, 173] However, proteases have more functional effects. Functional effects of proteolytic enzymes are reduction of mixing time; improvement of dough machinability; improvement of gas retention due to better extensibility; improved pan flow in bun and roll production; improvement of grain and crumb texture; improved water absorption; improved colour; and improved flavour.[174, 175]

When achievement of such changes in dough characteristics is the purpose of protease addition, it makes sense to add an endo-acting protease. Hydrolyzing internally located peptide bonds will have a much more dramatic rheological effect than a removal of a terminal amino acid by the action of an exo-acting peptidase.[176]

Apart from effects coming from added enzymes, there are numerous reports on effects from proteases coming from infections on wheat,[177, 178] from lactobacilli used in sourdough preparations[179] and from endogenous enzymes, for example in sprouted wheat.[172, 180] Infections can result in significant degradation of endosperm protein, lower amounts of storage protein, lower dough consistency, increasing resistance to extension and lower loaf volume.

6.6.2.1 *Bread flavour*

Limited proteolytic hydrolysis, as seen in sourdough, has a positive effect on bread flavour. Bread crumb flavour is to a large extent formed by 2-acetylpyroline, whereas bread crust is to a large extent determined by a limited number of volatile compounds formed during fermentation.[181] Many of these volatiles originate from fatty acid oxidation or from microbial metabolized amino acids. In sourdough, the level of free amino acids is much less than in normal bread[182] and the microbial conversion of certain amino acids (ornithine, leucine, phenylalanine) leads to improved bread flavour.[77, 181] Enhanced proteolysis in sourdough leads to the formation of higher levels of free amino acids and thus to improved sensorical

properties compared with yeasted dough.[183] During this proteolysis, mainly HMW glutenin subunits are broken down, leading to increased glutenin solubility and reduced ability for network formation.[184]

The effects of proteases are strongly dependent on bread-making methods used, on flour quality and on the presence of other functional ingredients. In a short process, the effects of a protease on mixing requirements were found to be negligible, whereas effects on volume and bread score were strongly dose dependent: at low dose rate there was a clear improvement in volume and bread score, whereas, at especially higher dose rates, overall bread score greatly reduced.[185] On the other hand, in a sponge and dough system, proteases greatly reduced mixing time.[186] In shelf stable bread systems, proteases reduced the firmness of the crumb more than other enzymes and also reduced moisture migration.[187] In both cases, effects of these proteases on dough volume were limited, but proteases had a strong reducing effect on crumb firmness and bread score.[185,186]

Most of the effects mentioned are derived from modification of the gluten proteins. Limited proteolytic hydrolysis with a degree of hydrolysis (DH) of 0–5%, leads to an increase in gluten solubility, in combination with an improvement of the emulsifying and foaming properties of the gluten. The released soluble peptides had decreased functional properties.[188]

Proteases can have major disadvantages. The action of the proteases is not limited in time, they continue after mixing and weaken the dough structure in time. This phenomenon increases the risk of weakening the dough and increases the stickiness of the dough. Sometimes their action is even enhanced by the pH drop during fermentation. The use of proteases in baking requires strict control of the bulk fermentation and proofing conditions of the dough. Almost all proteases are inactivated during baking. Especially care should be exercised when using neutral Bacillus proteases and papain, which should be dosed very carefully as overdosing will slacken the dough too much. This may result in dough collapse before baking or a lower bread volume and a more open crumb structure. Particularly in Europe, where the flours are weaker than in the US or Canada, the risk of overdosing a protease is very much present. Furthermore, proteases also increase stickiness because by hydrolytic action, water is released from the gluten. This means that in practice proteases are little used in bread making in Europe.

6.6.2.2 *Freshkeeping*

Proteases have also been investigated in relation to freshkeeping. As mentioned above, there are several ingredients (emulsifiers, fats, mono- and oligosaccharides) and processing tools (specific amylases) known to retard bread staling. As there was not always a good correlation between starch structure and staling, other flour constituents were also investigated. The role of flour proteins in the crumb firming process has been studied but it was found that they were less important than starch.[189]

Nevertheless, upon using an intermediate thermostable or thermostable protease in baked goods a pronounced effect on crumb softness and on retarding the staling of baked products was found.[190] The enzymes used had specific characteristics; no adverse effect on dough rheology, on crumb structure or the volume of the resulting bread; low activity at room temperature; and a relatively high optimum temperature. Such an activity pattern guarantees limited protein breakdown at temperatures encountered during mixing and proofing and higher breakdown in the early stage of the baking process. The enzymes used showed similar effects on a range of baked products.

6.7 OTHER ENZYMES

Besides amylases, xylanases, lipases, oxidases and proteases, several other (classes of) enzymes have been investigated for their effects in bread making and several other types mentioned above have been reported to have beneficial effects on one or more characteristics of dough or bread. And indeed some of them have been commercialized.

6.7.1 Transglutaminase

In the preparation of yeast-leavened goods from weak wheat flour, the dough often has an unsatisfactory stability. Such a dough has not been able to retain the carbon dioxide gas that is formed during fermentation. For this reason, it is generally common to add oxidizing compounds to the flour to improve the resistance to stretching.[191] Because of the effort to reduce the addition of chemicals to foodstuffs and instead to use natural auxiliaries, like enzymes, the problem arose of improving the resistance of dough without the addition of inorganic chemicals. It has been found that transglutaminase improves the resistance of dough, particularly yeast dough from wheat flour, in a manner comparable to potassium bromate.[192,193] Transglutaminase (TGase; synonym: Protein-Glutamine-γ-Glutamyltransferase and Protein-glutamine:amine γ-glutamyl-transferase; **EC 2.3.2.13**) is an enzyme accessible from various sources that is widespread in the animal and plant kingdoms. It is known that transglutaminase has a cross-linking effect on proteins independent of the redox system of the dough, not involving the thiol groups and disulphide bonds in the dough. The basic reaction is shown in Fig. 6.12.

However, the effect on the rheological properties of dough is similar to oxidation and the effects are the result from an increased number of disulphide bonds. The need for TGase for improvement of the stretching properties depends in individual cases on the nature of the flour. TGase can be added in different ways in the preparation of baked goods. The enzyme preparation can be used together with the remaining components of a bread improver system, but it can also be mixed with the flour at the mill. This has the advantage that the dosage can be based on the properties of the flour, that is on its natural gluten properties. In this way, a flour of consistent baking properties can always be supplied to the baker. Thus, TGase is preferably added to weak wheat flours in order to strengthen gluten properties.

The effect of TGase on the dough can be visualized in an extensogram (see Table 6.5).

Addition of TGase leads to strongly increased dough resistance and a reduced extensibility. Combining TGase with a protease can overcome too strong effects of the coupling enzyme. The results of TGase addition on bread quality can be seen in Table 6.6.

In addition to the desired increase in the dough resistance, a reduction in extensibility is sometimes observed, which leads to an earlier breakage of the dough when testing extensibility. It has been found that this undesired side effect can be excluded by combining

Fig. 6.12 Schematic representation of the transglutaminase reaction.

Table 6.5 Effect of transglutaminase on dough properties.

Dough property	No enzyme	2000 U TGase kg^{-1}	2000 U TGase kg^{-1} + 0.3 g protease kg^{-1}
Water uptake (%)	54.1	54.2	54.1
Dough resistance (EE)	230	510	400
Extensibility (mm)	182	120	170
Ratio of resistance/extensibility	1.3	4.25	2.35
Energy (cm^2)	80	75	122

transglutaminase with a protease. Furthermore, the volume of baked goods is increased and their crumb properties are improved by the use of a protease. The dough prepared using TGase can be worked up into high-quality bakery products under conventional conditions; wheat bread, rolls and variety breads are representative of such products.

6.7.1.1 *Transglutaminase in gluten-free bread*

Celiac disease (CD) is a chronic enteropathy caused by the intake of gluten proteins from widely prevalent food sources, such as wheat, rye, barley and possibly even oats. The ingestion of gluten causes an inflammatory response resulting in the destruction of the villous structure of the small intestine.[194] Currently, the only effective treatment for CD is the strict lifelong renunciation of gluten-containing foods.[195] Because cereal products, and especially bread, are part of the basic diet in many countries, there is a high demand for gluten-free breads.

In view of the fact that gluten is the major structure forming protein in wheat bread and that gluten is responsible for the viscoelastic properties, it is technically a big challenge to produce high quality gluten-free bread. Various ingredients (gums, dairy powders, rice, sorghum, starches) mimicking the properties of gluten have been evaluated.[107,196–200] Also enzymes (amylases, xylanases, proteases) have been evaluated in the manufacturing of gluten-free products.[185,201]

One of the main problems associated with gluten-free bread is obtaining a good structure. Transglutaminase can be a tool to improve the structure of gluten-free breads. The quality of these products is significantly better with the formation of a stable protein network. When the enzyme is used in combination with the right protein substrates, for example milk proteins, or egg proteins, protein networks are formed, resulting in improved volume, crumb structure and overall quality.[202]

Table 6.6 Effect of transglutaminase on bread characteristics.

No.	Additive/kg flour	Dough quality	Volume (mL/10 rolls)	Crumb porosity
1	None	Soft	1850	Open
2	500 U TGase	Woolly, solid	2000	Fine
3	2000 U TGase	Woolly, short	1750	Dense, solid
4	2000 U TGase + 0.3 g protease	Woolly	2200	Fine
5	500 U TGase + 0.2 g ascorbic acid	Woolly, solid	2100	Fine
6	1000 U TGase + improver	Woolly, solid	2300	Fine

6.7.2 Endoglycosidases

Type II endoglycosidases are a category of hydrolases which are capable of cleaving specific internal glycosidic linkages found in glycoproteins. These endoglycosidases cleave all or part of the carbohydrate moiety from a glycoprotein, depending on the location of the reactive glycosidic linkage in the glycoprotein. Examples of Type II endoglycosidases include endo-β-*N*-acetylglucosaminidases (Endo-D, Endo-H (**EC 3.2.1.96**), Endo-L, Endo-CI, Endo-CII, Endo-F-Gal and Endo-F), endo-α-*N*-acetylgalactosaminidase, endo-β-*N*-galactosidases, peptide-*N*-(*N*-acetyl-β-glucosaminyl), aspergine amidase F (PNGaseF **EC 3.5.1.52**) and glycopeptide *N*-glycosidase (Peptide *N*-glycosidase **EC 3.2.2.18**).

Wheat gluten proteins are to a certain extent also glycosylated. Glycans were detected on both gliadin and glutenin polypeptides. Covalently aggregated LMW glutenins were shown to contain *N*-glycans with xylose, which demonstrated their sorting in the Golgi apparatus.[203]

Tests done with purified endoglycosidases revealed enhanced dough relaxation.[158] This was explained by assuming better gluten network formation after removing side chains from gluten proteins which could hinder the building-up of a gluten network.

Since endoglycosidases are an intrinsic side activity of almost all xylanases, cellulases, glucanases and pectinases, there was limited commercial interest in large-scale production of such enzymes. The trend seen in the last decade to produce more and more enzymes from GMOs and even producing protein-engineered enzymes has resulted in the availability of 'mono-component' xylanases; that is xylanases which are purer as a result of the manufacturing process. This trend may lead to increased interest in these endoglycosidase side activities, since it is clear from the above that observed differences in performance of various xylanases may very well be the result of the presence or absence of non-xylanase side activities.

6.7.3 Cellulases

For cellulose breakdown the combined action of several enzymes is required. Cellulase (endo-1,4-β-D-glucanase **EC 3.2.1.4)** is the most relevant one. Five general types of cellulases, based on the type of reaction catalyzed, can be identified.

(1) Endocellulase breaks internal bonds to disrupt the crystalline structure of cellulose and expose individual cellulose polysaccharide chains; (2) exo-cellulase cleaves 2–4 units from the ends of the exposed chains produced by endocellulase, resulting in the tetrasaccharides or disaccharide such as cellobiose. There are two main types of exo-cellulases (cellobiohydrolases (CBH); **EC 3.2.1.91**) – one type working progressively from the reducing end, and one type working progressively from the non-reducing end of cellulose; (3) cellobiase or β-glucosidase hydrolyzes the endocellulase product into individual monosaccharides; (4) oxidative cellulases that depolymerize cellulose by radical reactions, as for instance cellobiose dehydrogenase and (5) cellulose phosphorylases that depolymerize cellulose using phosphates instead of water. The breakdown of cellulose is schematically shown in Fig. 6.13.

Most fungal cellulases have a two-domain structure with one catalytic domain, and one cellulose-binding domain, that are connected by a flexible link. This structure is adapted for working on an insoluble substrate and it allows the enzyme to diffuse two-dimensionally on a surface in a caterpillar way. However, there are also cellulases (mostly endoglucanases) that lack a cellulose-binding domain. These enzymes might have a swelling function.

Wholemeal bread formulations differ from those of standard bread as the former contains a higher level of both water-soluble and insoluble fibre ingredients. Soluble fibres consist of water extractable (WE) arabinoxylan, β-glucans and gums, whereas insoluble fibre is

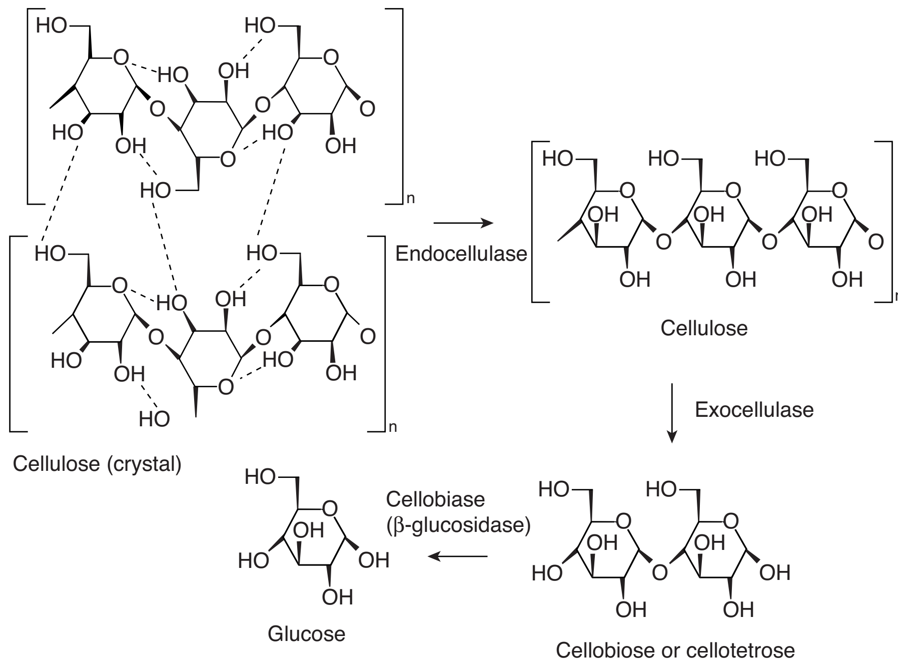


Fig. 6.13 Cellulose breakdown by endo- and exo-cellulases and cellobiase.

made up of lignin, cellulose and hemicellulose or water unextractable (WU) arabinoxylans. Wholemeal flour contains the entire seed of a plant and thus antioxidants, vitamins and fibres, which are beneficial to human health. Refined flour, used for standard white bread, has normally had the bran and the germ removed leaving only the endosperm.

The high fibre content of wholemeal bread may influence the dough consistency and weaken the gluten structure. This will ultimately lead to high water absorption, loss of extensibility and reduced fermentation tolerance. The final bread may show a reduced loaf volume, increased crumb firmness and a less pleasant taste and flavour. In addition, the crumb will be less white compared to normal bread.

Baking enzymes are therefore added to wholemeal formulations to improve dough and bread characteristics. In addition to the above-mentioned enzymes, cellulases are slowly finding their way into some bakery applications. The addition of cellulase enhances crumb structure, resulting in a more regular and fine crumb and, consequently, improved whiteness.[204]

The positive effect is due to break down of cellulose fibrils enabling better gluten development and improved proofing stability, increased dough tolerance and enhanced volume, texture and appeal in wholegrain bread types.

One of the relevant enzyme activities present in normal cellulase preparations is a CBH with bread improving activity.[205] The most important effects are an improved bread volume and an improved crumb structure, which are not accompanied by bad dough-handling properties due to stickiness of the dough (Table 6.7).

Like most sectors of the food industry, the bakery market has been presented with the opportunity of rising demand for healthy ‘wellness’ products promising nutritional benefits. Since consumption of whole grains has been linked to improvements in cardiovascular health as well as reducing the risk of certain cancers and lowering blood pressure, this move towards

Table 6.7 Effect of cellobiohydrolase on dough characteristics and loaf volume.

Addition	Dosage (ppm)	Dough quality	Volume (%)	Crumb structure
Reference		0	100	0
Xylanase	50	0	107	+
Xylanase	100	+	112	+
Cellobiohydrolase (CBH)	5	+	107	+
Cellobiohydrolase (CBH)	10	+++	110	++
Xylanase + CBH	50 + 5	+++	112	++
Xylanase + CBH	50 + 10	+++	115	+++

health-boosting foods has caused sales of wholegrain and high-fibre breads to rise rapidly in recent years with all major manufacturers offering wholegrain versions of their core bread brands to keep up with this demand.

6.7.4 Mannanases

β-Mannanase (1,4-β-D-mannan mannanohydrolase; **EC 3.2.1.78**) catalyzes the hydrolysis of β-1,4 mannose linkages of the backbone of β-mannans. These polysaccharides are found in various seeds and beans where they play an important role in the mechanical resistance and the swelling that occurs during germination. Mannans are also a major component of the hemicellulose fraction in soft woods.

There are several other types of enzymes that participate in the complete decomposition and conversion of the mannan, for example exo-β mannanase (1,4-β-D-mannan mannohydrolase; **EC 3.2.1.xx**–unassigned), exomannobiohydrolase (1,4-β-D-mannan mannobiohydrolase; **EC 3.2.1.100**) and β-mannosidase (**EC 3.2.1.25**).

Galactomannans and galactoglucomannans form a second group of hemicellulolytic structures present in plant cell walls. They are the major hemicellulose fraction of gymnosperms, in which they represent 12–15% of the cell wall biomass. Galactomannans are most commonly found in the family of Leguminoseae, in which they represent up to 38% of seed dry weight, but have also been identified in species of other plants such as Ebenaceae and Palmae. They consist of a backbone of β-1,4-linked D-mannose residues, which can be substituted by D-galactose residues via an α-1,6-linkage (Figs 6.14(a) and (b)). Depending on the source of the polysaccharide, mannose/galactose ratios can vary from 1.0 to 5.3.

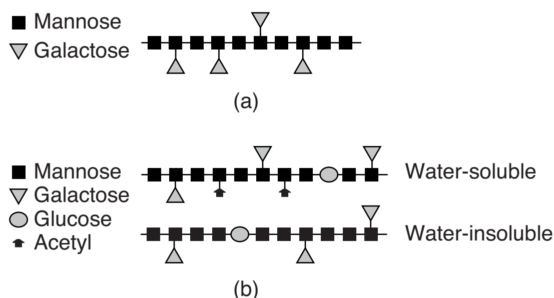


Fig. 6.14 (a) Schematic representation of the galactomannan structure. (b) Schematic representation of the galactoglucomannan structure.

Table 6.8 Effects of β-mannanase and guar on the anti-staling properties of bread.

Improver	Xylanase	Mannanase	Guar	Freshness (4 days)	Dough consistency
1	39.000	62	0	1	2
2	39.000	0	0.1%	2	3
3	39.000	62	0.1%	1	1

Galactoglucomannan is the major hemicellulolytic component of softwood. Two different structures can be identified within this group of polysaccharides. Both consist of a β-1,4-linked D-mannose backbone, which can be substituted by α-1,6-linked D-galactose. The galactoglucomannan backbone also contains β-1,4-linked D-glucose residues. Water-soluble galactoglucomannan has a higher galactose content than does water-insoluble galactoglucomannan and in addition contains acetyl residues attached to the main chain. Approximately, 20–30% of the backbone glucose and/or mannose residues are esterified with acetyl groups at C-2 or C-3.

Mannanases are useful in several industrial processes, such as the extraction of vegetable oil from leguminous seeds and the reduction of the viscosity of coffee extracts during manufacture of instant coffee. In the pulp and paper industry, mannanases can act synergistically with xylanases as biological pre-bleaching agents for soft wood pulp, allowing a significant reduction of environmental pollution compared with the use of chlorine-based chemical agents. However, in spite of these potentially interesting applications, the widespread use of mannanases is limited due to high production costs for the enzyme.

Mannanases are produced by microorganisms such as mould or yeast fungus as well as *Bacillus subtilis*, *Aeromonas*, *Enterococcus*, *Pseudomonas* and *Streptomyces*. Some higher plants or animals can produce mannanase. Microorganisms used for production of the mannanase are usually *Trichoderma* or *Aspergillus* sp. fungal strains.

Industrial applications of mannanases in bread making have been described.[76,206] The use of a β-mannanase in a baking improver in the preparation of baked goods improves the properties of dough and that of the final baked product. With regards to the dough, the tolerance can be improved, but also the dough flexibility, dough stickiness and general handling properties. With regards to the final baked product, mannanase retards staling and improves crumb structure.[206] Interesting is that the addition of a mannanase together with glucomannan and/or galactomannan such as guar of konjac gum, improves the properties of the dough and the baked products even further. This is shown in Tables 6.8 and 6.9.[206]

Table 6.9 Effect of specific enzymes on dough characteristics and shape.

Improver	Dough		Shape with proofing time		Specific volume with proofing time	
	Consistency	Stability	50′	65′	50′	65′
KBrO3	1	–	6	4	100	100
Xylanase (Xyl.)	4	+	7	5	104	103
Xyl. + glucose oxidase	2	+	7	5	106	105
Xyl. + β-mannanase	3	+	7	5	106	105
Xyl. + β-mannanase + glucose oxidase	2	++	7	6	107	107

6.8 CONCLUDING REMARKS

In the above, enzymes for baking have been discussed with regards to their classification, mechanism of action, effect and commercial availability. The word enzyme needs a little extra explanation. When talking about commercial enzyme preparations, these products are often referred to as, for example, lipase or xylanase or amylase. However, commercial preparations are hardly ever single enzyme preparations. In most cases commercial products are blends of enzymes. Besides the main enzyme activity, which often gives the preparation its name, a range of other enzymes can be present. These other enzymes are either present as natural side activities coming from the microorganism producing the main activity, or are deliberately added. This makes comparisons of commercial products very difficult, especially when looking only at the main activity. For a good comparison, it is absolutely necessary to test the products in the final application before a comparison is made based on activity, dose rate or product costs.

REFERENCES

1. Cura, J.A., Jansson, P.-E. and Krisman, C.R. (1995) Gelatinization of wheat starch as modified by xanthan gum, guar gum, and cellulose gum. *Starch* **47**, 207–209.
2. Atwell, W.A., Hood, L.F., Lineback, D.R., Varriano-Marston, E. and Zobel, H.F. (1988) Technology and methodology associated with basic starch phenomena. *Cereal Foods World* **33**, 306–311.
3. Schofield, J.D., Bottomley, R.C., Timms, M.F. and Booth, M.R. (1983) The effect of heat on wheat gluten and the involvement of Sulfhydryl-disulphide interchange reactions. *Journal of Cereal Science* **1**, 241–253.
4. Shewry, P. R. (1995) Plant storage proteins. *Biological Reviews* **70**, 375–426.
5. Wrigley, C.W. and Bietz, J.A. (1988) Proteins and amino acids. In: *Wheat Chemistry and Technology*, Vol. **1** (ed. Y. Pomeranz). AACC Inc., St. Paul, MN, pp. 159–275.
6. Finney, K.F. and Barmore, M.A. (1948) Loaf volume and protein content of hard winter and spring wheats. *Cereal Chemistry* **25**, 291–312.
7. Osborne, T.B. (1907) *The Proteins of the Wheat Kernel*. Publications of the Carnegie Institution Washington Judd and Detweiler, Washington, D.C.
8. Orth, R.A. and Bushuk, W. (1972) A comparative study of the proteins of wheats of diverse baking qualities. *Cereal Chemistry* **49**, 268–275.
9. Hoseney, R.C. (1994) *Principles of Cereal Science and Technology*, 2nd edn. Association of Cereal Chemists, Inc., St. Paul, MN, pp. 81–101, 229–273.
10. Kahn, K. (2006) Shelf life of bakery products. In: *Bakery Products, Science and Technology* (ed. Y.H. Hui). Blackwell Publishing Company, Ames, IA.
11. Belton, P.S. (1999) On the elasticity of wheat gluten. *Journal of Cereal Science* **29**, 103–107.
12. Dimler, R.J. (1963) Gluten, the key to wheat's utility. *Baker's Digest* **37**, 52–57.
13. Moonen, J.H.E., Scheepstra, A. and Graveland, A. (1986) Use of the SDS sedimentation test and SDS polyacrylamide gel electrophoresis for screening breeder's samples of wheat for bread making quality. *Euphytica* **31**, 677–690.
14. Payne, P.I., Nightingale, M.A., Krattiger, A.F. and Holt, L.M. (1987) The relationship between HMW glutenin subunit composition and the bread making quality of British-grown wheat varieties. *Journal of the Science of Food and Agriculture* **40**, 51–65.
15. Weegels, P.L., Hamer, R.J. and Schofield, J.D. (1996) Depolymerization and re-polymerization of wheat glutenin during dough processing I. Relationships between glutenin macropolymer content and quality parameters. *Journal of Cereal Science* **23**, 103–111.
16. Weegels, P.L., Hamer, R.J. and Schofield, J.D. (1997) Depolymerization and re-polymerization of wheat glutenin during dough processing II. Changes in composition. *Journal of Cereal Science* **25**, 155–163.

17. Bekes, F., Gras, P.W. and Gupta, R.B. (1994) Mixing properties as measurement of reversible reduction and oxidation of dough. *Cereal Chemistry* **71**, 44–50.
18. Jelaca, S.L. and Hlynca, I. (1971) Water binding capacity of wheat flour crude pentosans and their relation to mixing characteristics of dough. *Cereal Chemistry* **48**, 211–222.
19. Rattan, O., Izydorczyk, M. and Biliaderis, C.G. (1994) Structure and rheological behaviour of arabinoxylans from Canadian bread wheat flours. *Food Science and Technology* **27**, 350–355.
20. Sosulski, F., Krygier, K. and Hogge, L. (1982) Free, esterified and insoluble-bound phenolic acid. III. Composition of phenolic acids in cereal and potato flours. *Journal of Agricultural and Food Chemistry* **30**, 337–340.
21. Delcour, J.A., Vanhamel, S. and Hoseney, R.C. (1991) Physicochemical and functional properties of rye non-starch polysaccharides. II. Impact of a fraction containing water soluble pentosans and proteins on gluten-starch loaf volumes. *Cereal Chemistry* **68**, 72–76.
22. Michniewicz, J., Biliaderis, C.G. and Bushuk, W. (1991) Effects of added pentosans on physical and technological characteristics of dough and gluten. *Cereal Chemistry* **68**, 252–258.
23. Labat, E., Morel, M.H. and Rouau, X. (2000) Effects of laccase and ferulic acid on wheat flour dough. *Cereal Chemistry* **77**, 823–828.
24. Wang, M.W. (2003) Effect of pentosans on gluten formation and properties. PhD Thesis, Wageningen University.
25. Patil, S.K., Tsen, C.C. and Lineback, D.R. (1975) Water soluble pentosans of wheat flour. I. Viscosity properties and molecular weights estimated by gel filtration. *Cereal Chemistry* **52**, 44–56.
26. Geissmann, T. and Neukom, H. (1973) On the composition of the water soluble wheat flour pentosans and their oxidative gelation. *Lebensmittel-Wissenschaft und Technologie* **6**, 59–62.
27. Hoseney, R.C. and Faubion, J.M. (1981) A mechanism for the oxidative gelation of wheat flour water-soluble pentosans. *Cereal Chemistry* **58**, 421–424.
28. Izydorczyk, M.S., Biliaderis, C.G. and Bushuk, W. (1990) Physical properties of water-soluble pentosans from wheat. *Journal of Cereal Science* **11**, 153–169.
29. Rouau, X., El-Hayek, M.L. and Moreau, D. (1994) Effect of an enzyme preparation containing pentosanases on the bread making quality of flours in relation to changes in pentosans properties. *Journal of Cereal Science* **19**, 259–272.
30. Jelaca, S.L. and Hlynca, I. (1972) Effect of wheat flour pentosans in dough, gluten and bread. *Cereal Chemistry* **49**, 489–495.
31. Kim, S.K. and D'Appolonia, B.L. (1977a) Bread staling studies I: effect of protein content on staling rate and bread crumb pasting properties. *Cereal Chemistry* **54**, 207–215.
32. Kim, S.K. and D'Appolonia, B.L. (1977b) Bread staling studies II: effect of protein content and storage temperature on the role of starch. *Cereal Chemistry* **54**, 216–224.
33. Weegels, P.L., Marseille, J.P. and Hamer, R.J. (1992) Enzymes as processing aid in the separation of wheat flour into starch and gluten. *Starch* **44**, 44–48.
34. Jackson G.M. and Hoseney, R.C. (1986) Effect of endogenous phenolic acids on the mixing properties of wheat flour doughs. *Journal of Cereal Science* **4**, 79–85.
35. Neukom, H. and Markwalder, H.U. (1978) Oxidative gelation of wheat flour pentosans: a new way of cross-linking polymers. *Cereal Foods World* **23**, 374–376.
36. Figueroa-Espinoza, M.C., Morel, M.-H. and Rouau, X. (1998) Oxidative cross-linking of pentosans by a fungal laccase and horse radish peroxidase. Mechanism of linkage between feruloylated arabinoxylans. *Cereal Chemistry* **75**, 259–265.
37. Moore, A.M., Martinez-Munoz, I. and Bushuk, W. (1990) Factors affecting the oxidative elation of wheat water-solubles. *Cereal Chemistry* **67**, 81–84.
38. Oudgenoeg, G., Dirksen, E., Ingemann, S., Hilhorst, S.R., Gruppen, H., Boeriu, C.G., Piersma, S.R., Berkel, W.J.H., Laane, C. and Voragen, A.G. (2002) Horseradish peroxidase catalyzed oligomerization of ferulic acid on a template of a tyrosine containing tripeptide. *Journal of Biological Chemistry* **277**, 21332–21340.
39. Oudgenoeg, G., Hilhorst, R., Piersma, S.R., Boeriu, C.G., Gruppen, H., Hessing, M., Voragen, A.G. and Laane, C. (2001) Peroxidase mediated cross-linking of a tyrosine containing peptide with ferulic acid. *Journal of Agricultural and Food Chemistry* **49**, 2503–2510.
40. Vinkx, C.J.A., Van Nieuwenhove, C.G. and Delcour, J.A. (1991) Physicochemical and functional properties of rye non-starch polysaccharides III. Oxidative gelation of a fraction containing water-soluble pentosans and proteins. *Cereal Chemistry* **68**, 617–622.
41. Pomeranz, Y. and Chung, O.K. (1978) Interactions of lipids with proteins and carbohydrates in bread making. *Journal of the American Oil Chemists' Society* **55**, 285–289.

42. MacMurray, T.A. and Morrison, W.R. (1970) Composition of wheat flour lipids. *Journal of the Science of Food and Agriculture* **21**, 520.
43. Eliasson, A.-C. and Larsson, K. (1993) *Cereals in Bread Making. A Molecular Colloidal Approach*. Marcel Dekker Inc., New York, p. 376.
44. Morrison, W.R., Law, R.V. and Snape, C.E. (1993) Evidence for the inclusion complexes of lipids with V-amylose in maize, rice and oat starches. *Journal of Cereal Science* **18**, 107–109.
45. Chung, O.K. (1986) Lipid protein interactions in wheat flour, dough, gluten and protein fractions. *Cereal Foods World* **31**, 242–246.
46. Larsson, K. (1983) *Lipids in Cereal Technology*. Academic Press, London, pp. 237–251.
47. Gan, Z., Ellis, P.R. and Schofield, J.D. (1995) Amylose is not strictly linear. *Journal of Cereal Science* **21**, 215–230.
48. Kragh, K. (2002) Amylases in baking. In: *Recent Advances in Enzymes in Grain Processing* (eds C.M. Courtin, W.S. Veraverbeke and J.A. Delcour). Catholic University Leuven, Belgium, pp. 221–227.
49. Qi Si, J. (1996) New enzymes for the baking industry. *Food Technology Europe* **3**, 60–64.
50. Martinez-Anaya, M.A., Devessa, A., Andreu, P., Escriva, C. and Collar, C. (1999) Effects of the combination of starters and enzymes in regulating bread quality and shelf life. *Food Science and Technology International* **5**, 263–273.
51. van Duijnhoven, A.M. (2008) Personal Communication.
52. AFMB-CNRS-Universités Aix-Marseille I & II (1999) http://afmb.cnrs-mrs.fr/CAZY/
53. Qi Si, J. and Simmonsen, R. (1994) Functional mechanism of some microbial amylases antistaling effect. In: *Proceedings of the International Symposium on New Approaches in the Production of Food Stuffs and Intermediate Products from Cereal Grains and Oilseeds*. Beijing, China.
54. Pritchard, P. (1986) Studies on the bread-improving mechanism of fungal alpha amylase. *FMBRA Bulletin* **5**, 208–211.
55. van Dam, H.W. and Hille, J.D.R. (1992) Yeast and enzymes in bread making. *Cereal Foods World* **37**, 245.
56. Svensson, B. (1995) Protein engineering in the α-amylase family: catalytic mechanism, substrate specificity. *Plant Molecular Biology* **25**, 141–157.
57. Bowles, L.K. (1996) Amylolytic enzymes. In: *Baked Goods Freshness, Technology, Evaluation and Inhibition of Staling* (eds R.E. Hebeda and H.F. Zobel). Marcel Dekker Inc., New York, pp. 105–129.
58. Kulp, K. and Ponte, J.G. (1981) Staling of white pan bread: fundamental causes. *CRC Critical Reviews Food Science and Nutrition* **15**, 1–48.
59. Schoch, T.J. and French, D. (1947) Studies on bread staling. I: the role of starch. *Cereal Chemistry* **24**, 231–249.
60. MacRritchie, F. (1980) *Advances in Cereal Science and Technology III* (ed. Y. Pomeranz). American Association of Cereal Chemists, St. Paul, MN, Chapter 7.
61. Dragsdorf, R.D. and Varriano-Marston, E. (1980) Bread staling: X-ray diffraction studies on bread supplemented with α-amylases from different sources. *Cereal Chemistry* **57**, 310–314.
62. Martin, M.L. and Hoseney, R.C. (1991b) A mechanism of bread firming. II: role of starch hydrolysing enzymes. *Cereal Chemistry* **68**, 503–507.
63. Martin, M.L., Zeleznak, K.J., Hoseney, R.C. (1991a) A mechanism of bread firming. I: role of starch swelling. *Cereal Chemistry* **68**, 498–503.
64. Akers, A.A. and Hoseney, R.C. (1994) Water soluble dextrins from α-amylase treated bread and their relationship to bread firming. *Cereal Chemistry* **71**, 223–226.
65. Qi Si, J. (1994) Novo Nordisk applicant: use of laccase in baking. International Patent Application, WO 9428728.
66. Gerrard, J.A., Every, D., Sutton, K.H. and Gilpin, M.J. (1997) The role of maltodextrins in the staling of bread. *Journal of Cereal Science* **26**, 201–209.
67. Qi Si, J. and Lustenberger, C. (2002) Enzymes for bread, pasta and noodle products. In: *Enzymes in Food Technology* (eds R.J. Whitehurst and B.A. Law). CRC Press, Sheffield, pp. 19–57.
68. Anon. (2003) E.S.L. revolution/evolution. *Milling and Baking News*, March.
69. van Duijnhoven, A.M., Sturkenboom, M. and De Levita, P. (2005) Pan release agent. WO2005/094599.
70. Schieberle, P. (1990) The role of free amino acids present in yeast as precursors of the odorants 2-acetyl-pyrroline and 2-acetotetrahydropyridine in wheat bread crust. *Zeitschrift fur Lebensmittel Untersuchung und Forschung* **191**, 206–209.
71. Martinez-Anaya, M. and Jimenez, T. (1997) Functionality of enzymes that hydrolyses starch and non-starch polysaccharides in bread making. *Zeitschrift fur Lebensmittel Untersuchung und Forschung* **205**, 209–214.

72. Qi Si, J. (1988) Novamyl, a true antistaling enzyme. *Proceedings of IATA Meeting*, Valencia, Spain.
73. Anon. (2001) Novamyl is a maltogenic α-amylase. *Novozymes product sheet B547*.
74. Christophersen, C., Otzen, D.E., Norman, B.E., Christensen, S. and Schaefer, T. (1998) Enzymatic characterization of maltogenic alpha amylase, a thermostable α-amylase. *Starch* **50**, 39–45.
75. McCleary, B.V., Gibson, T.S., Allen, H. and Gams, T.C. (1986) Enzymic hydrolysis and industrial importance of barley glucans and wheat flour pentosans. *Starch* **38**, 433–437.
76. Maat, J., Roza, M., Verbakel, J., Stam, H., Santos da Silva, M.J., Bosse, M., Egmond, M.R., Hagemans, M.L.D., van Gorcom, R.F.M., Hessing, J.G.M and van den Hondel, C.A.M.J.J. (1992) Xylanases and their application in bakery. In: *Xylans and Xylanases* (eds G.B.J. Visser, M.A. Kusters-van Someren and A.G.J. Voragen). Elsevier Science Publishers, Amsterdam, Netherlands, pp. 349–360.
77. Gruppen, H., Kormelink, F.J.M. and Voragen, A.G.J. (1993) Water-unextractable cell wall material from wheat flour. III. A structural model for arabinoxylans. *Journal of Cereal Science* **19**, 11–18.
78. Rouau, X. and Moreau, D. (1993) Modification of some physicochemical properties of wheat flour pentosans by an enzyme complex recommended for baking. *Cereal Chemistry* **70**, 626–632.
79. Hamer, R.J. and Lichtendonk, W.J. (1987) Structure-function studies on gluten proteins. In: *Proceedings of the 3rd International Workshop on Gluten Proteins* (eds R. Lasztity and F. Bekezs). Budapest, Hungary, p. 227.
80. Courtin, C.M., Roelants, A. and Delcour, J.A. (2001) The use of two endoxylanases with different substrate selectivity provides insight into the role of endoxylanases in bread making. *Journal of Agricultural and Food Chemistry* **47**, 1870–1877.
81. Hamer, R.J. (1991) Enzymes in the baking industry. In: *Enzymes in Food Processing* (eds G.A. Tucker and L.F.J. Woods). Blackie, Glasgow, pp. 168–193.
82. Weegels, P.L. and Hamer, R.J. (1989) Predicting the baking quality of gluten. *Cereal Foods World* **34**, 210–212.
83. Wong, K.K.Y., Tan, L.U.L. and Saddler, J.N. (1988) Multiplicity of β-1,4-xylanase in microorganisms: functions and applications. *Microbiological Reviews* **52**, 305.
84. Jeffries, T.W. (1996) Biochemistry and genetics of microbial xylanases. *Current Opinion in Biotechnology* **7**, 337–342.
85. Biely, P., Vranska, M., Tenkanen, M. and Kluepfel, D. (1997) Endo-beta-1,4-xylanase families: differences in catalytic properties. *Journal of Biotechnology* **57**, 151–166.
86. Subramaniyan, S. (2000) Studies on the production of bacterial xylanases. PhD Thesis, Cochin University of Science and Technology, Kerala, India.
87. Bailey, M.J., Buchert, J. and Viikari, L. (1993) Effect of pH on production of xylanase by Trichoderma reesei on xylan and cellulose based media. *Applied Microbiology and Biotechnology* **40**, 224.
88. Subramaniyan, S. and Prema, P. (2003) Biotechnology of microbial xylanases: enzymology, molecular biology and application. *Critical Reviews in Biotechnology* **22**, 33–64.
89. Henrissat, B. and Bairoch, A. (1993) New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *The Biochemical Journal* **293**, 781–788.
90. Coutinho, P.M. and Henrissat, B. (1999) Carbohydrate-active enzymes: an integrated database approach. In: *Recent Advances in Carbohydrate Bioengineering* (eds H.J. Gibert, G. Davies, B. Henrissat and B. Svensson). The Royal Society of Chemistry, Cambridge, pp. 3–12. http://afmb.cnrs-mrs.fr/CAZY
91. Collins, T., Gerday, C. and Feller, G. (2005) Xylanases, xylanase families and extremophilic xylanases. *FEMS Microbiology Reviews* **29** (1), 3–23.
92. Collins, T., Meewis, M.A., Stals, I., Claeyssens, M., Feller, G. and Gerday, C. (2002) A novel family 8 xylanase, function and physicochemical characterization. *Journal of Biological Chemistry* **277**, 35133–35139.
93. Collins T., Gerday, C. and Feller, G. (2003) Xylanases, xylanase families and extremophilic xylanases. *Journal of Molecular Biology* **328**, 419.
94. van Petegem, F., Collins, T., Meewis, M.A., Feller, G. and van Beeumen, J. (2003) The structure of a cold adapted family 8 xylanase at 1.3 Å resolution, structural adaptations to cold and investigation of the active site. *Journal of Biological Chemistry* **278**, 7531–7539.
95. Courtin, C.M., Roelants, A. and Delcour, J.A. (1999) Fractionation reconstitution experiments provide insight into the role of endoxylanases in bread making. *Journal of Agricultural and Food Chemistry* **47**, 1870–1877.

96. Moers, K., Courtin, C.M., Brijs, K. and Delcour, J.A. (2002) A screening method for endo-beta-1,4 xylanase substrate selectivity. In: *Recent Advances in Enzymes in Grain Processing* (eds C.M. Courtin, W.S. Veraverbeke and J.A. Delcour). Catholic University Leuven, Belgium.
97. Guy, R.C.E. and Sahi, S.S. (2002) Comparison of effects of xylanases with fungal amylases in five flour types. In: *Recent Advances in Enzymes in Grain Processing* (eds C.M. Courtin, W.S. Veraverbeke and J.A. Delcour). Laboratory of Food Chemistry, Catholic University Leuven, Belgium.
98. Sprössler, B.G. (1997) Xylanases in baking. In: *1st European Symposium on Enzymes in Grain Processing* (eds S.A.G.F. Angelino, R.J. Hamer, W. van Hartingsveldt, F. Heidekamp and J.P. van der Lugt). TNO Food & Nutrition, Zeist, Netherlands, pp. 177–187.
99. Mathewson, P.R. (1998) Common enzyme reactions. *Cereal Foods World* **43**, 798–803.
100. Verger, R. and De Haas, G.H. (1973) Enzyme reactions in a membrane model. 1. A new technique to study enzyme reactions in monolayers. *Chemistry and Physics of Lipids* **10**(2), 127–136.
101. Brockerhof, H. and Jensen, G. (1974) In: *Lipolytic Enzymes* (eds J. Caro, M. Boudouard, J. Bonicel, A. Guidom and J. Desnuelle). Academic Press, New York.
102. Primo-Martin, C., Hamer, R.J. and de Jongh, H.H.J. (2006) Surface layer properties of dough liquor components: are they key parameters in gas retention in bread dough? *Food Biophysics* **1**, 83–93.
103. van Vliet, T., Janssen, A.M., Bloksma, A.H. and Walstra, J. (1992) Strain hardening of dough as a requirement for gas retention. *Journal of Texture Studies* **23**, 439.
104. Gan, Z., Angold, R.E., Pomeranz, Y., Shogren, M.D. and Finney, K.F. (1990) Response to shortening addition and lipid removal in flours that vary in bread making quality. *Journal of Cereal Science* **12**, 15–24.
105. Macritchie, F. and Gras, P.W. (1973) The role of flour lipids in baking. *Cereal Chemistry* **50**, 292–297.
106. Lundkvist, H., Arskog, P.B., Erlandsen, L., Ipsen, R. and Wilde, P (2007) Interfacial properties of dough liquor from lipase modified dough. In: *AACC C&E Spring Meeting*. Montpellier, France, 2–4 May.
107. Olesen, T. and Qi Si, J. (1994) Use of lipase in baking. International Patent Application, WO 94/0403035.
108. Qi Si, J. (1997) Synergistic effects of enzymes for bread making. *Cereal Foods World* **42**, 802–807.
109. Christiansen, L. (2006) Novozymes A/S (2001). Lipopan™ F BG Product Sheet.
110. Castello, P., Baret, J.L., Potus, J. and Nicolas, J. (2000) Technological and biochemical effects of exogenous lipases in bread making. In: *2nd European Symposium on Enzymes in Grain Processing* (eds T. Simoinen and M. Tenkanen). Espoo, Finland, pp. 193–200.
111. Castello, P., Jollet, S., Potus, J. and Nicolas, J. (1998) Effects of exogenous lipase on dough lipids during mixing of wheat flours. *Cereal Chemistry* **75**, 595–601.
112. Johnson, R.H. and Welch, E.A. (1968) Baked goods dough and method. US Patent Application, WO 3,368,903.
113. Poulsen, C. and Borch Søe, J. (1997) Effect and functionality of lipases in dough and bread. In: *1st Symposium of Enzymes in Grain Processing* (eds S.A.G.F. Angelino, R.J. Hamer, W. van Hartingsveldt, F. Heidekamp and J.P. van der Lugt). TNO Nutrition and Food Research Institute, Zeist, Netherlands, pp. 204–214.
114. Xu, F. (2005) Application of oxidoreductases: recent progress. *Industrial Biotechnology* **1**, 38–50.
115. Munro, A.W., Taylor, P. and Wilkinshaw, M.D. (2000) Structures of redox enzymes. *Current Opinion in Biotechnology* **11**, 369–376.
116. Dong, W. and Hoseney, R.C. (1995) Effects of certain bread making oxidants and reducing agents on dough rheological properties. *Cereal Chemistry* **72**, 58–64.
117. Bloksma, A.H. (1972) The relation between thiol disulfide contents of dough and its rheological properties. *Cereal Chemistry* **49**, 104–117.
118. Jorgensen, H. (1939) Further investigations into the nature of the action of bromate and ascorbic acid on the baking strength of wheat flour. *Cereal Chemistry* **16**, 51–60.
119. Bloksma, A.H. and Bushuk, W. (1988) Rheology and chemistry of dough. In: *Wheat Chemistry and Technology,* Vols. 1 and 2 (ed. Y. Pomeranz). AACC, St. Paul, MN.
120. Oort, M.G. van (1996) Oxidases in baking. *International Food Ingredients* **4**, 42–47.
121. Nicolas, J. and Potus, J. (2000) Interactions between lipoxygenase and other oxidoreductases in baking. In: *2nd European Symposium on Enzymes in Grain Processing* (eds T. Simoinen and M. Tenkanen). VTT Symposium, Espoo, Finland, pp. 103–120.
122. Wieser, H. (2003) The use of redox agents. In: *Bread Making: Improving Quality* (ed. S.P. Cauvain). Woodland Publishing Ltd., Cambridge, pp. 424–446.

123. Vermullapalli, V. and Hoseney, R.C. (1980) Glucose oxidase in bread making systems. *Cereal Chemistry* **75**, 439–442.
124. Primo-Martin, C., Valera, R. and Martinez-Anaya, M.A. (2003) Effect of pentosanase and oxidase on the characteristics of dough and the glutenin macropolymer (GMP). *Journal of Agricultural and Food Chemistry* **51**, 4673.
125. Mitani, M., Maeda, T. and Morita, N. (2003) Effects of various kinds of enzymes on dough properties and bread qualities. In: *Recent Advances in Enzymes in Grain Processing* (eds C.M. Courtin, W.S. Veraverbeke and J.A. Delcour). Laboratory of Food Chemistry, Leuven, Belgium, pp. 295–302.
126. Rosell, C.M., Wang, J., Aja, S., Bean, S. and Lockhart, G. (2003) Wheat flour proteins as affected by transglutaminase and glucose-oxidase. *Cereal Chemistry* **80**, 52–55.
127. Matheis, G. and Whitaker, J.R. (1987) A review: enzymatic cross-linking of food proteins applicable to foods. *Journal of Food Biochemistry* **11**, 309–327.
128. Haas, L.W. (1934) Bleaching agent and process of preparing bleached bread dough. United States Patent 1957334.
129. Hoseney, R.C., Rao, H., Faubion, J. and Sighu, J.S. (1980) Mixograph studies. IV. The mechanism by which lipoxygenase increases mixing tolerance. *Cereal Chemistry* **57**, 163–166.
130. Addo, K., Burton, D., Stuart, M.R., Burton, H.R. and Hildebrand, D.F. (1993) Soybean flour lipoxygenase isozyme mutant effects on bread dough volatiles. *Journal of Food Science* **58**, 583–585.
131. Delcros, J.F., Rakotozafy, L., Boussard, A., Davidou, S., Porte, C., Potus, J. and Nicolas, J. (1998) Effect of mixing conditions on the behaviour of lipoxygenase, peroxidase and catalase in wheat flour dough. *Cereal Chemistry* **75**, 85–93.
132. Junqueira, R.M., Rocha, F., Moreira, M.A. and Castro, I.A. (2007) Effect of proofing time and wheat flour strength on bleaching, sensory characteristics and volume of French breads with added soybean lipoxygenase. *Cereal Chemistry* **84**, 443–449.
133. Logan, J.L. and Learmonth, E.M. (1955) Gluten oxidizing capacity of soya. *Chemistry And Industry* **39**, 1220.
134. Grosch, W. (1986) Redox systems in dough. In: *The Chemistry and Physics of Baking: Materials, Processes and Products* (eds J.M. Blanshard, P.J. Frazier and T. Galliard). Royal Society of Chemistry, London, pp. 155–169.
135. Koch, R.B. (1956) Mechanisms of fat oxidation. *Bakers Digest* **30**, 48–53.
136. Dahle, L.K. and Sullivan, B. (1963) The oxidation of wheat flour. V. Effect of lipid peroxides and antioxidants. *Cereal Chemistry* **40**, 372–384.
137. Smith, D.E. and Andrews, J.S. (1957) The uptake of oxygen by flour dough. *Cereal Chemistry* **34**, 323–326.
138. Graveland, A. (1970) Enzymatic oxidations of linoleic acid and glycerol-1-monolinoleate in dough and flour water suspensions. *Journal of the American Oil Chemists' Society* **47**, 352–361.
139. Graveland, A. (1973) Analysis of lipoxygenase non volatile reaction products of linoleic acid in aqueous cereal suspensions by urea extraction and gas chromatography. *Lipids* **8**, 599–605.
140. Tait, S.P.C. and Galliard, T. (1988) Oxidation of linoleic acid in dough and aqueous suspensions of wholemeal flours: effect of storage. *Journal of Cereal Science* **8**, 55–67.
141. Levavasseur, L., Rakotozafy, L., Manceau, E., Louarne, L., Robert, H., Bartet, J-L., Potus, J. and Nicolas, J. (2006) Discrimination of wheat varieties by simultaneous measurements of oxygen consumption and consistency of flour during mixing. *Journal of the Science of Food and Agriculture* **86**, 1688–1698.
142. Tsen, C.C. and Hlynka, I. (1963) Flour lipids and oxidation of sulfhydryl groups in dough. *Cereal Chemistry* **40**, 145.
143. Tsen, C.C. (1965) The improving mechanism of ascorbic acid. *Cereal Chemistry* **42**, 86–96.
144. Bloksma, A.H. (1963) Oxidation by molecular oxygen of thiol groups in unleavened dough from normal and defatted wheat flours. *Journal of the Science of Food and Agriculture* **14**, 529–535.
145. Graveland, A. (1971) Modification of the Lipoxygenase reaction by wheat glutenin. PhD Thesis, University Utrecht, Utrecht.
146. Avram, E., Boussard, A., Potus, J. and Nicolas, J. (2003) Oxidation of glutathione by purified wheat and soybean Lipoxygenase in the presence of linoleic acid at various pH. In: *Recent Advances in Enzymes in Grain Processing* (eds C.M. Courtin, W.S. Veraverbeke and J.A. Delcour). Katholieke Universiteit, Leuven, Belgium.
147. Kiefer, R., Mattheis, G., Hofmann, G. and Belitz, H.D. (1982) *Zeitschrift Lebensmittel Untersuchung und Forschung*, **75**, 5–7.

148. Fukushige, H., Wang, C., Simpson, T., Gardner, H. and Hildebrand, D. (2005) Purification and identification of linoleic acid hydroperoxides generated by soybean seed lipoxygenases 2 and 3. *Journal of Agricultural and Food Chemistry* **53**, 5691–5694.
149. Nicolas, J., Richard-Forget, F., Goupy, P., Amiot, M.J. and Aubers, S. (1994) Enzymatic browning reactions in apples and apple products. *Critical Reviews in Food Science and Nutrition* **34**, 109–157.
150. Zawistowski, J., Biliaderis, C.G. and Eskin, N.A.M. (1991) Polyphenol oxidase. In: *Oxidative Enzymes in Foods* (eds D.S. Robinson and N.A.M. Eskin). Elsevier Applied Science Chemistry, London, pp. 217–273.
151. Kuninori, T., Nishiyama, J. and Matsumoto, H. (1976) Effect of mushroom extract on the physical properties of dough. *Cereal Chemistry* **53**, 420–428.
152. Takasaki, S. and Kawakishi, S. (1997) Formation of protein bound 3,4-dihydroxy-phenylalanine and 5-cysteinyl-3,4-dihydrophenylalanine as new cross-linkers in gluten. *Journal of Agricultural and Food Chemistry* **45**, 3472–3475.
153. Takasaki, S., Kawakishi, S., Murat, M. and Homma, S. (2001) Polymerization of gliadin mediated by mushroom Tyrosinase. *Lebensmittel Wissenschaft Technologie*, **34**, 507–512.
154. Tilley, K.A. (1999) Method of dough manufacture by monitoring and optimizing gluten protein linkages. US patent No 6.284.296.
155. Tilley, K.A., Benjamin, R.E., Bagorogoza, K.E., Okot-Kolber, B.M., Prakash, O. and Kwen, H. (2000) Tyrosine cross links: The molecular basis of gluten structure and function. *Journal of Agricultural and Food Chemistry* **49**(6), 2627–2632.
156. Hillhorst, R., Gruppen, H., Orsel, R., Laane, C., Schols, H.A. and Voragen, A.G.J. (2000) On the mechanism of action of peroxidase in wheat dough. In: *2nd European Symposium on Enzymes in Bread Making* (eds T. Simoinen and M. Tenkanen). Techn. Research Centre of Finland VTT, Espoo, Finland, pp. 127–132.
157. Reinikainen, T., Lantto, R., Niku-Pavoola, M-L. and Buchert, J. (2003) Enzymes for cross-linking of cereal polymers. In: *Recent Advances in Enzymes in Grain Processing* (eds C.M. Courtin, W.S. Veraverbeke and J.A. Delcour). Laboratory of Food Chemistry, Leuven, Belgium, pp. 91–99.
158. Oort, M.G. van, Hennink, H. and Moonen, H. (1997) Peroxidases in bread making. In: *First European Symposium on Enzymes in Grain Processing* (eds S.A.G.F. Angelino, R.J. Hamer, W. van Hartingsveldt, F. Heidekamp and J.P. van der Lugt). TNO Nutrition and Food Institute, Zeist, The Netherlands, pp. 195–203.
159. Dunnewind, B., Vliet, T. van and Orsel, R. (2002) Effect of oxidative enzymes on bulk rheological properties of wheat flour dough. *Journal of Cereal Science* **36**, 357–366.
160. Swaisgood, H.E. (1980) Oxygen activation by sulfhydryl oxidase and the enzyme's interaction with peroxidase. *Enzyme and Microbial Technology* **2**, 265–272.
161. Kaufman, S.P. and Fennema, O. (1987) Evaluation of sulfhydryloxidase as a strengthening agent for wheat flour dough. *Cereal Chemistry* **64**, 172–176.
162. Fok, J.J., Hille, J.D.R. and Ven, B. Van Der (1993) Yeast derivative to improve bread quality. European Patent 0588426A1.
163. Grosch, W. and Wieser, H. (1990) Redox reactions in wheat dough affected by ascorbic acid. *Journal of Cereal Science* **29**, 1–16.
164. Grant, D.R. and Sood, V.K. (1980) Studies in the role of ascorbic acid in chemical dough development. II. Partial purification and characterization of an enzyme oxidizing ascorbic acid in flour. *Cereal Chemistry* **57**, 46–49.
165. Every, D., Gilpin, M.J. and Larsen, N.G. (1995) Continuous spectroscopic assay and properties ascorbic acid oxidizing factors in wheat. *Journal of Cereal Science* **21**, 231–239.
166. Every, D., Gilpin, M.J. and Larsen, N.G. (1996) Ascorbic acid oxidase levels in wheat and relationship to baking quality. *Journal of Cereal Science* **23**, 145–151.
167. Cherkiatgumchai, P. and Grant, D.R. (1986) Enzymes that contribute to the oxidation of L-ascorbic acid in flour-water systems. *Cereal Chemistry* **63**, 197–200.
168. Grant, D.R. (1974) Studies in the role of ascorbic acid in chemical dough development. I. Reaction of ascorbic acid with flour water suspensions. *Cereal Chemistry* **51**, 584–592.
169. Sarwin, R., Laskawy, G. and Grosch, W. (1993) Changes in the levels of glutathione and cysteine during the mixing of dough with L-threo- and D-erythro-ascorbic acid. *Cereal Chemistry* **70**, 553–557.
170. Kanht, W.D., Murdy, V. and Grosch, W. (1975) Verfahren zur bestimmung der aktivität des glutathion-dehydrogenase (EC 1.8.5.1.). Vorkommen des enzymes in verschiedenen weizensorten. *Zeitschrift Lebensmittel Untersuchung und Forschung* **158**, 77–82.

171. Arnaut, F., De Meyer, K. and Van Haesendonck, I. (2005) Bakery products comprising carbohydrate oxidase. European Patent EP1516536.
172. McDonald, E.C. (1969) Proteolytic enzymes of wheat and their relation to baking quality. *Baker's Digest* **43**, 26–28, 30 and 72.
173. Mathewson, P.R. (2000) Enzymatic activity during bread baking. *Cereal Foods World* **45**, 98–101.
174. Lindahl, L. and Eliasson, A.-C. (1992) Influence of added enzymes on rheological properties of a wheat flour dough. *Cereal Chemistry* **69**, 542–546.
175. Stauffer, C.E. (1987) Proteases, peptidases and inhibitors. In: *Enzymes and Their Role in Cereal Technology* (eds J.E. Kruger, D. Lineback and C.E. Stauffer). AACC Inc., St. Paul, MN, pp. 166–169.
176. Stauffer, C.E. (1994) In: *The Science of Cookie and Cracker Production* (ed. H. Faridi). Chapman & Hall, New York, pp. 237–238.
177. Nightingale, M.J., Marchylo, B.A., Clear, R.M., Dexter, J.E. and Preston, K.R. (1999) Fusarium head blight: effect of fungal proteases on wheat storage proteins. *Cereal Chemistry* **76**, 150–158.
178. Rosell, C.M., Aja, S., Bean, S. and Lookhart, G. (2002) Effect of Aelia spp. and Eurygaster spp. damage on wheat proteins. *Cereal Chemistry* **79**, 801–805.
179. Thiele, C., Gänzle, M.G. and Vogel, R.F. (2002) Contribution of sourdough Lactobacilli, Yeast and cereal enzymes to the generation of amino acids in dough relevant for bread flavour. *Cereal Chemistry* **79**, 45–51.
180. Barbeau, W.E., Griffey, C.A. and Yan, Z. (2006) Evidence that minor sprout damage can lead to significant reductions in gluten strength of winter wheats. *Cereal Chemistry* **83**, 306–310.
181. Schieberle, P. (1996) Intense aroma compounds – useful tools to monitor the influence of processing and storage on bread aroma. *Advances in Food Science* **18**, 237–244.
182. Gobetti, M., Simonetti, M.S., Rossi, J., Cossignani, L., Corsetti, A. and Damiani, P. (1994) Free D- and L-amino acid evolution during sourdough fermentation and baking. *Journal of Food Science* **59**, 881–884.
183. Hansen, A., Lund, B. and Lewis, M.J. (1989) Flavour of sourdough rye bread crumb. *Lebensmittel Wissenschaft und Technologie* **22**, 141–144.
184. Loponen, J., Mikola, M., Sontag-Strohm, T. and Salovaara, H. (2002) Degradation of high molecular weight glutenin subunits during wheat sourdough fermentation. In: *Recent Advances in Enzymes in Grain Processing* (eds C.M. Courtin, W.S. Veraverbeke and J.A. Delcour). Lab. Food Chem, Kath. University Leuven, Leuven, Belgium, pp. 281–287.
185. Harada, O., Lysenko, E.D. and Preston, K. R. (2000) Effects of commercial hydrolytic enzyme additives on Canadian short process bread properties and processing characteristics. *Cereal Chemistry* **77**, 70–76.
186. Harada, O., Lysenko, E.D., Edwards, N.M. and Preston, K.R. (2005) Effects of commercial hydrolytic enzyme additives on Japanese style sponge and dough bread properties and processing characteristics. *Cereal Chemistry* **82**, 314–320.
187. Barrett, A.H., Marando, G., Leung, H. and Kaletunc, G. (2005) Effect of different enzymes on textural stability of shelf stable bread. *Cereal Chemistry* **82**, 152–157.
188. Linares, E., Larre, C., Lemeste, M. and Popineau, Y. (2000) Emulsifying and foaming properties of gluten hydrolysates with an increasing degree of hydrolysis: role of soluble and insoluble fractions. *Cereal Chemistry* **77**, 414–420.
189. Cluskey, J.E. (1959) Relation of the rigidity of flour, starch, and gluten gels to bread staling. *Cereal Chemistry* **36**, 236–246.
190. Arnout, F., Verte, F. and Vekemans, N. (2003) Method and composition for retarding staling of bakery products by adding a thermostable protease. European Patent EP1350432.
191. Pomeranz, Y. (1971) *Wheat Chemistry and Technology*. AACC, St. Paul, MN, p. 700.
192. Gottmann, K. and Sproessler, B. (1994) Baking products and intermediates. US Patent 5279839.
193. Gottmann, K. and Sproessler, B. (1995) Baking agent and process for the manufacture of dough and bakery products. European Patent EP 0492406.
194. Shan, L., Molberg, O., Parrot, I., Hausch, F., Filiz, F. and Gray, G.M. (2002) Structural basis for gluten intolerance in celiac sprue. *Science* **297**, 2275–2279.
195. Feighery, C. (1999) Fortnightly review: celiac disease. *British Medical Journal* **319**, 236–239.
196. Toufeili, I., Dahger, S., Shadarevian, S., Noureddine, A., Sarakbi, M. and Farran, M.T. (1994) Formulation of gluten-free pocket-type flat breads. Optimization of methylcellulose, gum Arabic and egg albumen levels by response surface methodology. *Cereal Chemistry* **71**, 594–601.

197. O'Brien, C.M., von Lehmden, S. and Arendt, E.K. (2002a) Development of gluten free pizzas. *Irish Journal of Agriculture and Food Research* **42**, 134–137.
198. O'Brien, C.M., Schober, T. and Arendt, E.K. (2002b) Evaluation of the effect of different ingredients on the rheological properties of gluten-free pizza dough. *AACC Annual Meeting*. Published online at http://www.scioc.org/aacc/meeting/2002/abstracts. AACC International, St. Paul, MN.
199. Gallagher, E., Gormley, T.R. and Arendt, E.K. (2003) Recent advances in the formulation of gluten-free cereal based products. *Trends in Food Science & Technology* **15**, 143–152.
200. Gallagher, E., Gormley, T.R. and Arendt, E.K. (2004) Crust and crumb characteristics of gluten-free breads. *Journal of Food Engineering* **56**, 153–161.
201. Guarda, A., Rosell, C.M., Benedito, C. and Galotto, M.J. (2003) Different hydrocolloids as bread improvers and antistaling agents. *Food Hydrocolloids* **18**, 241–247.
202. Moore, M.M., Heinbockel, M., Dockery, P., Ulmer, H.M. and Arendt, E.K. (2006) Network formation in gluten-free bread with application of transglutaminase. *Cereal Chemistry* **83**, 28–36.
203. Laurière, M. and Denery, S. (2005) Céréales et dérivés. In: *Méthodes D'analyses Immunochimiques Pour le Contrôle de Qualité Dans les IAA* (eds J. Daussant and P. Arbault). Lavoisier, Ed Tec et Doc, Paris, pp. 293–328.
204. Maat, J. and Roza, M. (1995) Cellulase bread improvers e.g., xylanase include an oxidase or peroxidase. European Patent EP0396162.
205. van Beckhoven, R.F.W.C. (2003) Bread improving composition. US Patent 6656513.
206. van Duijnhoven, A.M. (1993) Enzyme containing baking improver. European Patent EP0529712.

7 Enzymes in non-bread wheat-based foods

Caroline H.M. van Benschop and Jan D.R. Hille

7.1 INTRODUCTION

Wheat is one of the most important raw plant materials for food products around the world. For 2008, a production of 658 million tons is estimated.[1] Of this total production, 453 million tons will be for human food consumption and 118 million tons for feed. Human wheat consumption per head is around 67 kg per annum of which the major part is consumed as bread and paste products, with minor amounts in the form of biscuits, cookies, crackers, cakes, etc. In some parts of the world other cereals like rye and rice are the main staple food.

Currently most enzymes are used in the production of bread. However, application of enzymes is growing in other segments of cereal-based products. In this chapter, an overview will be given of the application of enzymes in wheat-based, non-bread products.

7.2 FUNCTIONALITIES OF ENZYMES IN WHEAT-BASED, NON-BREAD PRODUCTS

For centuries, baked and cooked wheat-based products have been present in our diets. Wheat flour derivatives are consumed all over the world in many different forms, varying throughout the continents and civilizations representing one of the most consumed foodstuffs.

Nowadays, a wide variety of products can be included in this foodstuff category, such as bread, cakes, muffins, doughnuts, cookies, crackers, biscuits, pasta, noodles, tortillas, breakfast cereals and many others. These products can be classified depending on product type (sweetened or unsweetened), method of leavening (biological, chemical or unleavened) or from a technical point of view, on the basis of their pH, moisture content and water activity.

Most enzymes applied in bread making can also be applied in the other wheat-based baking applications mentioned above. Depending upon the raw materials used in wheat-based products, amylases, hemicellulases, lipases, oxidases, cross-linking enzymes and proteases may be applied to improve the quality or modify the texture of the baked products.

7.3 APPLICATION OF ENZYMES IN CAKE AND MUFFIN PRODUCTION

Cakes and muffins are produced from the following basic ingredients: wheat flour, sugar, eggs and in the case of pound or high-ratio cake, also fat. Sponge and pound cakes are

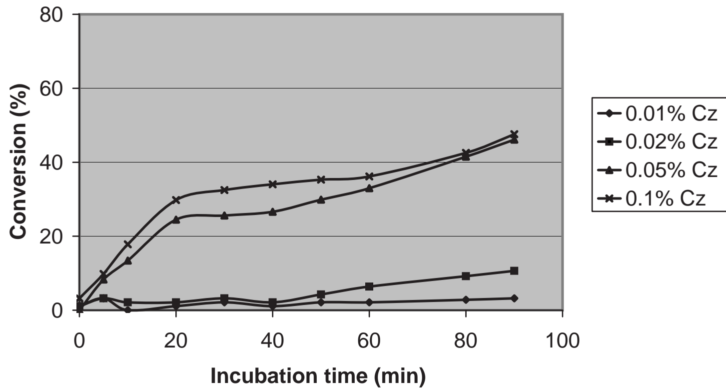


Fig. 7.1 Conversion of egg lipids by phospholipase A_2.

produced by mixing the constituents into a liquid batter and include air to form a foam. The air expands during baking and the foam transforms into a sponge because of the viscosity increase caused by the gelatinization of starch.[2]

A cake or muffin batter may be considered as an oil-in-water emulsion which can be stabilized by emulsifiers. Eggs and added emulsifiers may play this role. Eggs contain lipids consisting of triglycerides and lecithin. The latter fraction has surface-active properties and may act as an emulsifier to stabilize the oil-in-water emulsion.

The major constituents of egg lecithin are phospholipids consisting of phosphatidylcholine and phosphatidylethanolamine. These lipids have emulsifying properties which can be improved when part is hydrolyzed into the lyso-form. This hydrolysis is catalyzed by the action of phospholipase A_1 or A_2. Figure 7.1 shows the conversion rate of whole egg phosphatidylcholine (PC) by phospholipase A_2 at various dose levels and incubated at 20°C.

Figure 7.1 clearly shows that within the normal processing time required to produce batters, 2500–5000 CPU phospholipase A_2/kg whole liquid egg has enough time to hydrolyze approximately 30% of the lipid. The lysophosphatidylcholine (LPC) formed has stronger emulsifying properties than PC, which may help to improve cake quality and/or to reduce costs because of reduction in egg quantity in cake recipes.

Improvement of batter stability and baked sponge cake is shown in Fig. 7.2. In Figs 7.2(a) and (b) pictures are shown of batters heated for 5 min in a boiling water bath; (a) is the reference and in (b) 1250 CPU phospholipase A_2/kg whole egg has been added. Batter (b) is clearly more stable than reference (a).

Reference batter (a) and batter (b) were also baked in standard baking pans. Figures 7.3(a) and (b) show C-cell pictures of the crumbs of these cakes and in (c) and (d) microstructure images of reference and of phospholipase A_2 containing cakes are shown. Presence of phospholipase A_2 clearly results in formation of a more regular and fine crumb structure.

The use of phospholipase A_2 to improve the texture of sponge was described as early as 1987,[3] but in this patent the egg material is pre-incubated for 4 h at 50°C and afterwards for 30 min at 60°C before blending it with the other ingredients into the batter. In the method of

Fig. 7.2 Stability of sponge cake batter reference (a) plus Cakezyme™ (b) after 5 min heating in boiling water bath.

cake production described above, phospholipase is part of the ingredients and blended into the batter in a standard cake production set-up.

Emulsifiers are often part of cake and muffin recipes. They are added to support the incorporation of air and to improve dispersion of fat in the batter and will also stabilize expanding gas bubbles in the batter during baking. Replacement of these emulsifiers by a commercial lipase in the production of high-ratio layer cake was tested by Guy and Sahi.[4] Addition of this commercial lipase reduced the surface tension and surface viscosity at the air/water interface of batter. This indicates that surfactants were created which replaced

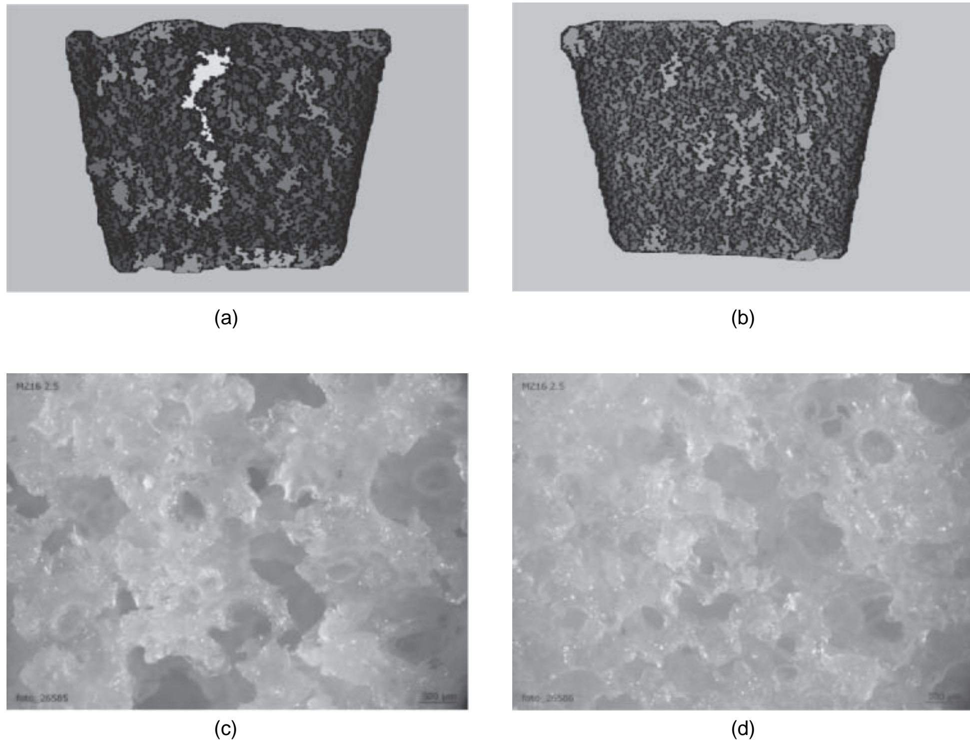


Fig. 7.3 In (a) and (b) C-cell pictures are shown of reference cake and of cake to which 1250 CPU kg^{-1} egg phospholipase A_2 was added. In (c) and (d) micro-images of (a) and (b), respectively are shown.

proteins at the air/water interface. Result after baking was an increase in cake specific volume and maintaining a fine crumb structure. Eating quality and perceived freshness over 14 days of chilled storage were improved.

When the amount of egg is reduced in a cake recipe, the quality of the cake in general will deteriorate. This deterioration can be counteracted by adding phospholipase A_1 or A_2 to the cake batter. This is seen by an increased cake volume and improved cake properties during storage, for example increased cohesiveness, increased springiness and increased elasticity.[5,6] In this patent, even a replacement of 50% egg by phospholipase A_2 is described, but to reach volume and quality of the reference cake, either soya protein (in a quantity corresponding to all dry material of the replaced egg) or whey protein (in a quantity corresponding to 50% of dry material weight of replaced egg) had to be added to the recipe.

Apart from lipolytic enzymes, starch-degrading enzymes have been applied in cake production. In 1980, starch-degrading enzymes were described to prevent cake staling.[7] In 2004, Danisco[8] rolled out a new product containing a highly specialized amylase that claimed to double the shelf life of cakes. In 2005, bacterial amylase was described as part of a cake powder conditioner, also containing calcium stearoyl lactate (CSL) and sodium stearoyl lactate (SSL) and glycerol mono stearate (GMS), which can improve the quality of cake in general and more specifically the softness of the crumb and the shelf life of the product.[9] In 2006, a patent by Novozymes was published describing maltogenic amylases which are more adapted to high sugar levels and thus more active in cake recipes.[10] Another recently developed enzyme is amylomaltase.[11] Amylomaltase or (α-(1,4))–(α-(1,4)) glucosyltransferase is a thermostable enzyme able to hydrolyze oligosaccharides from amylose and to couple these fragments to the ends of the branches of amylopectin. Amylomaltase has been applied to branch potato starch into a product forming a thermoreversible gel in water, which is able to form domains in complex foods like cake and behave like fat globules.[12] In other words, this product formed by amylomaltase can be used as a fat replacer and mouthfeel improver in cake recipes.

Proteases have been used to lower the viscosity of cereal flour suspensions and to avoid checking during biscuit baking (see Section 7.5), but are nowadays also applied to retard staling of the cake crumb. Arnaut *et al.*[13] show that the use of intermediate thermostable alkaline proteases, for example keratinase and thermitase, which had no perceivable influence on the dough rheology, had a pronounced effect on the softness and retardation of the crumb hardness, resulting in a prolonged shelf life. Specific proteases may also be applied to improve the flavour of cakes.[14,15]

7.4 APPLICATION OF ENZYMES IN PASTA AND NOODLE PRODUCTION

Pasta and noodles also play an important role in nutrition for the world's population. Both are popular for their ease of cooking and nutritional qualities.[16] Wet, dried and instant noodles are mainly consumed in China and South-East Asia, and pasta products are consumed more in North- and South-America and in Europe.

Pasta is produced by mixing semolina from durum wheat (*Triticum durum*) and water into a dry dough (25–35% moisture), and extruding this dough through a die to obtain the desired shape followed by drying to obtain a stable product.[17]

Noodles are produced by mixing flour from common wheat (*Triticum aestivum*) and salt (white noodles) or alkaline solution (yellow noodles) to form a crumbly dough[18] that is

compressed between a series of sheeting rolls to form a dough sheet. During this sheeting process the gluten network is developed, which contributes to the noodle texture. The sheeted dough is slit to produce noodles. The noodles are now ready for sale, or are further processed to prolong shelf life, to modify eating characteristics or to facilitate preparation by the consumer. The fresh noodles can be dried, or steamed and dried, or steamed and fried to obtain instant noodles.

Important characteristics for both pasta and noodles in dry form are mechanical strength, absence of checking (formation of hair line cracks in the product), smoothness of the surface and a bright colour. Dry pasta and noodles should retain their size and shape during packaging and shipment.[18] Checking may reduce the mechanical strength of pasta and noodles. Most probably checking is the result of improper drying conditions,[19] where the surface moisture evaporates too fast causing hardening of the surface. Subsequently the inner part of the strand dries, is unable to withstand the stress, and fracturing occurs.[20] In noodles, internal blisters have also been indicated as a cause of checking.[21]

In an early review by Matsuo[22] on the application of enzymes in pasta and noodle production, colour is described as the major quality factor. Pasta colour should be bright yellow and for fresh and dried noodles whiteness is preferred. For instant noodles colour is less important. Below, the influence of enzymes on pasta products produced from durum semolina will be considered first, followed by a discussion on the effects of enzymes on noodle products.

7.4.1 Enzyme effects on pasta products

The bright yellow colour of durum wheat products is a result of the natural carotenoid pigment content. Pigment loss is caused by oxidative degradation by endogenous lipoxygenase activity.[23] The phase mainly responsible for pigment loss is processing, particularly the dough mixing stage, when a substantial decrease in pigment content occurs. Lipoxygenase catalyzes the peroxidation of unsaturated fatty acids like linoleic acid to form conjugated monohydroperoxides.[24] These monohydroperoxides then react with carotenoid pigments, resulting in the loss of chromophores. Mixing under vacuum,[25] introduction of high temperature (HT) drying (60–85°C) and recently ultra high temperature (UHT) drying (85–110°C), and reducing processing time have improved pasta colour quality despite the danger of increased Maillard reactions because of high drying temperatures.[26]

As well as yellowness caused by carotenoids present in flour, another aspect of pasta colour is browning caused by naturally occurring compounds and enzymatic or non-enzymatic browning reactions.[22] Polyphenol oxidase has been implicated as a leading cause of discolouration in pasta and other wheat products.[27] Polyphenol oxidase catalyzes the hydroxylation of monophenols to *o*-diphenols (‘monophenolase’ activity) and the oxidation of *o*-diphenols to *o*-quinones (‘diphenolase’ activity). The quinones formed react with a number of functional groups, such as amines, thiols and phenolics, and form melanins, being complex coloured products.[28] However, in a recent study[29] it is concluded that although polyphenol oxidase contributes substantially to the darkening of pasta products, an additional unknown mechanism of darkening is most probably also involved. These discolouring mechanisms in wheat products may be of both enzymatic and non-enzymatic origin. One possible mechanism of enzymatic origin might be based on endogenous wheat peroxidase[30,31] but its role still remains obscure.

HT and UHT drying technologies have allowed the production of pasta of good quality from mediocre quality raw materials showing less gluten strength.[32] Edwards *et al.*[33] examined the viscoelastic properties of durum wheat semolina doughs of different strengths. They concluded from creep behaviour that high steady state viscosities of strong durum dough and relative inextensibility were consistent with strength in durum wheat being primarily a function of the density of physical cross-links present. For this reason, transglutaminase[34–37] has been described in many cases as cross-linking agent, but also oxidizing enzymes like glucose oxidase,[38] peroxidase,[39,40] dehydrogenases[41] and lactonohydrolases[42] have been described to improve gluten strength in pasta dough and overall quality of the end product.

HT and UHT drying can also offset the effects of wheat sprout damage. Sprout-damaged wheat contains increased levels of enzyme activities, for example α-amylase and protease. For both types of enzymes, conflicting results on effects in pasta production can be found in the literature.[21] However, no positive effects have been described and for that reason ultra high temperature drying is a good technique to inactivate these types of enzymes before they can act negatively on pasta products quality.

Endoxylanases have been described to have a significant influence on the presence of checking in pasta,[40] they are able to break down water-unextractable arabinoxylans having high water binding capacity. When endoxylanases are part of the recipe, the water content can be lowered and as a result checking is significantly reduced. Brown and Finley[43] explained the effect of this type of enzymes by their ability to reduce the pasta dough viscosity, allowing significantly reduced head pressures and/or significantly higher throughputs in commercial pasta extrusion systems.

The beneficial effects of lipase and the quality characteristics such as colour, bite, stickiness and cooking tolerance of non-durum pasta have recently been reviewed.[44] The application of lipase has also been described in durum-derived pasta.[45] Lipase addition extends shelf life and keeps the characteristic yellow colour over time. However, the remaining lipase activity in the pasta should be low (<100 LU kg^{-1}), which can be secured by a heat treatment of the dough before extrusion.

7.4.2 Enzyme effects on noodles

Noodle colour, appearance and cooked texture are very important to noodle producers and consumers. However, a wide variety of noodles are present in Asia with many local variations as a result of differences in culture, climate, region and a host of other factors. Each noodle type has its own unique colour and texture characteristics. Important factors responsible for noodle colour are flour colour, protein content, ash content, yellow pigment and polyphenol oxidase activity. Starch characteristics and protein content and quality play major roles in governing the texture of cooked noodles. However, the relative importance of starch and proteins varies considerably with noodles type. Starch pasting quality is the primary property determining the eating quality of Japanese and Korean noodles characterized by soft and elastic texture, while protein quantity and strength are very important to Chinese-type noodles that require firm bite and chewy texture.[46]

Noodles are produced by mixing wheat flour produced from a blend of soft, semi-hard and hard wheat, salt and/or alkaline salts and a low content of water (absorption range of 26–36%). These ingredients are mixed into a crumbly dough, which is sheeted over a number of sheeting rolls and afterwards cut into the noodle form.

A common problem is darkening of raw noodles or noodle dough sheets during production or shelf life. Polyphenol oxidase has also been implicated here as a leading cause.[29] Although considerable effort has been taken to reduce genetic levels of polyphenol activity in wheat germplasm, the problem is still there.[47,48] Treatment with fungal lipase helps to brighten noodle sheets or raw noodles during shelf life.[44]

Another common problem in most noodle varieties is speckiness, that is small dark spots, the quantity of which is mainly dependent on the level of flour extraction and ash content. By adding fungal lipase to the recipe the increase of the number of dark spots over storage time can be highly reduced.[44]

Lipase[44,49] or the combination of lipoxygenase and lipase[50] also makes boiled noodles firmer, smoother and less sticky, resulting in easier disentanglement and improved eating quality. The explanation for this effect is most probably the formation of monoglycerides by the action of triacylglycerol lipase. These monoglycerides form stable complexes with the α-helix of amylose molecules which inhibit the swelling of starch granules during cooking. Less amylose is leached out of the granules, resulting in a firmer texture and a less sticky and smoother surface. Instead of this triacylglycerol lipase activity other phospho- or galactolipase activity may be applied to obtain similar effects.[51]

The use of transglutaminase on its own (e.g. Ref. [52]) or in combination with extra vital wheat gluten[53] or with casein hydrolyzate[54] has been described in many (mainly Japanese) patents and articles. Blended with the flour or added as part of the dusting powder (e.g. Ref. [55]), transglutaminase is enabled to make internal cross-links between gluten and other protein fragments within the dough, resulting in more elastic and glutinous noodles having an improved texture, resilience and mouthfeel.

Glucose oxidase is also able to make cross-links between dough constituents. The use of glucose oxidase is described to improve the firmness, elasticity, surface properties and cooking tolerance of different types of noodles[56] (see Fig. 7.4). Low-quality wheat flours can be upgraded for production of noodles by adding glucose oxidase to the recipe.[57]

By adding glucose oxidase to noodle dough having a pH below 8, speck formation could be better controlled and noodle appearance could be maintained for a long period.[58]

Application of fungal α-amylase on the surface of pre-gelatinized noodles prevents clumping of the cooked noodles and improves its flavour and texture even after a long period

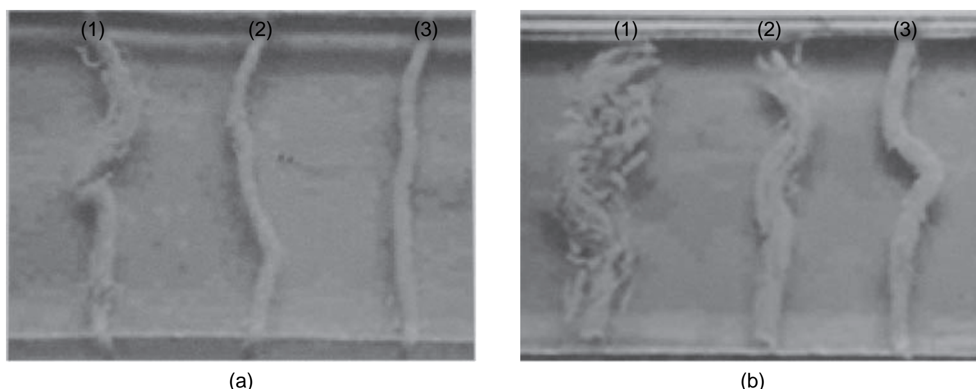


Fig. 7.4 Cooked instant noodles pressed between glass plates after 3 (a) and 7 (b) min cooking. (1) Reference, (2) + 45 SRU glucose oxidase/kg flour and (3) +75 SRU glucose oxidase/kg flour .

of storage.[59] Instead of α-amylase, glucoamylase may be used in the preparation of oil-fried noodles.[60]

7.5 APPLICATION OF ENZYMES IN BISCUIT, COOKIE AND CRACKER PRODUCTION

The word *biscuit* is of French origin and means 'twice cooked'. Nowadays, only a few products are baked twice, while most biscuits and cookies are baked only once.

In Europe, the word biscuit is used for low-moisture products that are made with high levels of sugar and shortening, products that are called cookies in the United States.[61]

The principal ingredients used in the manufacture of biscuits and cookies are wheat flour, fat and sugar. Water plays an important role in the biscuit-making process but is largely removed during baking. Biscuit manufacturing generally includes several steps: mixing, resting time, machining and finally baking.

In general, there are two types of biscuit dough: hard dough and short dough (Europe) also known as hot doughs and rotary doughs (USA), respectively.

The difference is determined by the amount of water required to make dough that has a satisfactory handling quality for making dough pieces for baking.[62]

Hard dough resembles bread dough by being more tough and extensible. Gluten development takes place and the dough shows viscoelastic behaviour. Due to the frictional forces of developing gluten, heat will be generated, hence the name hot dough.[63] Product examples are Petit Beurre and Marie biscuits.[64] Short dough on the other hand contains much less water and relatively high levels of sugar and fat. The consistency looks more like wet sand and there will be no gluten or heat development. The initial structure of the biscuit will be formed by the fat that holds the flour and sugar syrup together. Later, during baking and the following days, structure formation will be due to and dependent on the extent of sugar crystallization.[63] Product examples are wire-cut biscuits, extruded biscuits and deposited biscuits,[64] like gingerbreads and shortbread.

Only minor starch gelatinization takes places in both types of biscuits.[63] Most biscuits are made with soft or weak flour. Strong flours tend to bind up the mixture and do not allow the oven spread, which is desirable in most biscuit types.[65] If the gluten is too strong, more water is required during dough preparation and the biscuit will rise too much. After sheeting and cutting, the dough pieces will contract and the biscuits will show shrinkage, resulting in uneven bottom or surface.[66] Irregular dimensions of biscuits are caused by the strong elasticity of wheat gluten, particularly glutenins, which need to be hydrolyzed. However, this should be carried out very carefully; too strong hydrolysis will liquefy the dough, making sheeting and laminating impossible; conversely, if glutenins are only moderately degraded, gluten elasticity will remain too high and the dough pieces can contract, yielding irregular sizes of biscuits.[67]

Sodium metabisulphite (SMS) is currently used in the baking industry to soften biscuit dough. In particular, SMS is used in the industry to reduce shrinking of dough pieces and irregular sizing of baked products. SMS is known to react with gluten proteins preventing them from forming inter covalent S—S bridges.[68] The effect of sulphite in dough is almost immediate and results in inextensible and inelastic dough. It has also been proposed that sulphite might activate wheat proteases which enhance the breakdown of the gluten structure.[62]

Table 7.1 Comparison on the use of bacterial protease, L-cysteine and SMS in a Marie-type biscuit.

		Biscuit characteristics	
Processing aid	**Dose**	**Length (cm)**	**Density (g/cm^3)**
Reference	0	29.2	1.30
Bacterial protease	16 800 PC kg^{-1}	29.5	1.21
L-cysteine	80 ppm	29.5	1.21
SMS	220 ppm	29.7	1.22

L-Cysteine hydrochloride can be used as an alternative to SMS and is permitted for use in biscuit production. However, due to its relative high cost in use compared with SMS it is not routinely used.[69]

The use of protease to modify gluten quality has been known for quite some time. Standardized protease tablets from *Aspergillus sp.* are available and can be used in cracker sponges in order to increase dough extensibility. This allows cracker manufacturers tight control over dough consistency.[67]

Compared to sulphite, proteases work in a different way since they hydrolyze the inner peptide linkages of gluten proteins, whereas SMS increases extensibility by breaking the disulphide bonds. The texture of the biscuits obtained will also be more open and tender.

Compared to sulphite, enzymes are pH, temperature and time dependent; therefore dosage should be higher in the case of cool doughs than with hot doughs. Additionally, pH increase due to carbonate present in the biscuit dough may lower the enzymatic activities.

Different types of proteases can be used in order to hydrolyze the gluten network; however, the most common practice is the use of a bacterial protease, for example originating from *Bacillus amyloliquefaciens*.

The comparison of SMS, L-cysteine and bacterial protease in a Marie-type biscuit is shown in Table 7.1. Marie-type biscuits have been prepared according to a typical recipe. After 25 min kneading at 35°C, the dough has been laminated, cut-off and baked for 5 min at 280°C. Finished biscuits have been cooled down for 30 min at room temperature before final dimensions have been measured. As can be seen in Table 7.1, the final biscuit dimensions obtained from dough containing bacterial protease match the dimensions obtained from dough prepared with 80 ppm L-cysteine, and closely similar to the dough prepared with 220 ppm SMS.

However, microbial proteases will not be easy to manage with their continuous action over time, not allowing the manufacturers a degree of freedom with regard to the resting time of dough.

In 1997, it was proposed by Souppe *et al.*[70] that the use of an oxidation-sensitive protease, such as papain, in combination with an oxidizing enzyme (such as glucose oxidase) producing an oxidizing agent, can enable biscuit manufacturers to mimic the effect of sulphite in dough.

By using a protease that may be only active at the beginning of the dough preparation, shrinking of the dough may be reduced and more regular sizes of baked products, such as biscuits, can be obtained. The action of the protease can then be substantially decreased when the concentration of the oxidizing agent has reached such a level that inactivation of the protease will take place. This will be of particular benefit when left-over dough is fed back into the system as rework.

From Fig. 7.5(b), it is clear that papain hydrolyzes the gluten to such a degree that the resulting dough is not suitable for biscuit baking. The combination of papain and glucose

oxidase (Fig. 7.5(c)) results in a quick decrease in dough consistency to a desired level. This level remains more or less constant over time. The overall results show that glucose oxidase was able to reduce the action of papain over time.

More even baking can also be achieved by improving the properties of the dough through the addition of hemicellulose and cellulose-degrading enzymes. It has been proposed by Haarasilta *et al.*[71] that the enzymes make the dough softer, requiring less water, less energy input, finally resulting in increased factory output.

α-Amylases only play a minor role in biscuit manufacturing. Due to the fact that α-amylase is able to produce dextrins from damaged starch, they will play a role in the enzymatic browning during baking, resulting in darker biscuits.

Crackers belong to the category of hard dough biscuits and can be either chemically leavened or fermented. Soda crackers, or saltines, are a traditional type of fermented cracker produced from laminated dough and involving a two-stage sponge and dough process. During the lengthy fermentation time, the bacteria will produce acids allowing the pH of the sponge to drop from about pH6 to pH4. This will activate flour protease present, which will modify the gluten to become more extensible and thus easier to sheet. Further to this, the fermentation will also contribute to the creation of a desirable taste and flavour. The sponge will then be added to the remaining ingredients (the pH will raise to pH7–8 due to the addition of sodium bicarbonate) and the dough will undergo a final fermentation step, allowing yeast fermentation to continue. The final dough will be sheeted and laminated into several layers and formed to individual crackers. The soda crackers are baked at high temperature (230–315°C) for approx. 2.5–6 min.

Snack crackers are also produced from laminated dough, but with added flavouring and less fermentation time, in a so-called one-stage 'direct' process. Due to the short fermentation time, 3–4 h in the S&D process versus 0.5–2 h in a direct process, L-cysteine and sodium metabisulphite are often used as reducing agents in order to partially break down the gluten and relax the dough.[70]

As described above, enzymatic alternatives for the use of metabisulphite have been examined and often include a protease. Bacterial proteases are frequently used; however, these require good process control due to their continuous hydrolyzing action. Papain is an interesting protease to use since it has a strong hydrolytic action on glutenins but can be spontaneously stopped by natural oxidation of the dough. Possibly, addition of glucose oxidase generates hydrogen peroxide from glucose present in the dough which inactivates papain in an irreversible way. This allows the use of papain in the dough with quite a great security and without fearing any adverse process effects.

As shown in Table 7.2, the use of protease alone approaches the performance of SMS, whereas, as expected, glucose oxidase alone does not. Most likely, a combination of both enzymes will result in similar effect as SMS. The glucose oxidase will stop the activity of the protease, as has been shown in Fig. 7.5.

Table 7.2 Comparison on the use of papain, glucose oxidase and SMS in cracker dough.

		Biscuit characteristics		
Processing aid	**Dose**	**Length (cm)**	**Width (cm)**	**Thickness (cm)**
Papain	7680 NFU kg^{-1}	4.9	6.6	0.67
Glucose oxidase	750 SRU kg^{-1}	4.3	7.2	0.65
SMS	1200 ppm	4.9	6.8	0.63

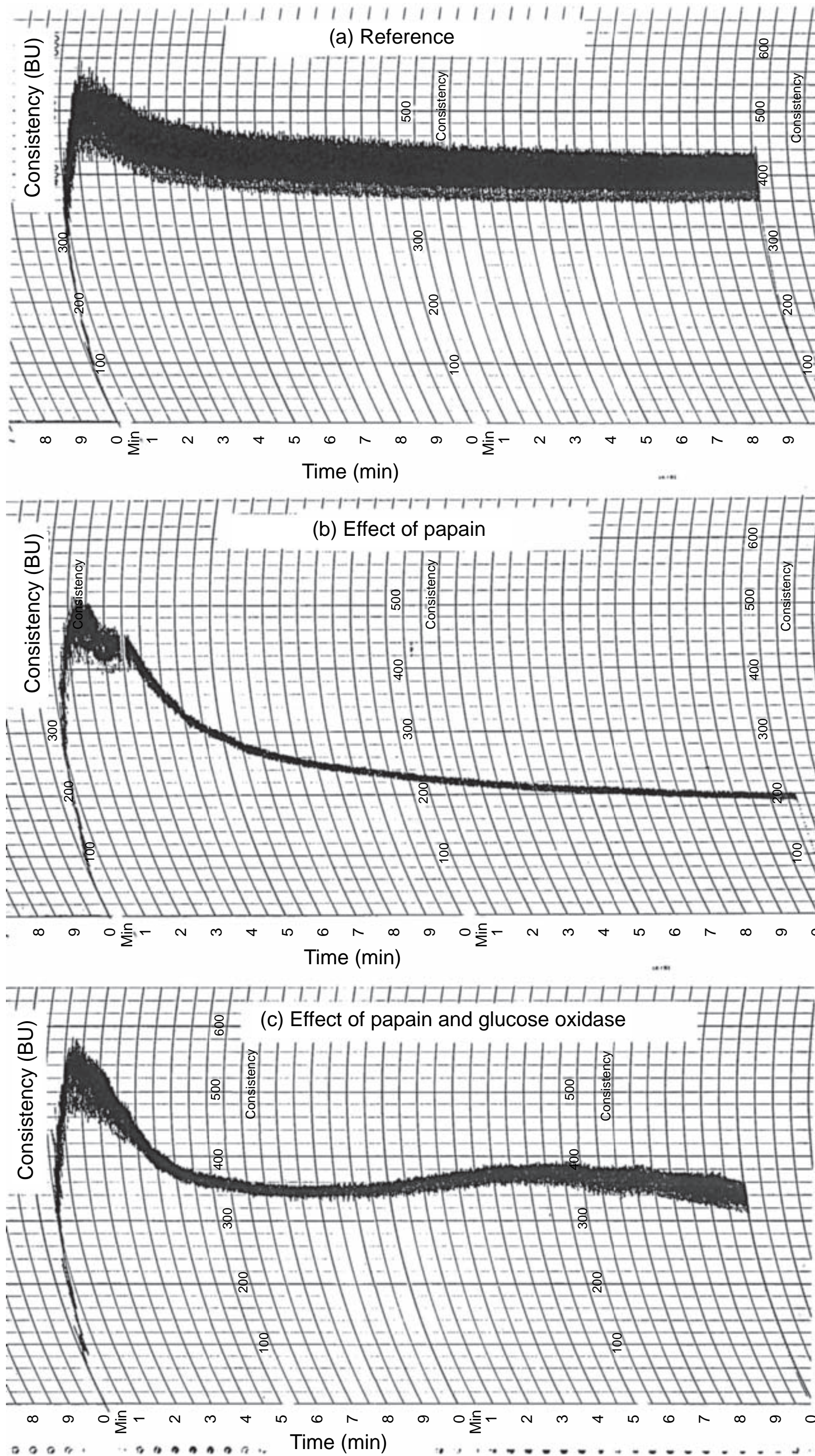


Fig. 7.5 (a) Reference, (b) effect of papain and (c) combination of papain and glucose oxidase.

The use of hemicellulases in cracker dough can potentially be also very useful. The partial breakdown of the water-extractable hemicellulose fraction will lower the water binding capacity. Hence, more water will be available and softer dough will be achieved. Consequently less water is needed to prepare the dough. Furthermore, a reduction in baking time will be achieved, as well as an improvement in quality through more even baking resulting in reduced checking.

This has also been confirmed by Nabisco,[72] who are claiming that the use of a pentosanase will reduce checking in crackers by reduction of the water content and will be particularly useful in low fat and/or high-fibre formulations. Dough containing low level of fat or high level of fibre do require higher amount of water to be added to the process in order to achieve good machinability. This water also needs to be removed during baking, resulting in longer baking times. The addition of hemicellulases will result in lower water binding capacity, hence more water will be available for easier processing.

Also, the addition of (fungal) α-amylase will potentially prevent checking as well as creating a leavening effect and improved flavour development. The amylase will act on the damaged starch granules, thereby providing food for yeast to generate carbon dioxide, while at the same time liberating water from the damaged starch. This will improve the distribution of water throughout the dough, creating more uniformity, hence less problems with checking after baking.[72]

7.6 APPLICATION OF ENZYMES IN WAFERS

Wafer biscuits are made from mainly wheat flour and water to which other minor ingredients may be added. The manufacturing process consists of preparing a batter, typically 40–50% flour and other ingredients such as fat/oil, emulsifiers, sugar, egg, salt, sodium bicarbonate and/or yeast. The batter is usually deposited and cooked between a pair of plates for a short amount of time at high temperature (e.g. around 2 min at 160°C). The wafer sheets produced will contain a low moisture level and will be further cooled down and processed according to requirements of the end product.[73] A low batter viscosity and an even dispersion of all ingredients are essential for uniform wafers with a homogeneous structure.[74] The formation of gluten strands is undesirable as these will block sieves and depositor nozzles.[75]

Proteases, especially bacterial proteases, can be added to the wafer batter in order to prevent gluten development and liquefy the gluten, resulting in a uniform mixture with optimum flow properties. Liquefied protein also binds less water giving the opportunity to add less water to the batter. Consequently, a lower water level in the batter will result in less baking time. This effect is strengthened by the addition of endoxylanases able to break down hemicellulose. The endoxylanase, preferably originating from *Trichoderma sp.*, is able to hydrolyze the backbone of the arabinoxylan resulting in a moderate release of water due to the decrease in water binding capacity of the hemicellulose.

Wafer batter test results, shown in Fig. 7.6, clearly indicate that the addition of bacterial protease originating from *B. amyloliquefaciens* in combination with endoxylanase originating from *Trichoderma longibrachiatum* immediately lowers the batter viscosity. This indicates that the water binding capacity has decreased and water has been released. This will allow the manufacturer to reduce the water proportion by 5–10% (as a function of the flour grade), which consequently will lead to a reduced energy requirement for evaporation and thus to a saving of energy. Furthermore, the quality of the wafer also increases by changes in

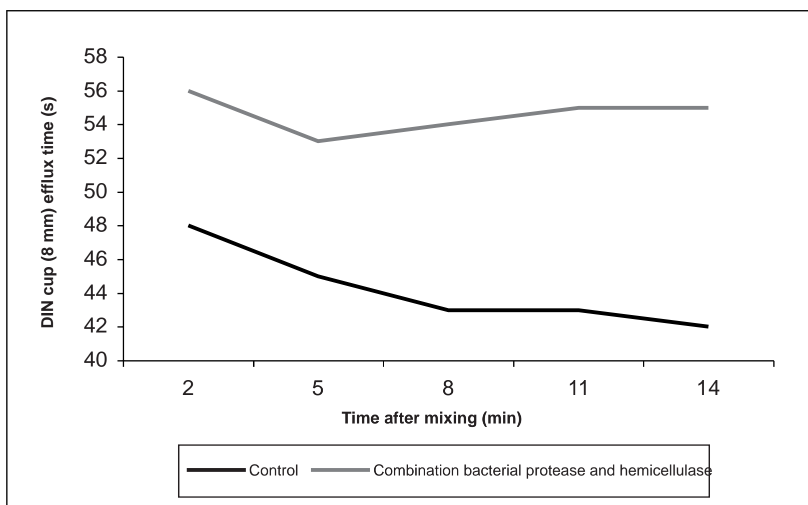


Fig. 7.6 Wafer batter viscosity with and without bacterial protease and endoxylanase

texture. Due to reduced water quantity, less water needs to be evaporated, resulting in a more dense structure whereby the wafers will increase in crunchiness and become more firm. The application of bacterial protease and xylanase has become widely accepted.[76]

In 2004, a patent by Nestec S.A. was published describing the use of a thermalstable α-amylase to manipulate textural attributes of flour-based food products like wafers, without resulting in an increased batter viscosity. Furthermore, the stickiness of the batter is not changed and the batter remains easily processable.[73]

7.7 USE OF ENZYMES IN WHEAT FLOUR TORTILLA

Tortillas are unleavened, flat, round breads made from wheat or corn. They are an important part of the diet in Mexico and Central America and a fast increasing part of the diet in North America. In fact, currently in the USA there is already more wheat flour used for tortilla manufacturing than for the production of white bread.

Flour tortillas are made of wheat flour, water, shortening, and salt, preservatives, leavening agents, reducing agents and emulsifiers. The bread has a circular, flat shape and usually varies in diameter from 100 to 700 mm and in thickness from 1 to 5 mm.[77] Tortilla dough is gluten structured and most of the tortillas contain chemical leavening agents. During mixing, small air bubbles are formed and distributed evenly throughout the dough.

The texture of tortillas depends upon the retention of gasses by the gluten matrix.[78] Good quality flour tortillas are soft, opaque and flexible without cracking when folded.[79]

Tortilla staling can be significantly reduced by the addition of a low activity level of bacterial amylases. Also the flexibility will significantly improve by the addition of bacterial amylase. In the past, it was proposed that bacterial amylase will affect the structure of starch through hydrolysis of the amylopectin, and the anti-staling effect could be possibly due to the interference of the hydrolysis products with amylopectin retrogradation.[77]

Recent insights have shown that staling of tortillas involves the starch in the amorphous phase and does not significantly interfere with the amylopectin crystallization. It is proposed

that bacterial α-amylase partially hydrolyzes the amylose, starch bridging the crystalline region and protruding amylopectin branches. Starch hydrolysis decreases the rigid structure and plasticity of starch polymers during storage. The flexibility of tortillas results from the combined functionalities of the amylose gel and amylopectin solidifying the starch granule during storage.[80]

7.8 APPLICATION OF ENZYMES IN BREAKFAST CEREALS

Ready-to-eat breakfast cereals have become well established on breakfast tables almost all over the world. They have been defined as ‘processed grain formulations suitable for human consumption without further cooking in the home’.[81]

Hence, the most important raw material in any breakfast cereal is grain. Most commonly used are corn, wheat, oats, rice and barley. Other ingredients often included in the manufacturing process are salt, sweeteners, flavouring agents, colouring agents, vitamins, minerals and preservatives.

The manufacturing process of breakfast cereals involves several stages. Depending on the type of breakfast cereal, the grain will be used in total, or may require further processing which often includes a crushing step of the whole grain between large metal rollers in order to remove the outer layer of bran. The whole grain or coarse grain fractions are mixed with the rest of the raw ingredients including water, in order to be cooked to the required state. The cooked mass will undergo a drying step to the point of achieving a soft but solid mass which can be shaped easily, often after a tempering period which allows the grains to cool and stabilize the moisture content of each individual grain.[82]

Due to advances in technology, extrusion is the most frequently used process for cooking and for partial forming of cereal products.[83] A major advantage of this technique is the ability to achieve precise process requirements, resulting in much shorter overall processing time.

Wheat can serve as a substrate to several types of enzymes. Consequently, the use of enzymes during the manufacturing process of ready-to-eat breakfast cereals has been known for quite some time. Fritze *et al.*[84] report a process to saccharify the starch contained in the cereal to form dextrose by enzymatic degradation using a, preferably thermostable, α-amylase. The final product is claimed to have a good flavour and the dextrose produced is absorbed directly as sugar by the body. However, the process includes saccharification of the major starch content making it difficult to provide the typical matrix-forming properties required for a cereal dough.[85] Producing fructose provides a greater level of sweetness for a given amount of starch conversion; hence more starch or high molecular weight dextrins may be retained for their matrix forming ability and improved machinability into cereal shapes.

In 1989, Maselli *et al.*[86] reported the use of a glucoamylase in combination with glucose isomerase and optional α-amylase in the method for making cereal products naturally sweetened with fructose.

The glucoamylase is able to form dextrose by using the starch present in the endosperm. Consequently, a portion of the dextrose can be converted to fructose by the use of glucose isomerase. In combination with these enzymes, optionally, thermostable α-amylase can be used to convert the starch to dextrins. Best results will be obtained by adding the α-amylase prior to the addition of glucoamylase and glucose isomerase.

The amount of fructose produced by the enzymatic conversion of the starch is sufficient to provide a sweet taste in combination with the other reducing sugars produced during the enzymatic treatment.

The use of an 'R-enzyme' has been reported by Antrim and Taylor[87] in the production process of shredded wheat. The R-enzyme, also known as starch debranching enzyme or pullulanase, will accelerate starch retrogradation and allow for a shortening of the holding step.[88]

7.9 MISCELLANEOUS

7.9.1 Asparaginase to reduce acrylamide content in wheat-based baked products

Acrylamide is known to be neurotoxic and has been classified as a probable carcinogenic compound for humans, group 2A, by the International Agency for Research on Cancer (IARC) in 1994.[89] For this reason, the finding of high levels of acrylamide in carbohydrate-rich foods processed at high temperatures as reported by the Swedish National Food Administration in 2002[90] was taken as a serious concern worldwide. It became clear that a wide range of cooked foods – prepared industrially, in catering, or at home – contain acrylamide at levels in between a few parts per billion (ppb or μg/kg) and in excess of 3000 ppb. This includes staple foods like bread, fried potatoes and coffee as well as specialty products like potato crisps, biscuits, cookies, crisp bread and a range of other heat-processed products.[91] The main formation mechanism of acrylamide in foods is the reaction of reducing sugars with free asparagine in the context of the Maillard reaction. In many cooking processes, the Maillard cascade is the predominant chemical process determining colour, flavour and texture of cooked food. This reaction is based on highly complex reactions between amino acids and sugars, that is common nutrients present in all relevant foods. The cooking process per se – baking, frying, microwaving – seems to be of limited influence. It is the thermal input that is crucial: that is the temperature and heating time to which the product is subjected. Acrylamide is formed at temperatures above 120°C.

Consequently, any intervention to reduce acrylamide formation has to take into account that it is very difficult to decouple acrylamide formation from the main Maillard reaction processes. In baked products, the formation of acrylamide is closely related to the combination of moisture content and baking temperature/time. If baking temperatures could be kept low and moisture content high, less acrylamide would be formed during baking even though longer baking times would be required. However, many products baked according to this procedure suffer significant changes to overall product quality (colour, flavour, texture, etc.).[92,93]

Reduction of pH in semi-sweet biscuits by addition of citric acid has shown to result into 20–30% reduction of acrylamide[94] but addition of acidifiers in most cases will have an impact on the organoleptic properties of the final product.[95] Model experiments have also shown that in certain bakery products lower pH in combination with fermentation can lead to an increase of another undesired process chemical, that is 3-monochloropropanediol (3-MCPD).[96]

In a number of baked products ammonium bicarbonate is used as a baking salt. However, ammonium bicarbonate (and other ammonium salts) has shown to be strongly supportive to acrylamide formation. Replacement by sodium bicarbonate results in lowering the acrylamide content; however, product quality changes dramatically in most cases.[97,98]

In 2004, it was shown that the enzyme asparaginase is performing very well in reducing the acrylamide content without any change in product quality.[99,100] Up to 95% reduction could be obtained in certain types of baked products without any change in the production process. Asparaginase catalyzes the conversion of asparagine into aspartic acid; aspartic acid

is therefore not able to form acrylamide. This enzyme became commercially available in 2007[101,102] and was added to the CIAA Acrylamide 'Toolbox' [103] in the same year.

Further details of the use of asparginase for acrylamide reduction may be found in Chapter 4.

REFERENCES

1. Dawn (2008) Wheat production to drop slightly, http://www.dawn.com/2008/05/23/nat10.htm
2. Guy, R.C.E. (1995) In cereal processing: the baking of bread, cakes and pastries, and pasta production. In: *Physico-Chemical Aspects of Food Processing* (ed. S.T. Beckett). Blackie A&P, Glasgow, pp. 258–274.
3. Notomi, T., Ichimura, T., Furukoshi, O. and Kamata, M. (1987) Manufacture of sponge cakes with improved texture using phospholipase-treated egg. Patent Application JP63258528.
4. Guy, R.C.E. and Sahi, S.S. (2006) Application of a lipase in cake manufacture. *Journal of the Science of Food and Agriculture* **86**, 1679–1687.
5. Haesendonck, I. and Kornbrust, B.A. (2008) Method of preparing a cake using phospholipase. Patent Application WO 2008/025674.
6. Mastenbroek, J., Hille, J.D.R., Terdu, A.G. and Sein, A. (2008) Novel method to produce cake. Patent Application WO 2008/092907.
7. Nippon Shinyaku Co (1980) Prevention of cake staling. Patent Application JP58032852.
8. Danisco Media Relations (2004) New Danisco enzyme keeps cakes fresh, 31 August.
9. Liu, X. (2005) Cake powder conditioner containing calcium stearoyl lactate and sodium stearoyl lactate. Chinese Patent Application 1830265.
10. Beier, L., Friis, E. and Lundquist, H. (2006) Method of preparing a dough-based product. Patent Application WO 2006/032281.
11. Kaper, T., Leemhuis, H., Uitdehaag, J.C.M., Van Der Veen, B.A., Dijkstra, B.W., Van Der Maarel, M.J.E.C. and Dijkhuizen, L. (2007) Identification of acceptor substrate binding subsites +2 and +3 in the amylomaltase from *Thermus thermophilus* HB8. *Biochemistry* **46**, 5261–5269.
12. Claassen, V. (2008) White biotechnology: challenges and opportunities, http://www.bio.org/ind/wc/08/breakout_pdfs/20080429/Track4_Marquette/Session6±230pm400pm/Claassen_Marquette_Tue.pdf
13. Arnaut, F., Vekemans, N. and Verte, F. (2007) Method and composition for the prevention or retarding of staling and its effect during the baking process of bakery products. Patent Application EP1790230.
14. Edens, L. and Hille, J.D.R. (2005) Method to improve flavor of baked cereal products. Patent Application WO 2005117595.
15. Boot, J., Boot, J.H.A., Deutz, I.E.M., Ledeboer, A.M., Leenhouts, C.J. and Toonen, M.Y. (1993) Proline imino-peptidase polypeptide-genetically prepared and useful for modifying flavour of food products. Patent Application EP700431.
16. Brennan, C.S., Kuri, V. and Tudorica, C.M. (2004) Inulin-enriched pasta: effects on textural properties and starch degradation. *Food Chemistry* **86**, 189–193.
17. Abecassis, J., Abbou, R., Chaurand, M., Morel, M.-H. and Vernoux, P. (1994) Influence of extrusion conditions on extrusion speed, temperature, and pressure in the extruder and on pasta quality. *Cereal Chemistry* **71**, 247–253.
18. Hoseney, R.C. (1994) Pasta and noodles. In: *Principles of Cereal Science and Technology*, 2nd edn (ed. R.C. Hoseney). AACC Inc., St. Paul, MN, pp. 321–334.
19. Hummel, C. (1966) *Macaroni Products: Manufacture, Processing and Packaging*. Food Trade Press, London, pp. 250–264.
20. Feillet, P. and Dexter, J.E. (1996) Quality requirements of durum wheat for semolina milling and pasta production. In: *Pasta and Noodle Technology* (eds J.E. Kruger, R.B. Matsuo and J.W. Dick). AACC Inc., St. Paul, MN, pp. 95–131.
21. Sugisawa, K., Matsui, F., Yamamoto, Y., Nakanaga, R., Takeda, N., Fujii, Y. and Hirano, Y. (1982) A method for producing dried instant noodles containing less than 15% moisture as a final product. US Patent Application 4483879.
22. Matsuo, R.R. (1987) The effect of enzymes on pasta and noodle products. In: *Enzymes: Their Role in Cereal Technology* (eds J.E. Kruger, D. Lineback and C.E. Stauffer). AACC, St. Paul, MN.
23. Fares, C., Maddalena, V., De Leonardis, A. and Borrelli, G.M. (2001) Lipoxygenase influence on durum wheat quality characteristics. *Tecnica Molitoria* **52**, 231–235.

24. Irvine, G.N. and Winkler, C.A. (1950) Factors affecting the color of macaroni. 11. Kinetic studies of pigment destruction during mixing. *Cereal Chemistry* **27**, 205–209.
25. Burov, L.M., Medvedev, G.M. and Ilias, A. (1974) Lipoxygenase, carotenoids and the colour of macaroni products. *Khlebopekanaya i Konditerskaya Promyshlennost* **11**, 25 (Chem. Abstr. 82, 56221h (1975)).
26. Dexter, J.E. and Marchylo, B.A. (2000) Recent trends in durum wheat milling and pasta processing: impact on durum wheat quality requirements. In: *Proceedings of the International Workshop on Durum Wheat, Semolina and Pasta Quality: Recent Achievements and New Trends*. INRA, Montpellier, France, 27 November, pp. 77–101.
27. Morris, C.F. (1995) Breeding for end-use quality in the Western U.S.: a cereal chemist's view. In: *Proceedings of the 45th Australian Cereal Chemistry Conference* (eds Y.A. Williams and C.W. Wrigley). Royal Australian Chemical Institute, North Melbourne, pp. 238–241.
28. Whitaker, J.R. and Lee, C.Y. (1995) Recent advances in chemistry of enzymic browning: an overview. In: *Enzymatic Browning and its Prevention* (eds C.Y. Lee and J.R. Whitaker). American Chemical Society, Washington, DC, pp. 2–7.
29. Fuerst, E.P., Anderson, J.V. and Morris, C.F. (2006) Delineating the role of polyphenol oxidase in the darkening of alkaline wheat noodles. *Journal of Agricultural and Food Chemistry* **54**, 2378–2384.
30. Fraignier, M.-P. (1999) A study of durum wheat peroxidases. Involvement in enzymatic browning of pasta products. PhD Thesis, Université de Montpellier, Montpellier, France.
31. Feillet, P., Autran, J.-C. and Verniere, C.-I. (2000) Pasta brownness: an assessment. *Journal of Cereal Science* **32**, 215–233.
32. Malcolmson, L.J., Matsuo, R.R. and Balshaw, R. (1993) Textural optimization of spaghetti using response surface methodology: effects of drying temperature and durum protein level. *Cereal Chemistry* **70**, 417–423.
33. Edwards, N.M., Peressini, D., Dexter, J.E. and Mulvahey, S.J. (2001) Viscoelastic properties of durum wheat and common wheat dough of different strengths. *Rheologica Acta* **40**, 142–153.
34. Hondo, K., Ishii, C., Soeda, T. and Kuhara, C. (1996) Stabilised transaminase preparation obtained by drying solution containing protein material and enzyme useful in food products, e.g. sausages, ice cream, yoghurt, bread and spaghetti, stable for a long time at room temperature. Patent Application WO 9611264.
35. Motoki, M. and Kumazawa, Y. (2000) Recent research trends in transglutaminase technology for food processing. *Food Science and Technology Research* **6**, 151–160.
36. Kuraishi, C., Yamazaki, K. and Susa, Y. (2001) Transglutaminase: its utilization in the food industry. *Food Reviews International* **17**, 221–246.
37. Takács, K., Gelencsér, É. and Kovács, E.T. (2008) Effect of transglutaminase on the quality of wheat based pasta products. *European Food Research and Technology* **226**, 603–611.
38. Resmini, P. and De Bernardi, G. (1973) Proteic denaturation induced by the glucoxidase enzyme in macaroni production. In: *Proceedings of the Symposium on Genetics and Breeding of Durum Wheat* (ed. G.T. Scarascia-Mugnozza). Università di Bari, Italy, p. 539.
39. Si, J. and Qi Si, J. (1994) Microbial peroxidase dough/bread or pasta – improvers – giving better volume, freshness, structure, softness, stability, and dough stickiness. Patent Application WO 9428729.
40. Ingelbrecht, J. (2001) Arabinoxylans in durum wheat: characterization, behaviour during spaghetti processing and influence on spaghetti processing and quality. PhD Thesis, Katholieke Universiteit Leuven, Belgium.
41. Wagner, P. and Xu, F. (1999) Methods for preparing an improved dough and/or baked product, e.g. bread, roll, cookie, and pasta. Patent Application WO 199957986.
42. Xu, F. (2001) Preparing dough useful for preparing baked products such as bread, roll, pasta, tortilla taco, cake, biscuit, cookie, pie crust, and steamed and crisp bread, involves incorporation of lactonohydrolases into dough. Patent Application WO 2001035750.
43. Brown, P.H. and Finley, J.W. (2002) Enzymic improvement of pasta processing. US Patent Application 2002102328.
44. Qi Si, J. and Drost-Lustenberger, C. (2002) Enzymes for bread, pasta and noodle products. In: *Enzymes in Food Technology* (eds R.J. Whitehurst and B.A. Law). Sheffield Academic Press, Sheffield, pp. 19–56.
45. Halden, J.P., Realini, A., Juillerat, M.A. and Hansen, C.E. (2001) Pasta manufacturing process. US Patent Application 6326049.
46. Hou, G. (2001) Oriental noodles. *Advances in Food and Nutrition Research* **43**, 143–193.

47. Guttieri, M., McLean, R., Stark, J.C. and Souza, E. (2005) Managing irrigation and nitrogen fertility of hard spring wheats for optimum bread and noodle quality. *Crop Science* **45**, 2049–2059.
48. McLean, R., O'Brien, K.M., Talbert, L.E., Bruckner, P., Habernicht, D.K., Guttieri, M.J. and Souza, E.J. (2005) Environmental influences on flour quality for sheeted noodles in idaho 377s hard white wheat. *Cereal Chemistry* **82**, 559–564.
49. Katakura Kagaku Kogyo Kenkyusho KK (2001) Cereal processed food such as noodles with improved disentanglement. Patent Application JP2001327257.
50. Nisshin Flour Milling Co (2001) Preparation of noodles for use as foodstuff. Patent Application JP2001169738.
51. Christiansen, L., Ross, A. and Spendler, T. (2002) Production of fried flour-based product, e.g. noodles. WO Patent Application 2002065854.
52. Murofushi, K., Kajio, F., Fujita, A. and Hirasawa, F. (1997) Processes for the production of noodles by machines. US Patent Application 6197360.
53. Yamazaki, K., Sakaguchi, S. and Soeda, T. (1999) Enzyme preparations and process for producing noodles. US Patent Application 6432458.
54. Yamazaki, K., Naruto, Y. and Soeda, T. (1996) Transglutaminase in the preparation of noodle. Patent Application JP08051944.
55. Tanaka, K. and Kanaya, M. (1997) Dusting powder containing transglutaminase for producing noodles. Patent Application JP11009209.
56. Feng, W. (2000) Application of glucose oxidase in noodle processing. *Shipin Gongye Keji* **21**, 67–68.
57. Gao, H. and Zhang, S. (2005) Effect of glucose oxidase compound conditioner on baking quality of wheat flour. *Shipin Gongye Keji* **26**, 64–67.
58. Nisshin Flour Milling Co (1999) Noodles having good appearance with controlled speck formation. Patent Application JP11137196.
59. Endo, S., Daihara, H., Okayama, T.M. and Akashi, H. (2000) Production of cooked noodle. Patent Application JP2000106836.
60. Endo, S. and Yamada, T. (2000) Wheat flour composition for oil-fried instant noodles. Patent Application JP2000333629.
61. Cookies and Biscuits (1997) Cookie and biscuit production. In: *Lallemand Baking Update*, Vol. **2**(19) .
62. Manley, D. (1998) Ingredients. In: *Biscuit, Cookie and Cracker Manufacturing Manuals*. Woodhead Publishing Limited, Cambridge.
63. Fok, J.J. (1990) *Internal Memo; Biscuit, wat is dat en wat kunnen we ermee*. Gist-brocades, Delft, The Netherlands.
64. http://www.haas.com/en/products/haas/biscuit-plants/hard-dough-biscuits.html
65. Manley, D. (2000) Part 3: types of biscuits. In: *Technology of Biscuits, Crackers and Cookies* 3rd edn. Woodhead Publishing Ltd., Abington, Cambridge.
66. Popper, L. (2002) Enzymes for cookies and wafers. *Baking +Sweets* **04**/02, 23–25.
67. Matz, S.A. and Matz, T.D. (1978) A procedure for partial purification of proteases. In: *Cookie and Cracker Technology*, 2nd edn (ed. S.A. Matz). Avi Publishing Co, Westport, CT, p. 131.
68. Staufer, C.E. (1994) Redox system in cracker and cookie dough. In: *The Science of Cookie and Cracker Production* (ed. H. Faridi). Chapman & Hall, New York/London, Chapter 6, pp. 237–238.
69. Oliver, G., Wheeler, R.J. and Thacker, D. (1996) Semi-sweet biscuits. 2. Alternatives to the use of sodium metabisulphite in semi-sweet biscuit production. *Journal of the Science of Food and Agriculture* **71**, 337–344.
70. Souppe, J. and Naeye, T.J.-B. (1997) A novel enzyme combination. Patent Application EP0796559.
71. Haarasilta, S., Pulllinen, T., Tammersalo-Karsten, I., Vaisanen, S. and Franti, H. (1993) Methods of improving the production process of dry cereal products by enzyme addition. Patent Application US5176927.
72. Slade, L., Levine, H., Craig, S. and Arciszewski, H. (1994) Reduced checking in crackers with pentosanase. Patent Application US5362502.
73. Nicolas, P. and Hansen, C.K. (2004) Flour based food product comprising thermostable alpha-amylase. Patent Application EP1415539.
74. Popper, L. *Enzymes-Best Friends of Flours*. Mühlenchemie GmbH, Germany. http://muehlenchemie.de/downloads-expertenwissen/mc-enzyme-popper-eng.pdf
75. Manley, D. (1998) *Biscuit Cookie and Cracker Manufacturing Manuals*. Manual 1; Ingredients. Woodhead Publishing Limited, Cambridge, p. 22.
76. van Wakeren, J. and Popper, L. (2004) Replacing sodium metabisulfite with enzymes in hard biscuit dough formulations. *Cereal Foods World* **49**(2), 62–64.

77. Arora, S. (2003) The effect of enzymes and starch damage on wheat flour quality. Master Thesis, Texas A&M University.
78. McDonough, C.M., Seetharaman, K., Waniska, R.D. and Rooney, L.W. (1996) Microstructure changes in wheat flour tortillas during baking. *Journal of Food Science* **61**, 995–999.
79. Waniska, R.D. (1999) Perspectives on flour tortillas. *Cereal Foods World* **44**, 471–473.
80. Alviola, J.N. and Waniska, R.D. (2008) Determining the role of starch in flour tortilla staling using alpha-amylase. *Cereal Chemistry* **85**, 391–396.
81. Fast, R.B. (2000) Manufacturing technology of ready-to-eat cereals. In: *Breakfast Cereals and How They Are Made* (eds R.B. Fast and E.F. Caldwell). AACC, St Paul, MN.
82. Secrest, R. (1995) Cereal. In: *How Products Are Made*, Vol. **3** (eds J.L. Longea and N. Schlager). Gale, Detroit.
83. Buhler, A.G. (2000) Breakfast cereals: production plants from a single source. In: *Breakfast Cereals*. http://www.buhlergroup.com
84. Fritze, H., Koenemann, K., Koenemann, R. (1991) Process for producing a foodstuff of cereal. Patent Application US42541450.
85. Fulger, C.V. and Gum, E.K. (1987) Process for preparing an all grain, enzyme-saccharified cereal and product produced. Patent Application US4656040.
86. Maselli, J.A., Neidleman, S.L., Antrim, R.L. and Johnson, R.A. (1989) Method for making cereal products naturally sweetened with fructose. Patent Application US4857339.
87. Antrim, R.L. and Taylor, J.B. (1990) R-Enzyme-treated breakfast cereal and preparation process. Patent Application CA2016950.
88. Spendler, T. and Nielsen, J.B. (2003) Enzymatic treatment of starchy food products for shortening the tempering step. Patent Application WO/2003/024242.
89. International Agency for Research on Cancer (1994) *Acrylamide. IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans*. IARC, Lyon, France, pp. 389–433.
90. Tareke, E., Rydberg, P., Karlsson, P., Eriksson, S. and Törnkvist, M. (2002) Analysis of acrylamide, a carcinogen formed in heated foodstuffs. *Journal of Agricultural and Food Chemistry* **50**, 4998–5006.
91. CIAA (2005) The CIAA acrylamide “toolbox” Rev. 6. 23 September, http://www.ciaa.be
92. Taeymans, D., Ashby, P., Blank, I., Gondé, P., van Eijck, P., Lalljie, S., Lingnert, H., Lindblom, M., Matissek, R., Müller, D., O’Brien, J., Stadler, R.H., Thompson, S., Studer, A., Silvani, D., Tallmadge, D., Whitmore, T. and Wood, J. (2004) A review of acrylamide: an industry perspective on research, analysis, formation, and control. *CRC Critical Reviews in Food Science and Nutrition* **44**, 323–347.
93. CIAA Technical Report (2005) ‘Acrylamide status report December 2004’. A summary of the efforts and progress achieved to date by the European Food and Drink Industry (CIAA) in lowering levels of acrylamide in food. Brussels, http://www.ciaa.be
94. FIAL (2005) FIAL meeting. ETH Zürich.
95. Stadler, R.H. (2006) The formation of acrylamide in cereal products and coffee. In: *Acrylamide and Other Hazardous Compounds in Heat-Treated Foods* (eds K. Skog and J. Alexander). Woodhead Publishing, Cambridge, pp. 23–40.
96. RHM Technologies (2005) RHM Technologies presentation at the UK FSA Process Contaminants meeting. 19 April, London.
97. Graf, M., Amrein, T.M., Graf, S., Szalay, R., Escher, F. and Amadò, R. (2006) Reducing the acrylamide content of a semi-finished biscuit on industrial scale. *LWT – Food Science and Technology* **39**, 724–728.
98. Amrein, T.M., Schönbächler, B., Escher, F. and Amadò, R. (2004) A method for the determination of acrylamide in bakery products using ion trap LC-ESI-MS/MS. *Journal of Agricultural and Food Chemistry* **52**, 4282–4288.
99. Boer, L. de (2004) Reduction of acrylamide formation in bakery products by application of Aspergillus niger asparaginase. In: *Using Cereal Science and Technology for the Benefit of Consumers*. Proceedings of 12th International ICC Cereal and Bread Congress, 24–26 May 2004, Harrogate, Part 10.
100. Vass, M., Amrein, T.M., Schönbächler, B., Escher, F. and Amadò, R. (2004) Ways to reduce the acrylamide formation in cracker products. *Czech Journal of Food Sciences* **22**, 19–21.
101. DSM Food Specialties (2007) Preventase™ – The proven solution to acrylamide, http://www.dsm.com/en_US/html/dfs/preventase_welcome.htm
102. Novozymes (2007) Acrylaway® – A natural solution to a natural problem http://www.acrylaway.novozymes.com/en/MainStructure/Acrylaway±applications/index.html
103. CIAA Acrylamide Toolbox key updates (2007) http://www.ciaa.be/documents/brochures/CIAA_Acrylamide_Toolbox_Oct2006.pdf

8 Brewing with enzymes

Eoin Lalor and Declan Goode

8.1 INTRODUCTION

The principal raw materials that are needed to brew beer are water, malted barley, adjuncts (barley, maize, rice, wheat and sorghum), hops and yeast. The brewing process involves extracting and breaking down the carbohydrate and protein from malted barley and adjunct sources. This results in a carbohydrate/protein-rich solution that can be used as a source of nutrients for yeast fermentation.

The major biological changes that occur in this process are catalyzed by naturally produced enzymes from the barley and yeast, respectively. Barley that has been malted has all the necessary enzymes needed to degrade starch, β-glucans, pentosans, lipids and proteins, which are the major components of interest to the brewer.

When poor quality malt or high levels of adjunct material are used in the process, then the addition of commercial enzymes is necessary. Commercial enzymes give the brewer the freedom to use cheaper, poorer quality raw materials, to enhance process yields and to enhance the quality characteristics of the final beer.

The aim of this chapter is to provide the reader with a step-by-step overview of the main enzyme-dictated steps involved in the beer production process (Table 8.1) from the malting of barley grain through to the final stabilization of the beer in the package. The reader is referred to broader brewing texts[1–3] for further explanations of the science and technology behind each step.

8.2 MALTING: THE TRANSFORMATION OF RAW BARLEY TO AN ENZYME-RICH PACKAGE

Raw barley cannot be used for brewing in its native form without (1) malting (the controlled germination of the grain, to stimulate endogenous enzyme production) or (2) the intervention of added exogenous enzymes. This section will focus primarily on the malting processes.

The malting process mimics the natural germination of barleycorns which would otherwise happen in the field but with two significant differences (1) growing conditions are manipulated to encourage enzyme production by the aleurone, while minimizing yield losses due to respiration and growth of the embryo and (2) the germinated corn is gently kilned to preserve enzymatic activities where possible.

The purpose of malting is to activate the endogenous phytohormones and enzymes of the barley to make it more amenable to starch/carbohydrate extraction, which is further facilitated

Table 8.1 Summary of main brewing process steps simplified.

Process area	Main step	Process description	Key objectives/results
Malting	Steeping	Grain is soaked in water. Water is absorbed by the grain. Hydration of grain embryo and stimulation of grain for start of germination	▶ Activities of embryo are awakened ▶ Enzymes will be distributed evenly throughout the kernel
Malting	Germination	Grain is placed in shallow vessels. Water is removed. Temperature is maintained at 14–16°C and a relative humidity (RH) of 100%. Aleurone layer is stimulated to produce enzymes. Enzymes act to degrade cell wall structures and ensure modification	▶ Enzymes are stimulated from the aleurone layer ▶ Hydrolysis of proteins, carbohydrates, hemicellulose and lipids in barley grain ▶ Degree of modification is controlled
Malting	Kilning	The temperature of the malt is raised from 15 to 85°C (lager/pale malt) in a controlled manner (temperature, airflow, RH, time)	▶ Germination process is halted at desired malt quality ▶ Green malt is converted to stable, storable product ▶ Colour and flavour are developed ▶ Enzymes are stabilized and preserved ▶ Unwanted flavours are removed
Malt preparation	Milling	Grain is broken into small fragments When a lauter tun is used, overall aim is to break starchy endosperm to as fine particles as possible, whilst retaining husk layer as a filtration aid for lautering (roller milling). When a mash filter is used, the aim is to grind the whole grain kernels to as fine particles as possible (hammer milling)	▶ Small particles mean full accessibility for the malt enzymes to act on the grain components ▶ Size of particles will have influence on mashing time, extract yields, brewhouse yields, mash filtration time, beer filterability, etc.
Brewhouse processing	Mashing	Milled grain is mixed with water at various temperatures, for specific rest times under gentle agitation. Temperatures are optimized for the malt enzymes. A typical example would be: 50°C for proteolysis, 62°C for gelatinization/ liquefaction, 72°C for saccharification and 78°C for mashing-off and malt enzyme inactivation	▶ Solubilization and dissolution of grain components ▶ Break down of grain cell wall structure ▶ Extraction and hydrolysis of starch, sugars, proteins and non-starch polysaccharides ▶ Fermentable sugar profile is established ▶ Nitrogenous components are established for yeast fermentation
Brewhouse processing	Lautering	Mash is transferred to either a lauter tun or a mash filter. Wort (aqueous solution) is separated from the spent grains (78°C). Grain bed is sparged with hot water to extract as much extract as possible	▶ Degree of clarity of wort is established ▶ Extract level of wort is established

Table 8.1 *(Continued)*

Process area	Main step	Process description	Key objectives/results
Brewhouse processing	Boiling	Wort is boiled at 100°C, hops are added during the boil	▶ Evaporation of water ▶ Coagulation of proteins ▶ Isomerization of hop bitter components ▶ Inactivation of enzymes ▶ Wort sterilization ▶ Evaporation of undesirable volatiles ▶ Formation of flavour compounds and colour development
Brewhouse processing	Whirlpool/ cooling/yeast addition	Boiled wort is whirl pooled. Insoluble material is separated from the wort solution. Wort solution is cooled to yeast pitching temperature (<20°C)	▶ Coagulated proteins and insoluble hop material are removed ▶ Temperature is reduced to allow for yeast addition ▶ Yeast is added to sterile cooled wort
Fermentation	Primary/ secondary	Wort components of sugars and proteins are utilized by yeast to form alcohol, CO_2 and flavour components. Fermentation temperatures for lager type beers are 8–15°C, for ale fermentations 14–20°C. After primary fermentation, beer is gradually/slowly cooled to -2°C. Yeast falls out of suspension and can be more easily removed. Cold temperatures encourage precipitation of proteins and beer clarification	▶ Alcohol level is established ▶ Flavour profile of beer is established ▶ Carbonation level is established ▶ At the end of fermentation, yeast flocculates and can be easily separated ▶ Cold maturation temperatures will influence beer clarity
Filtration	Beer filtration	Coarse filtration using diatomaceous earth (kieselguhr) filter, fine filtration 10, 1.5 and 0.2 μm	▶ Product is stabilized, clarified
Finishing	Pasteurization	Flash pasteurization (72°C *[c2] 30 s, carried out before packaging, common for large pack such as beer kegs) or tunnel pasteurization (62°C * 30 min, carried out after packaging, common for small packs such as bottles)	▶ Product is stabilized from a microbiological perspective
Packaging	Kegging/ bottling/ canning	Beer is filled into final beer packages such as kegs, bottles, cans	▶ Product is ready for market distribution

by milling. This is given the general term modification. For successful modification, a number of conditions must be satisfied during the conversion of barley into malt:

- Endosperm cell walls must be degraded sufficiently for the malt to be easily milled and for starch grains to become available for enzymatic attack. β-Glucans must be degraded sufficiently to prevent a viscous wort that would otherwise slow down wort run-off.
- Sufficient amylolytic enzymes must be produced so that the endosperm starch can be attacked during mashing.
- Sufficient storage proteins must be enzymatically degraded to amino acids to support yeast growth during fermentation.
- Grainy/vegetable-like flavours associated with raw grain must be dissipated and replaced by malt flavours.
- Barleycorns must retain their individual integrity and be dried sufficiently to become chemically and biologically stable, while retaining enzymatic activities.

The full malting process will not be the subject of this chapter. The reader is thus referred to more comprehensive texts[1–4] covering the technologies, equipment and science in more detail. This section will primarily focus on the enzyme-mediated processes, which occur during the main process steps of malting. Malting can be broken down into three main parts: (1) steeping, (2) germination and (3) kilning.

8.2.1 Steeping

During the steeping process, barley is submerged in water at approximately 12–15°C. This increases the moisture content of the grain from 10% to ~45%. To ensure that an anaerobic state does not become established, either the water is removed allowing air-rests to take place or alternatively it is purged with air during steeping. This is vital to maintain the metabolic processes within the embryo. A typical steeping programme may last 40–68 h depending on the number and duration of air-rests. During steeping, water is absorbed through the micropyle of the grain. The main purpose of steeping is to hydrate the embryo and awaken its activities. It also serves to distribute the enzymes uniformly throughout the grain kernel and thus ensure a more even distribution of modification.

8.2.2 Germination

Following sufficient hydration, the grain is now ready for germination. Grain is removed from the steep water and placed in shallow vessels. Temperatures are kept constant at 14–16°C and the relative humidity (RH) is maintained at 100% to prevent moisture loss from the grain. The germinating barley is turned regularly to prevent matting of growing rootlets that develop as the grain germinates. This also serves to control the germinating bed temperature and facilitate CO_2 removal, which would otherwise inhibit the metabolic processes of the grain. The hydrated embryo respires using internal lipid and sugar reserves, and produces gibberellins. Respiration in the aleurone cells increases as they use internal reserves to synthesize enzymes (Table 8.2). The scutellum can also produce enzymes but needs externally supplied nutrients. It is these enzymes which, as they diffuse from the aleurone cells into the hydrated parts of the endosperm, bring about the series of changes termed modification. The most important part of the modification process is enzymatically

Table 8.2 Main enzyme types involved in the barley malting process.[a]

Enzyme	Substrate	Activity	Source	Significance
Carboxypeptidase (commonly referred to as β-glucan solubilase)	Endosperm cell walls	Removes the outer layer of protein in the endosperm cell wall	Detectable in raw barley Also produced by the aleurone	Makes β-glucan more accessible to glucanase enzymes
Xyloacetylesterase	Endosperm cell walls	Releases acetate from arabinoxylan	Detectable in raw barley Also produced by the aleurone	Plays a role in releasing β-glucan and making it more accessible to glucanase enzymes
Feruloyl esterase	Endosperm cell walls	Releases ferulate from arabinoxylan	Detectable in raw barley Also produced by the aleurone	Has a limited effect on making β-glucan more accessible to glucanase enzymes
Xylanse	Endosperm cell walls	Responsible for hydrolyzing the [c3]	Detectable in raw barley Also produced by the aleurone	Hydrolyzes pentosans surrounding the glucan layer in the barley cell walls, releasing the glucan and making it more accessible to enzymic attack
α-L-arabinofuranosidase	Endosperm cell walls	α-L-arabinofuranosidase	Detectable in raw barley Also produced by the aleurone	Hydrolyzes pentosans surrounding the glucan layer in the barley cell walls, making the glucan layer more accessible to enzymic attack
Endo-β, 1-3 glucanase	Solubilized β-glucan	β 1-3 linkages	Aleurone	Reduces wort viscosity. Improves mash filtration and final beer filtration
Endo-β, 1-4 glucanase	Solubilized β-glucan	β 1-4 linkages	Aleurone	Reduces wort viscosity. Improves mash filtration and final beer filtration

(*Continued*)

Table 8.2 (*Continued*)

Enzyme	Substrate	Activity	Source	Significance
Endo-β-glucanase	Solubilized β-glucan	Mixed linkages	Aleurone	Reduces wort viscosity. Improves mash filtration and final beer filtration
Endopeptidase	Insoluble hordein	Cleaves protein chains	Aleurone	Breaks protein matrix around starch grains. Improves mash extraction. Improves foam stability
Exopeptidase	Soluble oligopeptides derived from hordein	Remove amino acids from ends of peptide molecules	Detectable in raw barley	Carboxypeptidase amino acids for barley embryo and eventually yeast growth
			Present prior to germination	
β-Amylase	Starch grains	α-1,4 linkages (two glucose units at a time from the end of the chain)	Detectable in raw barley	Rapid starch solubilization
			Present prior to germination	Improves fermentability
α-Amylase	Starch grains	Starch internal α-1,4 linkages	Aleurone	Fermentability
α-Glucosidase	Starch	α-1,4 linkages	Detectable in raw barley	Fermentability
		α-1,6 linkages	Some present prior to germination, but most from aleurone	
Limit dextrinase	Starch	Cleaves amylopectin α-1,6 linkages	Detectable in raw barley Some present prior to germination, but most from aleurone	Fermentability

[a] Many other endogenous enzyme reactions occur during malting which involve acid and alkaline phosphatases, peroxidases, catalases, polyphenolicoxidases, lipoxygenase, phospholiases, phytase and others.

induced cell wall breakdown. Cell wall breakdown will be discussed in more detail later in the chapter.

During germination, endogenous enzymes (Table 8.3), some directly influenced by phytohormones such as gibberellic acid (GA3), hydrolyze proteins, β-glucans, arabinoxylan and hemicellulose in the barley cell wall. The main effect of gibberellic acid is to stimulate enzyme development within the aleurone of the germinating barley. Gibberellins are one of the five main groups of hormones that coordinate plant growth. As well as affecting the growth of mature plants, gibberellins are vital for germination of seeds. One of the first responses of the embryo, once it is hydrated sufficiently, is the production and secretion of gibberellins. These diffuse to the aleurone cells, via the scutellum, which are stimulated to

Table 8.3 Enzymes whose production is influenced by gibberellic acid.

Aleurone enzyme
α-Amylase
Endo-β, 1,3:1,4 glucanase
Endoprotease
Pentosanase
Limit dextrinase
Cellobiase (β-glucosidase)
Laminaribiase

produce the enzymes associated with germination. The action of gibberellic acid in barley is to increase the production of the messenger RNA, which is responsible for the formation of specific enzymes, from the DNA of the aleurone cell nuclei. It does this by switching on specific genes that had previously been suppressed by other substances.

8.2.2.1 *Cell wall breakdown during germination*

The cell walls in the starchy endosperm of the barley kernel comprise 75% β-1,3 β-1,4 glucan, 20% arabinoxylan and 5% protein, plus traces of other constituents such as acetic and ferulic acids. The cell wall polysaccharides are detrimental to brewing performances if they are not sufficiently hydrolyzed during malting and later again during mashing. A simple model (Fig. 8.1) has been proposed[5] to explain both the structure of the barley cell wall and the range

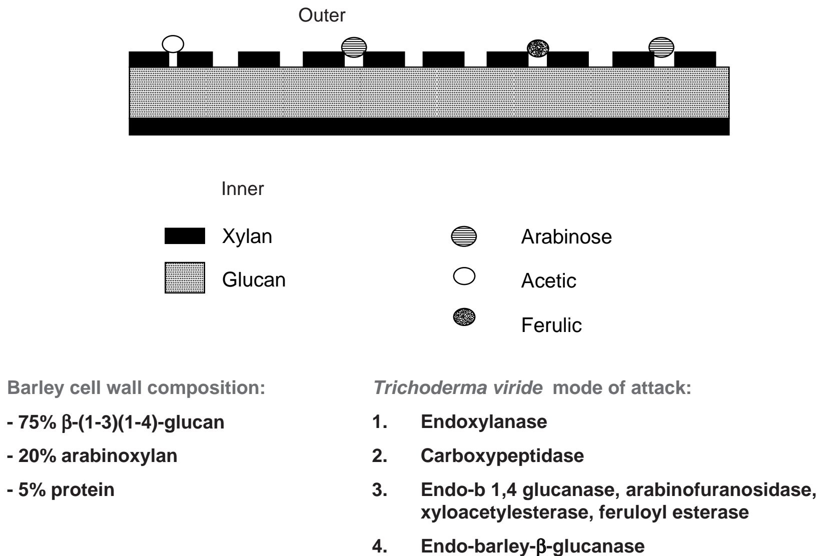


Fig. 8.1 Model of barley cell wall structure, barley cell wall composition and *Trichoderma viride* mode of attack (adapted from Ref. [5]).

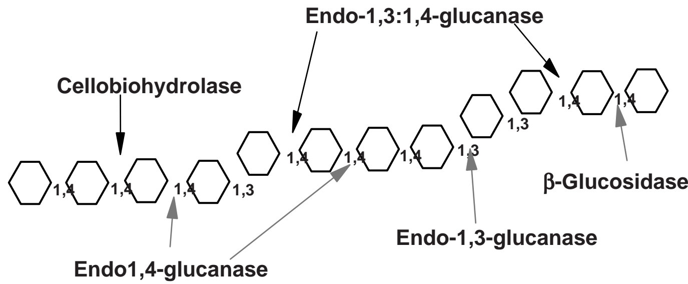


Fig. 8.2 Enzymatic hydrolysis of β-glucan.

of complex enzymes and processes involved in its breakdown. In several studies,[5,6] it was shown how *Trichoderma viride* (which commonly occurs in nature) acts to naturally decompose organic material. When *T. viride* was grown on denatured crude barley cell walls as sole source of carbon and energy, it produced a range of enzymes. The first enzyme to be switched on in substantial quantities was endoxylanase. This highlights the prior need to remove pentosan before glucan. A carboxypeptidase was also measured early on in the study. This will act to break protein structures in the barley cell wall and thereby help to release β-glucan. Endo-β 1,4-glucanase, arabinofuranosidase and general esterase enzymes (xyloacetylesterase, feruloyl esterase) were next in sequence to be detected. The arabinofuranosidase releases arabinose from the xylan backbone, while it has been proposed that xyloacetylesterase and feruloyl esterase release acetate and ferulate respectively from arabinoxylan. These enzymes therefore increase the accessibility of the β-glucan to enzyme attack. Of particular significance is their elaboration before the development of endo-barley-β-glucanase. The endo-β-glucanases act to break down the β 1-3 and β 1-4 linkages in the barley β-glucan (Fig. 8.2). This enhances the solubility of β-glucan and reduces potential downstream brewing difficulties that are associated with under-modified or poorly malted grains.

Using whole barley kernels, it has been shown[7] that during malting, the order of synthesis of enzyme development also supports the model proposed by the *T. viride* study;[5] namely the early synthesis of xylanase and carboxypeptidase, followed next by β-glucanase and arabinofuranosidase and lastly by α-amylase. This also supports the model of an arabinoxylan sheath, covering a β-glucan core in the cell walls, which must first be hydrolyzed in order to give glucanases better access to the cell wall core.

To further explain the proposed model[5] in Fig. 8.1: a layer of pentosan is located in the outer regions of the wall, rendering it accessible to xylanolytic enzymes and ensuring that the solubilization of β-glucan is restricted. The covering of β-glucan by pentosan, however, is not complete. This allows glucanases to access their substrate, with the low molecular weight digestion products readily leaking back through the pentosan cover. It allows for calcoflour to stain β-glucan and for a certain amount of soluble β-glucan to be accessible to solvating water in the absence of enzyme activity. Enzymes that remove the arabinosyl-, acetyl- and feruloyl-plugs increase this accessibility still further. A further more substantial layer of pentosan is hidden within the wall. This model gives a very good indication of the complexity of the barley cell wall structure. It also serves to give a visual understanding of the difficulties encountered when mashing with unmalted adjunct material and under-modified malt. It provides a simple explanation of why commercial enzyme complexes that contain arrays of glucanases, xylanases and cellulases are better to reduce brewhouse-processing difficulties than, for example, a purified β-glucanase.

8.2.2.2 *Protein breakdown during germination*

Protein hydrolysis during germination has two main consequences: (1) the removal of the protein matrix surrounding the starch granules and (2) the provision of nitrogenous nutrients for the embryo to grow. The mobilization of these reserves can be separated into three different phases: (1) proteins in the scutellum and aleurone are broken down to small peptides and amino acids which are mainly used to synthesize the hydrolytic enzymes secreted into the endosperm. (2) Proteins in the endosperm are hydrolyzed, initially by acid proteases secreted by the scutellum. Later, during germination, gibberellin-induced sulfhydryl endoproteinases and carboxypeptidases are secreted by the aleurone. The degradation of endosperm proteins does not proceed to completion but results in a mixture of amino acids and small peptides. (3) Small peptides and amino acids are absorbed by the scutellum where the peptides are hydrolyzed to amino acids. Approximately 70% of the amino nitrogen found in wort is produced during germination. This amino nitrogen commonly referred to as free amino nitrogen (FAN), is necessary for optimal yeast performance in the downstream process of fermentation.

8.2.2.3 *Lipid breakdown during germination*

Lipid breakdown is important in providing the initial source of energy for synthesis by the embryo of the acrospire and rootlets. Stored triglycerides are degraded to diglycerides and monoglycerides, fatty acids and glycerol. Lipoxygenase enzymes are already present in barley. However, their levels also increase during germination. They are implicated in beer flavour staling and can exert a detrimental effect during milling and mashing in the presence of oxygen.

8.2.2.4 *Starch breakdown during germination*

Starch is only attacked to a minor degree during germination despite the presence of several starch-degrading enzymes in the kernel. This is because these enzymes are deprived of any significant period of activity during germination by the following factors:

1. The initial protection of starch by the glucan/protein matrix within endosperm cells.
2. The sequential mode of production of enzymes.
3. The enzymes have pH and temperature optima different to the conditions within the grain.
4. The termination of enzyme development by kilning.

The limited action that these enzymes have during germination is on the small starch granules. Barley starch granules are of two different granular sizes. The large and smaller granules are referred to as A- and B-type granules, respectively. In general, B-type granules are those with less than 6 μm in diameter (more often 2–4 μm in diameter); these constitute 80–90% of the total number of starch granules but generally only 10–15% of the total starch weight. Large granules (A-type) range in size from 10 to 30 μm but generally lie in the 15–20 μm range; they constitute a small proportion (10–20%) of the total number of starch granules but a high proportion (85–90%) of the total weight of starch. During malting, the small starch granules are broken down. The breakdown is slow because the starch is not gelatinized and it seems that amylopectin is degraded to a greater extent than amylose. It is thought that the

presence of α-glucosidase assists the action of α and β amylases on the starch granules. At the end of germination, when viewed under an electron microscope, many of the small starch granules will have disappeared and pitting (small holes) will be apparent on the large starch granules. The small amount of soluble sugars produced can be measured as cold-water extract. This provides a measure of modification.

8.2.2.5 *Exogenous gibberellic acid applied during germination*

The action of externally applied gibberellic acid (abbreviated as GA or GA3) early in germination has the effect of reducing dormancy and of accelerating and accentuating the effects of naturally produced gibberellins. The gibberellic acid must be applied early during germination to yield maximum benefits. It is usually sprayed evenly on the malt during casting or transfer of the steeped malt to germination vessels. Gibberellic acid does not stimulate the production of all enzymes equally. Production of starch and protein-degrading enzymes is increased more than endo-β-glucanase enzymes. Care must be taken when applying gibberellic acid, since excessive addition can accelerate nitrogenous modification but can leave cell walls incompletely degraded with the consequent possibility of poor wort run-off.

8.2.3 Kilning

The purpose of kilning is to dry the green malt (40–45% moisture), thereby reducing the moisture content in a controlled manner to ∼5%. It also serves to terminate the development of the embryo. This ensures that valuable brewers extract is not expended. Depending on the beer to be brewed, the colour and enzyme activity can be controlled by the kilning temperature. In general, malts with darker colour have lower endogenous enzyme activity as they are produced by kilning at higher temperatures.

In summary the purpose of kilning is to:

- halt the germination process at the optimum malt quality;
- convert green malt into a stable, storable product (low in moisture) that is amenable to milling;
- develop colour and flavour;
- stabilize and preserve enzymes (Table 8.4);
- remove unwanted flavours.

Table 8.4 Stability ranking of main enzyme families during kilning.

Enzyme	Stability
α-Glucosidase	Least stable
β-Glucanase	Destroyed during any kilning
Limit dextrinase	Little activity in well-kilned malts
Endopeptidase	Activity retained in lager malt
β-Amylase	Some activity lost during kilning
β-Glucan solubilase	Survives kilning
α-Amylase	Most stable during kilning

The objectives of kilning are achieved through the application of heat, airflow and RH. In essence, it can be described as a balance of drying and cooking, which will vary with the type of malt being produced. The temperatures during kilning are raised from 15°C to 85°C (pale/lager malt) in a controlled manner (controlled temperature, RH, airflow and time). Modern practice for all but coloured and speciality malts is to dry the green malt rapidly at a low temperature using a high airflow in order to preserve the enzyme activity. Malt enzymes are preserved by properly controlled drying, although activity is reduced during the higher temperatures of curing. The higher the temperature and the longer the period of curing, the greater the destruction of enzymatic activity. High enzymatic malts used for distilling are not usually heated above 55–60°C. This is done to preserve as much enzyme activity as possible.

8.2.4 Commercial enzymes applied during the malting processes

Exogenous enzymes can also be applied during the malting process. There are two main possibilities: (1) the inoculation of microbial cultures onto the grain during malting and (2) the direct application of commercial enzymes onto the grain. In both cases, there are a number of possibilities for point of application. The products can be (a) added to steeping water, (b) sprayed on during germination or (c) applied post kilning to the grain. Most commonly applied exogenous enzymes belong to the glucanase, cellulase and xylanase families. The benefit for the maltster is twofold. First, the enzymes can act during the process to accelerate modification and second, enzymes applied post kilning will increase enzyme activity for subsequent easier processing performance during the brewery extraction processes.

8.3 BREWHOUSE PROCESSING

8.3.1 Milling

On receipt of malt or adjunct into the brewery, the first step in its processing is milling. Milling is the process where grain is broken down into small fragments. This provides a greater surface-to-volume ratio, therefore ensuring good hydration during mashing and good access of the malt enzymes to their substrates. The quality and size distribution of the milled grain fragments affect:

- the mashing process and the saccharification time;
- the brewhouse yield;
- fermentation;
- beer filterability, beer colour, taste and the overall character.

The overall aim of milling is to break the starchy endosperm into fine particles while retaining the outer coat husk layer of the barleycorn with little or no starchy endosperm adhering. This husk fragment is used as a filtration aid during the mash separation process of lautering. In cases where mash filters are used, the whole grain is hammer milled to produce a fine powder that can be easily extracted. More detailed descriptions of milling technologies and equipment are covered sufficiently in broader brewing textbooks.[1–3]

Table 8.5 Endogenous malt-derived enzymes, pH and temperature-stable properties.

Enzyme	pH Optima	Temperature optima (°C)
α-Amylase	5.2	67
β-Amylase	5.5	62
Proteases	5.5	52
β-Glucanases	6.0	56
Endo β-1,4-glucanase	4.5–4.8	37–45
Endo barley β-glucanase	4.7–5.0	40
Endopeptidase	3.9–5.5	45–50
Carboxypeptidase	.9–5.5	45–50
Amino peptidase	4.8–5.2	50

8.3.2 Mashing

The aim of mashing, which follows the milling process, is to yield as much extract and as good an extract as possible from the grain. Most of the extract is produced by the action of enzymes, which are allowed to act at the optimal temperature specifics required. The brewer will optimize liquor to grist ratios, mashing in temperature, water chemistry (pH and salt contents) and mashing time and temperature stands to take advantage of the optimal temperature specifics of both endogenous malt enzymes and exogenous commercial enzymes, when added.

The four main enzymatic reactions occurring during mashing are (1) the hydrolysis of proteins into peptides and free amino acids, (2) the degradation of β-glucan chains, (3) the hydrolysis of pentosans (into arabinose and xylose) and (4) the breakdown of gelatinized starch into fermentable carbohydrates (glucose, fructose, sucrose, maltose and maltotriose). These conversions happen due to the action of proteolytic, glucanolytic, pentosanolytic and amylolytic enzyme classes, respectively. To cover the optimal activity temperature range of each enzyme group (Table 8.5), mashing is usually operated by successive rests at increasing temperatures. A simple representation of this is; a first rest at 50°C for hydrolysis of protein, β-glucans and pentosans, a second rest at 63°C for the action of β-amylase, a rest at 70°C for optimal activity of α-amylase, followed by heating at 78°C to promote enzyme inactivation.

8.3.2.1 *Protein degradation*

Protein is a major component of all beers. It can typically represent 5 g L^{-1} in a final product. In malted barley, protein can typically represent 9–11% of dry weight. Protein plays a key role in foam formation and foam stabilization of a beer, has a positive influence on the mouthfeel of a beer and is necessary for adequate yeast nutrition. Amino acids and di–tri peptides play a key role in the metabolism of yeast, thus having an indirect influence on the flavours developed during fermentation. Protein also plays a role in both the colloidal stabilization and de-stabilization of beer. Protein will interact with grain-derived polyphenols, which leads to haze formation in the final beer. Protein in the form of isinglass finings (positively charged marine-derived collagen), when applied to the green beer, will interact with negatively charged yeast cells and promote faster yeast settling/sedimentation and a more compact yeast precipitant.

The function of proteolytic enzymes during mashing is to hydrolyze the large chain protein molecules, to make starch molecules more accessible to enzymatic attack and to produce sufficient levels of amino acids and di–tri peptides for an optimal fermentation. Likewise,

proteolysis will reduce haze problems that could be caused by proteins and can also influence the foam stability of beer. While about 95% of starch from malt is solubilized by the end of mashing, only about 30–40% of the malt protein is solubilized. The principle groups of enzymes involved in the breakdown of malt proteins are endoproteases and exopeptidases. Endoproteases randomly break the large protein molecules into relatively large polypeptide chains. The exopeptidases attack the polypeptides from a particular end stripping off small units to produce amino acids. The endopeptidases have a relatively low optimum temperature (Table 8.5) and hence with high temperature mashing most of the protein breakdown will have taken place during the malting process. Exopeptidases are able to withstand higher temperatures (Table 8.5) and release the amino acids from the polypeptide chains. There are two principle groups of endopeptidase enzymes (1) carboxypeptidases which attack the proteins from the carboxyl end and (2) aminopeptidases which attack the proteins from the amino end. Carboxypeptidase is detectable in raw barley but is not present in significant quantities. It is rapidly produced during steeping and is active at normal mash pH. Amino peptidase is much less active at mash pH and does not play a significant role in protein breakdown during mashing.

Most proteolysis will already have occurred during malting. It is impossible to completely compensate for a nitrogen deficiency in malt by introducing a prolonged mash stand at 50°C without adding exogenous enzymes. By mashing in at lower temperatures (∼45°C), the brewer will get the benefit of endogenous malt protease activity. This allows for the release of amino acids from proteins, thereby increasing the wort amino acid level to the desired target level (typically 150 mg L^{-1} for a 12 °P wort). Amino acids and peptides, as well acting as yeast nutrients, will also impact on the colour and flavour characteristics of the final product by undergoing complex Maillard and Strecker degradation reactions. Excess proteolysis can have a negative impact on foam stability of the final beer, by reducing the level of foam positive proteins. Too little proteolysis will have a negative impact on the colloidal stability of the beer and the fermentation characteristics. The amino acid and peptide profile of the final wort before fermentation has a major consequence on the flavours produced by the yeast during fermentation.

When large amounts of unmalted cereals/adjuncts are used in the process, it is normal to add exogenous proteases to supplement the limited amount present in the process. Typical examples of industrial processes where significant amounts of exogenous proteases are used to increase wort FAN levels are (high level) barley brewing and sorghum brewing (Table 8.6). However, care should be taken when using proteases, as this can affect excessive colour formation (through Maillard reactions), and may affect the foam potential of the final beer.

Table 8.6 Effect of protease addition to a 100% raw sorghum brew. All enzyme products mentioned are Kerry Bio-Science Products

Enzymes[a] used in process	Wort FAN (mg/L standardized at 12°P)
Hitempase Bioferm FA Conc	38.5
Hitempase Bioferm FA Conc Bioprotease P1	96.4

[a] All enzyme products mentioned are Kerry Bio-Science products.
Hitempase: high temperature-stable bacterial amylase for stach liquefaction; Bioferm FA conc: fungal amylase for starch saccharification; Bioprotease P1: neutral protease.

8.3.2.2 *β-Glucan and other non-starch polysaccharide degradation*

Besides starch-based oligosaccharides, there are a number of non-starch barley polysaccharides. As already mentioned, the most significant non-starch polysaccharide in barley and malt is the β-glucan molecule (Fig. 8.2). Generally, it is accepted that the β-glucan content of barley is 5% by dry weight, while β-glucans and gums constitute 1–3% of the malt weight and occur at concentrations of 200–800 mg L^{-1} in wort. β-Glucans make up more than 70% of the barley cell wall, while arabinoxylan represents 20%. The β-glucan molecule has a distinctive linear structure with roughly 70% β-1,4 linkages and 30% β-1,3 linkages. In its native un-hydrolyzed form, β-glucan has a molecular weight of $>10^6$ Dalton. Most β-glucan is water soluble, but a proportion is bound covalently to cell wall proteins. If there is sufficient degradation of the cell walls, then enzymic access to the protein and the starch will be restricted and the extract from the malt reduced. Likewise, intact β-glucans and high molecular weight β-glucan fragments will increase wort viscosity, will cause mash filtration problems, increase beer filtration times and may result in an undesirable glucan haze in the beer. If arabinoxylan is not attacked sufficiently during malting or mashing, then access to the β-glucan molecules is impeded, and extraction and filtration difficulties can occur. Arabinoxylan consists of a xylan backbone with arabinose side chains. Also present in ester form are ferulic acid and acetic acid. In terms of enzyme hydrolysis, acetyl esterase and feruloyl esterase will cleave acetyl and feruloyl groups from the pentosan; an arabinofuranosidase can split side chains from the xylan backbone and a xylanase will digest the xylan backbone. Although much of the necessary enzymatic hydrolysis occurs during malting, there is inevitably some survival of cell wall material. This will be exacerbated if poorly modified malt or unmalted adjuncts such as barley and wheat are used.

8.3.2.3 *Commercial enzymes for cell wall breakdown*

Fungal glucanases (Table 8.7) are seen to be the most stable in the most relevant pH range and consequently are the most widely used in the brewing industry. However, it must be pointed out that even low levels of β-glucan do not guarantee trouble free brewing. It is the size (apparent molecular weight in solution) of the β-glucan polymers that plays a major role in wort lautering, beer haze and filtration problems. When faced with such β-glucan-related problems, it is possible to add commercially produced glucanase, hemicellulase and xylanase enzymes, which will aid in the breakdown of these persistent gums and improve brewhouse operations and beer processing further downstream. The example shown in

Table 8.7 Glucanase characteristics.

Source	Barley malt	*Bacillus subtilis*	*Aspergillus oryzae*	*Aspergillus niger*	*Trichoderma spp*
pH optimum	4.5–5.3	4.5–7.0	4.0–6.0	3.0–6.0	4.5–7.0
Temp optimum range °C	40–45	45–55	45–55	45–65	45–70
Degrades					
Barley β-glucan	+	+	+	+	+
Laminarin	+	−	+	+	+
CMC	+	−	+	+	+
Lichenin	+	+	+	+	+

Source: Kerry Bio-Science.

Table 8.8 Effect of glucanase enzyme on brewhouse and beer filtration plant operation. Kerry Bio-Science.

Brewhouse	**Malt (tonnes)**	**Wort (hl)**	**Lautering time (h)**	**°Plato**
Control	10	750	2.45	15
Trial	10	764	2.15	15
Filtration plant	Filter type	Filtered beer (hl) one pre-coat	Filter run (h)	Kieselguhr consumption g/hl
Control	Candle	5 080	2.45	165
Trial	Candle	11 400	2.15	79

Trial: Glucanase enzyme (*Source*: Kerry Bio-Science).

Table 8.8 clearly demonstrates the beneficial effects of the addition of an exogenous glucanase in the brewhouse when poor quality malt was used. As well as delivering extract yields in the brewhouse in a reduced time, there were significant benefits with respect to filtration plant operation and kieselguhr usage.

8.3.2.4 *Starch conversion and amylolysis*

Starch is the most abundant component of a barley kernel. It represents 54–65% of the total dry weight of a barley kernel. Starch is composed of two main parts, amylose and amylopectin. Starch conversion (malt contains ca. 60% starch of which 25% is amylose and 75% is amylopectin) is carried out by a range of amylolytic enzymes. Amylose consists of glucose units linked by α-1,4 linkages, while amylopectin consists of both α-1,4 and α-1,6 linkages, thereby making it a branched polymer (Fig. 8.3).

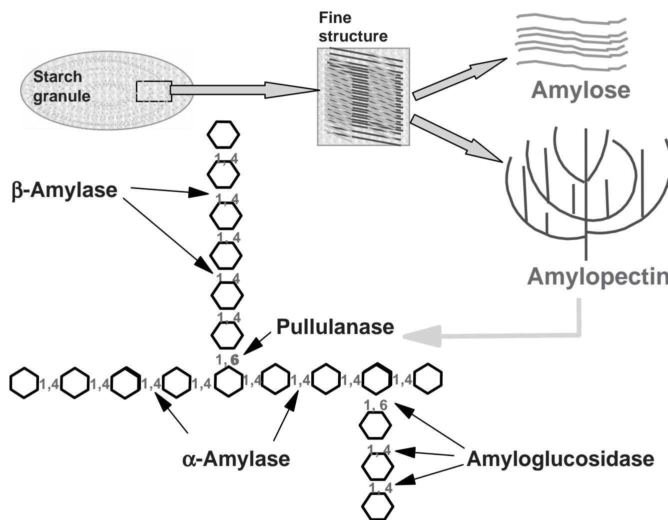


Fig. 8.3 Enzymatic mode of starch hydrolysis.

The diastatic power of malt is an expression of the amount and balance of the four amylolytic enzymes produced during germination:

1. α-Amylase which solubilizes the starch polymers and releases extract.
2. β-Amylase which releases maltose and increases wort fermentability.
3. Limit dextrinase which debranches amylopectin and increases wort fermentability.
4. α-Glucosidase which releases glucose and aids wort fermentability.

Gelatinization
After completion of the proteolytic stage, the mash is raised to a higher temperature by decoction mashing or by programmed infusion mashing. At this point, three very important processes take place, gelatinization, liquefaction and saccharification. The gelatinization temperature of starch is influenced not only by the type of starch (Table 8.9) but also by the size and structure of its starch granules. As was previously mentioned, barley starch granules are reported to be of two different granular sizes. The large and smaller granules are referred to as A- and B-type granules, respectively. It is also well reported that the smaller the starch granules, the higher their temperature of gelatinization. The reported negative impacts of small starch granules are that, due to higher temperatures of gelatinization, they are less digestible during mashing. In addition, they can impede wort filtration by cross-linking with other polymers. In most cases, raw unmalted barley has higher levels of small starch granules than malted barley since, during the malting process, the small granules are preferentially degraded. In addition, at higher unmalted barley levels a greater proportion of smaller milled grain components are present for mashing.

Liquefaction/saccharification
The next stage is liquefaction/saccharification. Liquefaction refers to the liquefying capacity of the α-amylase and saccharification refers to the enzymatic production of fermentable sugars. Prior to this stand, the starch in the mash remains ungelatinized, so it is not readily available to be hydrolyzed by enzymes. The starch of barley malt gelatinizes at 61–65°C. Once gelatinized, the starch is digested rapidly by the α- and β-amylases present in the malt. Their combined activities result in the production of large quantities of fermentable sugar, especially maltose. α-Amylase randomly hydrolyzes starch to dextrins while β-amylase attacks the starch and dextrins from the non-reducing ends, stripping off pairs of glucose molecules (maltose). The saccharification stage seldom lasts longer than 30 min. At temperatures above 60°C, which are required to obtain gelatinization of malt, the malt amylases, especially β-amylase, are not very stable. During saccharification at 65°C, β-amylase is inactivated almost completely within 30 min. α-Amylase is also inactivated significantly, but some residual enzyme activity is still present at the end of saccharification. The saccharification stage therefore represents a compromise between the need for higher temperatures to obtain gelatinization and lower temperatures to preserve enzyme activity. The chosen temperature results in optimal sugar formation but incomplete starch degradation. Starch degradation must be completed by raising the mash temperature again so that the finishing temperature is at least 70°C but below 80°C. Smaller or larger proportions of fermentable sugars will be formed in the wort, giving beers with different ratios of alcohol to non-fermented dextrins.

8.3.2.5 *Mashing programmes*

During mashing, the milled grain is mixed batchwise with water at different temperatures for different times. This is referred to by the brewer as a mashing programme. The following is an outline of a typical mashing programme used by a brewery using 100% malted barley.

Table 8.9 Physicochemical composition of some adjunct materials (adapted from Moll,[1] Goode and Arendt.[8]

Cereals	Moisture	Extract (% dry)	Gelatinization temperature (°C)	Fat (% dry)	Proteins (% dry)	Starch (% dry wt of cereal)	Amylose	Starch granule sizes (μm)
Maize grits	11–13	88–93	62–75	0.8–1.3	9–11	71–74	24–28	1–5 and 10–20
Maize starch	8–12	101–106	62–74	<0.1	0.2–0.3	71–74	24–28	1–5 and 10–20
Rice grits	10–13	89–94	61–78	0.2–0.7	6–9	57–88	14–32	2–10
Sorghum grits	10–12	75–82	68–75	0.5–0.8	6–10	70–74	24–28	0.8–10
Wheat starch	10–14	101–107	52–75	0.2–0.4	0.4–0.5	67–69	25–28	<10 and 10–35
Barley	12–16	75–80	57–65	2–3	9–14	54–65	20–24	2–3 and 12–32
Triticale	8–14	70–75	55–70	2–4	13–16	63–69	28–29	5 and 22–36
Rye	10–15	76–80	55–70	1.5–2.0	8–16	58–62	23–25	2–3 and 22–36
Oats	10–16	45–50	55–62	3–7	8–18	40–63	19–28	2–14
Millet	10–13	79–84	67–77	3–7	10–14	61–70	17–25	0.8–10
Potato starch	10–12	101–105	56–69	<0.1	0.05	65–85	20–23	0.8–30
Manioc (cassava)	8–11	87–97	52–70	0.3–0.6	9–12	85–87	15–17	9–20

Source: Kerry Bio-Science.

The first step is mashing at 50°C. At this mashing temperature, the proteolytic enzymes (carboxypeptidase, amino peptidase and dipeptidase) together with endo-β-1,4-glucanases and pentosanases are most active. With an increase in mash temperature from 50°C to 63°C, the viscosity increases rapidly at ~58°C. This temperature of viscosity increase is noted as being the point of the gelatinization of the barley starch (gelatinization temperature). The most frequently reported gelatinization temperatures for many bulk samples of normal barley starch lie in the range 53–58°C. During gelatinization, the starch granules take up warm water, soak and swell, causing a rapid increase in viscosity. Amylolytic enzymes (α- and β-amylase) can therefore act on this accessible starch substrate. Heating causes inactivation of the proteases and β-glucanases. Following this, the mash temperature is maintained at 63°C. Due to the action of β-amylase, maltose production continues. Further, enzymatic breakdown of the gelatinized starch occurs due to the combined action of the α- and β-amylases. The mash temperature is then raised to 70°C; β-amylase is less active at this point. Enzyme inactivation is an interaction between time and temperature. With an increase in time at temperatures ≥70°C, β-amylase is inactivated. The α-amylases further break down the gelatinized starch and high molecular weight dextrins into low molecular weight dextrins and glucose. The temperature is then raised to 78°C where α-amylase inactivation is reported to occur.

8.3.3 Biological acidification during mashing

Studies have also shown that biological acidification of mash and wort can result in improved mash and wort characteristics. Some reports even claim that ultimately a smoother tasting beer can be produced. More importantly, it has been shown that when employed in high adjunct mashes it can compensate for decreased endogenous grain enzyme activities. It has been shown that at a barley adjunct level of 20%, biological acidification of the mash with *Lactobacillus amylovorus* stock wort resulted in improved extraction, fermentability, FAN and reduced wort β-glucan levels. This could be attributed not only to a lowering of mash-in pH to 5.4, but also to the additional proteolytic and amylolytic enzyme activities that the biologically acidified stock brought into the mash. Thus, biological acidification can offer the adjunct brewer an alternative natural way of bringing additional enzyme activities into the mash. It is commonly applied in countries operating under Rheinheitsgebot (beer purity laws), which specifies that beer can only be made from malted barley, malted wheat, yeast, hops and water. Because the acidified stock is malted barley-derived and the inoculated strains have been isolated from the natural micro flora present on the surface of malt, a biologically acidified stock is therefore considered a natural malt-derived ingredient.

8.3.4 Enzymes in lautering/mash filtration

Lautering and other forms of mash filtration are performed at mashing-off temperatures of 75–78°C. Almost all malt enzymes are already inactivated at these temperatures. A small amount of residual α-amylase activity will be present when a relatively low mash filtration temperature is used. During sparging of the spent grains in the filter bed, water with a temperature of 75–78°C is used. Higher water temperatures are preferred for the reasons of reduced wort viscosity, increased rate of wort separation and to some extent increased extract recovery. Temperatures in excess of 80°C are not advised as these may lead to extraction of excess polyphenols, leading to astringency in flavour and potential shelf life stability problems with the final product. If large quantities of adjunct or under-modified

malt are used, starch may be released late in the mashing cycle. These are the smaller starch granules previously mentioned in the text. Naturally occurring amylolytic enzymes only show a limited amount of activity at this stage during mashing. Therefore, the risk of carry-over of starch polymers into the filtered wort, especially when sparging water with temperatures above 75°C (where α-amylase is rapidly inactivated) is applied, is high. Lower sparging temperatures possibly minimize these problems, but have a negative effect on wort viscosity. Addition of heat-stable exogenous α-amylase (from *Bacillus spp.*) (Table 8.10) during mashing can reduce this wort-starch problem. At the same time, it allows the brewer to use higher sparging temperatures with the accompanying benefits of maximum run-off speeds and optimal extraction.

Table 8.10 Brewhouse enzymes for increased process ability (all trade-names refer to Kerry Bio-Science products) (adapted from Goode and Lalor[11]).

Problem	Symptom	Process aid/enzyme description	Key benefits
Deactivation or insufficient concentration of malt amylase	Starch positive wort and slow fermentation	Bioferm is a fungal endoamylase. It can be used in the mash (or in fermentation) to reduce starch to low molecular weight dextrins and maltose	▶ Improves wort fermentability ▶ Production of maltose in high adjunct brewing ▶ Eliminates starch hazes ▶ Improves beer filtration
Deactivation or insufficient concentration of malt enzymes	Slow or incomplete fermentation	Amylo is a fungal amyloglucosidase. It sequentially hydrolyzes both exo α-1,4 and α-1,6 glycosidic linkages and therefore is used to degrade starch polymers and maltose to glucose. The addition of Amylo in the brewhouse to the cold wort or beer (in the fermenter) converts dextrin to glucose and results in high alcohol/'low carb.' beer	▶ Increases wort glucose levels ▶ Low carbohydrate beer production ▶ Improved alcohol yield
Poor starch hydrolysis/liquefaction	High mash or cereal cooker viscosities and starch positive wort	Hitempase is an endo-acting bacterial α-amylase which randomly hydrolyzes the α-1,4 glycosidic linkages in amylose and amylopectin resulting in the production of dextrins. It is characterized by its stability at extremely high temperatures (~105°C)	▶Improves starch liquefaction and subsequent yields in cereal cooker ▶ Control of starch positive worts in kettle

(*Continued*)

Table 8.10 (*Continued*)

Problem	Symptom	Process aid/enzyme description	Key benefits
Non-starch polysaccharides released during mashing are insufficiently hydrolyzed	Poor mash or beer filtration and β-glucan haze	Bioglucanase is an endo-β glucanase preparation and has a pure lichenase activity and hydrolyzes 1,4 glycosidic linkages next to a 1,3 linkage, in mixed linked β-glucans	▶ Reduces mash and wort viscosity
		Biocellulase contains cellulase, hemicellulase and β-glucanase activities. The activities present in Biocellulase are effective in the degradation of the complex carbohydrates found in plant cell walls	▶ Eliminates β-glucan gums and hazes
			▶ Improves filtration ▶ Increases extract yield ▶ Compensates for malt quality and season to season variations ▶ Improves beer shelf life
Presence of a significant percentage of unmalted barley or use of other adjuncts	Insufficient nitrogen available for yeast growth and slow fermentation	Bioprotease is a neutral proteolytic enzyme	▶ Optimizes FAAN (Free Alpha Amino Nitrogen) levels in high adjunct brewing
			▶ Improves mash lautering/filtration and extract yield ▶ Improves filterability of the wort
Poor extract yield or ineffective wort filtration in high adjunct brewing	Presence of starch and β-glucan residues in the final product	Promalt is an enzyme cocktail providing amylase, glucanase, cellulase and protease activities	▶ A single addition of amylase, glucanase, cellulase and protease
			▶ Improves extract yield and fermentability ▶ Gives consistent brewhouse performance ▶ Improves wort stability/clarity

8.4 ADJUNCT BREWING

Brewing adjuncts (Tables 8.9 and 8.11) are materials other than malted barley that bring additional sources of carbohydrate and protein into wort. While adjunct material can be derived from any carbohydrate source, the five main cereals that are currently used as a base

Table 8.11 Common brewing adjuncts available (adapted from Goode and Arendt[8]).

Whole cereal	Barley, wheat, sorghum, triticale, maize, millet, buckwheat
Grits	Maize, rice, sorghum, barley
Flaked	Corn, rice, barley, oats
Torrified/micronized	Corn, barley, wheat
Extrusion cooked	Maize, rice, sorghum, wheat
Flour/starch	Corn, wheat, rice, potato, cassava, soya, sorghum
Syrup	Corn, wheat, barley, potato, sucrose
Malted cereals	Wheat, oats, rye, sorghum, millet
Malted pseudo cereals	Buckwheat, quinoa

for brewing adjuncts are barley, maize, rice, sorghum and wheat. Table 8.9 gives an outline of the main adjunct types, which are currently available to the international brewing industry. The type of adjunct available to an individual brewer largely depends on the geographical location of that brewery. Likewise, the physicochemical properties of that adjunct (Table 8.9) will dictate its addition rates to a grist recipe, its time of addition and how it will be processed. This section will cover the basics of adjunct brewing. The reader is referred to broader brewing texts[8] for a more comprehensive review of adjuncts and their role in brewing.

8.4.1 Brewing with raw barley as adjunct

The use of barley over other cereal adjuncts offers significant advantages to the brewer. Since its starch has a similar gelatinization temperature (53–58°C) to malted barley (61–65°C) it can be easily be incorporated into conventional malted barley mashing procedures. Its endogenous β-amylase ensures maltose production during mashing. Likewise, the presence of a husk can aid mash filtration through a traditional lauter tun.

As covered earlier in the chapter, the endosperm cell wall structure of raw barley presents a challenge to the brewer. Careful selection and application of commercial enzymes can alleviate the difficulties encountered. This will ensure good yields, easy process ability and good quality beer. Raw barley grain is abrasive and difficult to mill, resulting in a high percentage of fine material which can give problems during lautering. Due to its low levels of essential enzymes (α-amylase, proteases and β-glucanases) together with a relatively inaccessible starchy endosperm, high inclusions of unmalted barley (>20%) in the mash (without the aid of commercial enzymes) can lead to problems such as low extract yields, high wort viscosities, decreased rate of lautering, fermentation problems and beer haze problems. In recent years, an increased knowledge of the structural complexity of the barley starchy endosperm cell wall membranes[5–7,9] together with their native enzyme inhibitors has enabled a more specific approach by enzyme producers to increase process ability of raw barley adjunct.

When brewing with barley as adjunct together with malted barley, increases in the level of barley adjunct can result in decreases in extract recovery, wort α-amino nitrogen, fermentability and increases in wort viscosity and β-glucan levels if commercial enzyme levels are not optimized. While increases in wort amino acid levels result from inclusions of higher levels of malted barley, the endogenous malt enzymes exhibit very poor raw barley protein and starch hydrolyzing ability. Likewise, the endogenous malt amylases have been reported to exhibit very poor raw barley starch hydrolyzing ability. As the level of malt is increased, their raw barley hydrolytic effects decrease.

Table 8.12 α-Amylase characteristics.

	Aspergillus oryzae	***Bacillus spp.***	**Barley malt**	**Porcine pancreas**
pH range(optimum)	4.8–5.8	5.0–7.0	4.0–5.8	6.0–7.0
pH stability range	5.5–8.5	4.8–8.5	4.9–9.1	7.0–8.8
Temp. optimum range (°C)	44–55	60–95	50–65	45–55
Effective temp. range	Up to 60	Up to 100[a]	Up to 70	Up to 55

[a] Because of the extreme thermostability of bacterial amylases, they are typically used in brewhouse operations in the cereal cooker to aid in the gelatinization and liquefaction of starches from such adjunct sources as sorghum, rice and maize.

While hydrolysis of barley adjunct (∼20%) can be achieved by using the enzyme capacity of malted barley, high adjunct levels may dilute the malt enzymes to a limiting level. These are then required to be augmented or replaced by commercial enzyme preparations. Exogenous proteolytic activity is required to modify endosperm structure and to facilitate saccharification, to release bound β-amylase and to adjust the ratio of soluble nitrogen necessary for yeast growth. The most suitable preparation reported is one containing only bacterial neutral protease from *Bacillus subtilis*. Addition yields an increase in total soluble nitrogen, FAN, wort colour and extract recovery. Addition of β-glucanase, xylanase and cellulase from *Bacillus*, *Aspergillus*, *Penicillium* or *Trichoderma* sources (Table 8.7) has been found to improve filtration, when under-modified malt or unmalted barley is used. When mashing with 100% raw barley substrate and commercial enzymes, exogenous β-glucanase (*B. subtilis*) has little impact on mash filtration, but was found to reduce high molecular weight wort β-glucan level. High heat thermo-labile α-amylase derived from *B. subtilis* is widely used for the degradation of gelatinized starch and high molecular weight dextrins to yield molecular weight dextrins and fermentable sugars. An alternative is to use high heat thermostable bacterial α-amylase from *Bacillus licheniformis*, which is inactivated only at temperatures close to boiling. Thus, it can hydrolyze a difficult starch even though swelling and gelatinization occur at higher temperatures than usual. When mashing with 100% barley adjunct, exogenous α-amylase (*B. subtilis*) addition has the greatest positive impact on mash separation. Increasing the level of exogenous α-amylase results in higher wort glucose and maltotriose levels and lower wort maltose levels. Optimal addition of an exogenous high heat-stable α-amylase (*B. licheniformis*) (Table 8.12) in combination with an exogenous α-amylase (*B. subtilis*) is necessary for complete starch conversion and maximum extract recovery from the raw barley substrate.

8.4.2 Brewing with maize or rice as adjunct

The double-mashing/decoction system was developed to deal with grists containing large proportions of rice or maize grits (25–60%) and to use the nitrogen and enzyme-rich malts that were available. The adjunct mash containing the grist and a small proportion of enzyme-rich malt or bacterial α-amylase is mashed in at about 35°C in the cereal cooker. The stirred mash is heated to about 70°C and after remaining at this temperature for 20 min is brought to 85–100°C. It is held at this temperature for 45–60 min to ensure that any starch that has not been liquefied is gelatinized. Meanwhile, the malt mash has been mashed in at 35°C. After a stand of about an hour, the adjunct mash is pumped in with mixing, so that

the final temperature of the mash is around 65°C. The whole process may take 3.5 h. It is important to remember that the actual time and temperature programme vary between breweries depending on the ratio of adjuncts, the quality of adjuncts, modification of the malt, exogenous enzyme quality, process equipment and capabilities/capacities available to the brewer.

8.4.3 Brewing with sorghum as adjunct

Sorghum, like barley, can be used in many forms including malted grain sorghum, sorghum grits, extruded dehulled sorghum and unmalted whole grain sorghum. Each has its own advantages and disadvantages. Limited endosperm cell wall degradation, low extract yields, poor wort separation and poor beer filtration are obstacles which have been widely reported when sorghum is used in lager production.

Brewing beer with unmalted sorghum as adjunct involves many technical considerations such as the capacity of the cooker, energy costs and the high gelatinization temperature of sorghum (71–80°C). A proper liquefaction step resulting in a low viscosity mash is suggested regardless of the proportion of sorghum adjunct to barley malt. This can be achieved by heating the sorghum to 80–100°C in the presence of a thermostable bacterial α-amylase. Brewing beer with sorghum adjuncts (at levels ≤50%) has the same limitations known in producing beer containing rice/maize as adjunct. If the sorghum is of a good quality, there should be no lautering problems as the 50% content of malt has sufficient husk material to ensure the formation of the necessary filter bed in the lauter tun. In all cases when mashing with unmalted sorghum, efficient amylolytic hydrolysis of starch will only occur if the starch has been effectively gelatinized. When brewing with low levels of unmalted sorghum (5–10%) as adjunct to barley malt, the endogenous enzymes of the malted grain can be sufficient to maintain adequate extract recovery, wort FAN and fermentability levels. However, when increasing the amount of sorghum adjunct, a decrease in wort filtration, colour, viscosity, attenuation limit, FAN and an increase in pH can be expected. Addition of commercial enzymes can alleviate these problems. The inclusion of a heat-stable α-amylase is essential for efficient saccharification. The inclusion of a fungal α-amylase can improve filtration rates to that of 100% malted barley mashes, while the addition of a bacterial protease increases the amount of nitrogen solubilization and peptide degradation.

A typical mashing regime involves mashing in hammer-milled unmalted sorghum at 50°C with a liquor: sorghum ratio of 3:1. The pH can be adjusted to 6.5–7.0 by the addition of calcium hydroxide to give a calcium level of 50–150 mg L^{-1}. A blend of neutral protease (for FAN production), thermostable α-amylase (for liquefaction) and a range of β-glucanases (to open up endosperm cell walls) can be added at this point. After 30 min at 50°C (the protein rest period), the temperature can be slowly increased to 85°C and maintained at that temperature for 30 min in order to liquefy the starch. The malt mash can be prepared with water at 20°C. After 15 min, the cold mash is combined with the hot liquefied sorghum mash or part of this and maintained for a further 60 min at 50°C. More enzymes are then added for starch saccharification. A fungal α-amylase can be added which hydrolyzes α-1,4 linkages of starch and dextrins, thereby producing maltotriose, oligosaccharides and large amounts of maltose. At the same time a new enzyme mixture of neutral protease, cellulase and amyloglucosidase may be added. After 60 min at 50°C, the combined mash is mixed with the rest of the 85°C sorghum mash, and the temperature is raised to 75°C. After 20 min at this temperature, the mash can be transferred to the mash filter. It is important to remember

that the actual time and temperature programme vary between breweries depending on the ratio of adjunct to malt, adjunct grain quality, modification of the malt, exogenous enzyme quality, process equipment and capabilities/capacities available to the brewer.

Though it is more common for some African-based brewers to brew with 50% unmalted sorghum and 50% unmalted maize, brewing with 100% unmalted sorghum is possible. Because unmalted sorghum contains no enzymes, a considerable amount of exogenous enzyme must be added. The potential for brewing a high-quality beer could be improved by (1) adjusting the mash-in liquor to give a calcium content of 200 parts per million, (2) adjusting the mash-in pH to 6.5, (3) using a mashing programme with temperature/time stands of 50°C × 50 min, 80°C × 10 min, 95°C × 40 min and 60°C × 30 min, (4) using a heat-stable α-amylase added at the end of the 50°C stand, a neutral protease added at mash-in and a fungal α-amylase added at the start of the 60°C, (5) adjusting the pH to 5.5 prior to the 60°C stand. The addition of calcium prevents the thermal inactivation of α-amylase by extending the pH range of the enzyme. Stabilization of the added α-amylase can result in increased liquefaction and therefore in increased extraction of the grist. It can also mean that inclusion of calcium ions in the mashing liquor could allow the same amount of extract recovery, but with a lower proportion of added exogenous α-amylases in the mash. With the incorporation of a 10-min stand at 80°C, significant increases in the levels of filterability, extract recovery and FAN have been observed. The pH adjustment from pH 6.2 to pH 5.5 prior to the 60°C heating step, optimized pH conditions for the fungal amylase whilst increasing mash bed permeability. The inclusion of an amyloglucosidase can significantly increase attenuation levels.

8.4.4 Other potential sources of adjunct

While the use of the traditional adjunct sources in brewing is already very well established (Table 8.11), there are many other sources of carbohydrate, some of which are already used in commercial brewing. Other sources have such physicochemical make-up and architecture that they could have potential for incorporation as brewing adjuncts. Examples are sugar beet, sugar cane, potato, millet, oats, rye, cassava, chick peas, mung beans, quinoa, buckwheat, amaranth, soya bean, banana, honey and the milk sugar lactose. With the current difficulties in world grain supply, these alternative adjunct sources represent very interesting prospects for brewing purposes. Exogenous enzymes can play a major role in ensuring extractability of these sources for brewing purposes.

8.5 ENZYME APPLICATIONS AND THEIR ROLE DURING FERMENTATION

8.5.1 Enzymatic processes in yeast fermentation

Enzymes (both endogenous and exogenous) and the expertise of the brewer to control enzymatic-mediated processes have up to now played the major role in determining the quality of the wort which goes forward for fermentation. The pre-boiled cold sterile wort to which the pitching yeast will be added (to start fermentation) will contain no residual enzymatic activity. However, a new source of enzyme transformation is ready to kick in – enzyme systems brought through the yeast. Through enzyme-mediated processes within yeast cells, sugar molecules are transformed into ethanol and CO_2, and amino acids are transformed

in yeast proteins. The formation of ethanol occurs via the Embden–Meyerhof–Parnas pathway (also called the glycolytic pathway) where, theoretically, 1 g of glucose will yield 0.51 g of ethanol and 0.49 g of CO_2.

Brewers yeast types are of the genus *Saccharomyces*. In wort, brewers yeast absorbs dissolved sugars, simple nitrogenous matter (amino acids and very simple peptides), vitamins, ions, etc., through their outside cell membrane (the plasma membrane). Then they employ a structured series of reactions known as metabolic pathways to use these substances for growth and fermentation. The two main types of beer, lager and ale are fermented with *Saccharomyces uvarum* (*carlsbergensis*) and *Saccharomyces cerevisiae*, respectively. *S. cerevisiae* has the ability to take up a wide range of sugars, for example glucose, fructose, mannose, galactose, sucrose, maltose, maltotriose and raffinose. Unlike *S. cerevisiae*, strains of *S. uvarum* (*carlsbergensis*) possess the MEL gene, which means that through an extracellular enzyme (α-galactosidase or melibiase), they are able to utilize melibiose (glucose–galactose). Wort components like dextrins, β-glucan and soluble proteins are not metabolized by strains of brewer's yeast.

Wort contains the sugars sucrose, fructose, glucose, maltose and maltotriose together with dextrins. The initial step in the utilization of any sugar by yeast is usually either its intact passage across the cell membrane or its hydrolysis outside the cell membrane followed by entry into the cell by some or all of the hydrolysis products. Maltose and maltotriose are examples of sugars that pass intact across the cell membrane whereas sucrose is hydrolyzed by an extracellular enzyme and the hydrolysis products are taken up into the cell. Maltose (50–60% of wort fermentable sugar) and maltotriose (20% of wort fermentable sugar) are the major sugars in brewers wort and consequently, a brewers yeast's ability to use these two sugars is vital and depends upon the correct genetic complement. It is probable that brewer's yeast possesses independent uptake mechanisms (maltose and maltotriose permease) to transport the two sugars across the cell membrane into the cell. Once inside the cell, both sugars are hydrolyzed to glucose units by the α-glucosidase (maltase) system. Once the sugars are inside the cell, they are converted via the glycolytic pathway into pyruvate. There are many more enzymatic-induced processes occurring during yeast metabolism. This is not the subject of this chapter and thus the reader is referred to more specific texts[10] covering the broad area of brewing fermentation.

8.5.2 Exogenous enzymes applied during fermentation

Exogenous enzymes (Table 8.10), apart from the application areas described below, are applied in fermentation as tools to help possible difficulties occurring later in the process. In the case of downstream beer filtration problems, β-glucanase can be added to the fermenter (or during maturation) to assist in degradation of residual glucans, which would otherwise cause the filters to block. This is a last option technique, as fermentation temperatures are low, hence enzyme activities are lower. It is more economical to apply the enzymes during the brewhouse stages of the process. Another area for enzyme application during fermentation is to reduce haze problems in the final beer. This will be covered in the next section.

If filtration problems are due to protein, starch or non-starch polysaccharides or combinations of these materials, then this can be detected via laboratory tests applying enzyme, filtration and flow rate tests to the wort. A visual representation of this is shown in Fig. 8.4. In this case, β-glucanase was very effective in restoring filtration to normal rates. The control beer had a β-glucan level of 300 mg L^{-1} β-glucan. With the addition of β-glucanase at

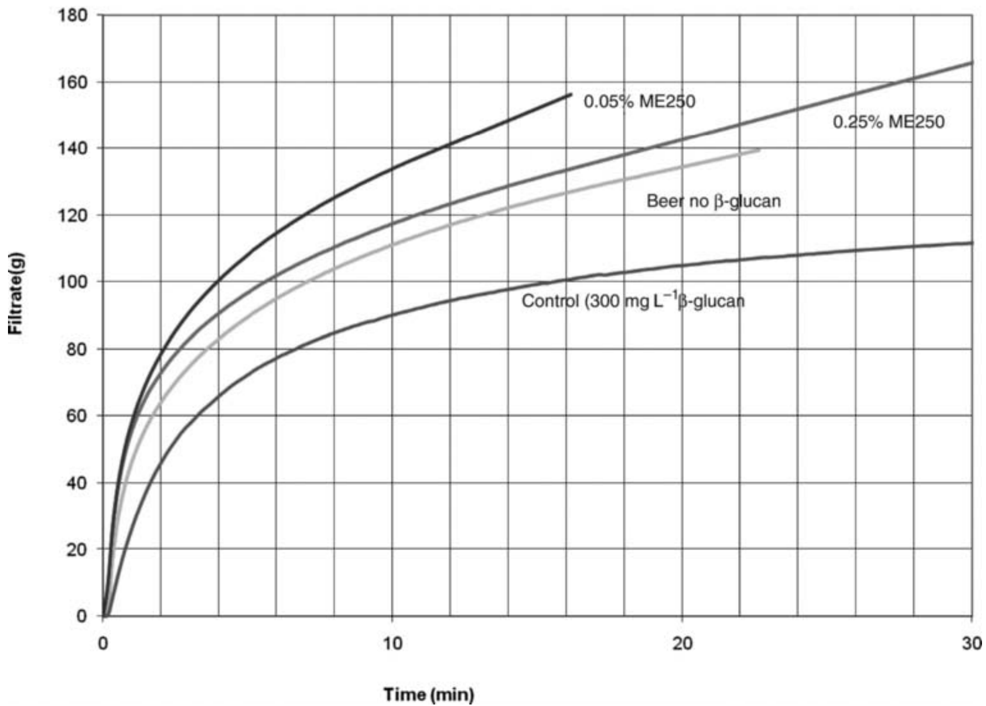


Fig. 8.4 Effect of β-glucanase on beer filtration. (Also see Colour Plate 1.)

a level of 0.025%, the filtration performance of the green beer was restored. Similarly, the filtration rates were improved further at higher dose rates. Likewise, with this approach the effects of proteases, hemicellulases and amylases can be demonstrated.

8.5.3 Low carbohydrate beer production

Low carbohydrate beer, commonly referred to as 'lite' beer, can be produced through the application of enzymes. In this case, enzymes can be applied during wort production or during fermentation. The α-1,6 bonds in amylopectin are not hydrolyzed by the α-amylase or β-amylase. Although malt is able to produce all the enzymes needed to break down starch into fermentable sugars, the malt limit dextrinase is unable to break down ungelatinized intact granules. As the limit dextrinase is temperature labile, it is degraded easily during kilning and also during mashing. This means that in normal beers the maximum fermentability is 70–82% (apparent degree of fermentation). Together with other beer components like proteins and tannins, this unfermentable dextrin material is mainly responsible for the mouthfeel, fullness and sweetness of beer. Together with the alcohol component, it contributes to the caloric value of beer. A small increase in fermentability can be achieved by adding a fungal α-amylase to ensure the complete breakdown of the α-1,4 links. A greater increase in fermentability can be obtained by adding an amyloglucosidase. This preferentially hydrolyzes the α-1,4 linkages and also slowly hydrolyzes the α-1,6 linkages to produce glucose. Low caloric or

low/zero carbohydrate beer (typically 3–7 g carbohydrate/serving) can be produced in this manner (in practice usually a combination of a fungal α-amylase and an amyloglucosidase is used). The enzyme can be added during mashing processes (fungal α-amylase and an amyloglucosidase) or directly to the fermenter (amyloglucosidase). Because of the short contact time during mashing, higher dosage rates are required than when the enzyme is added during fermentation. The enzymes are fully degraded during high temperature mashing and boiling. However, the amyloglucosidase is stable to normal beer pasteurization conditions and this means that when the enzyme is added during fermentation, residual enzyme activity will be present in the final beer package. Benefits of addition during fermentation are lower dosage rate requirements and the possibility of spoon-feeding the yeast with simple sugars (glucose and maltose). In this way, yeast glucose inhibition is prevented. This would occur if the yeast were provided with too much free glucose. Glucose inhibition of yeast will slow down or could inhibit fermentation. Distillers will most commonly add the enzyme during fermentation for the reasons mentioned above and also because they are not concerned with inactivating the enzyme before distillation.

One of the most troublesome problems for a brewer is that of 'hanging fermentation', where complete attenuation is not reached within normal fermentation time (or not at all). Hanging fermentation can be caused by a number of factors:

- Unbalanced wort carbohydrate spectrum; insufficient levels of fermentable sugars because of incomplete enzymatic saccharification during mashing. Usually this is related to the use of adjuncts or under-modified malt.
- Failure of the yeast, although still in suspension, to utilize all of the fermentable wort sugars. This is usually due to the inability of the yeast to take up and metabolize maltotriose. This can be the result of a spontaneous mutation which can occur in most brewing strains.
- Deficiency of yeast nutrients like amino acids, vitamins, cofactors such as zinc (a common problem with high brewing).
- Premature flocculation of the yeast culture in the fermenting wort.

Table 8.13 shows a typical balanced wort sugar spectrum. The problem of an unbalanced wort carbohydrate composition can be controlled during fermentation with the help of exogenous α-amylase (acting on α-1–4 bonds) or a pullulanase-type enzyme (some varieties acting on both α-1–4 and α-1–6 bonds), which will correct a possible incomplete saccharification during mashing. Exogenous amyloglucosidase may also be used to save batches of beer if the problem of the inability to metabolize maltotriose (yeast mutation) occurs. Maltotriose will then be degraded to smaller sugars that can subsequently be fermented. A possible shortage of amino acids (yeast food) can be overcome in the brewhouse by ensuring a better protein

Table 8.13 Typical carbohydrate composition of 12°P wort.

Sugar	**% in wort**	**Fermentable**
Fructose	0.1–0.2	+
Glucose	0.9–1.3	+
Maltose	5.8–6.8	+
Sucrose	0.2–0.4	+
Maltotriose	1.4–1.7	+
Dextrins	1.9–2.4	–

breakdown in the mashing cycle, either by prolonged protease rests at the start of mashing or the use of exogenous exo-peptidases.

8.6 BEER STABILIZATION

Beer haze can be of both biological and non-biological origin. Biological haze can be caused by contaminating microbial sources and therefore will not be the subject of this chapter. Non-biological haze can be caused by polysaccharides such as under-degraded β-glucan and starch. As addressed earlier in the chapter, optimal use of both endogenous and exogenous enzymes during brewhouse processing can control the formation of hazes due to these polysaccharides. If haze is detected in beer, the brewer has the possibility of applying very simple techniques to troubleshoot and identify the origin and nature of the turbidity (Fig. 8.5). One such method is the application of exogenous enzymes to turbid beer samples. Haze caused by starch, glucans and proteins can be quickly identified by applying amylases, glucanases and proteases, respectively.

The most common form of beer haze is the one caused by cross-linkage of the high molecular weight protein (hordein) of malt. This glycoprotein fraction contains high proportions of hydrophobic amino acids, which combine with polyphenols, which principally consist of proanthocyanidins and catechins (flavanoids). Small amounts of carbohydrate and trace mineral ions are also involved. Initially the haze that develops in the early stages of the beer shelf life is reversible and will disappear at higher temperatures. The polyphenols combine slowly with protein to form a chill haze when the beer is cooled. This haze redissolves when the beer is warmed up. Oxygen and specifically the process of oxidation play an important part in the polymerization of the polyphenols to produce an irreversible permanent haze.

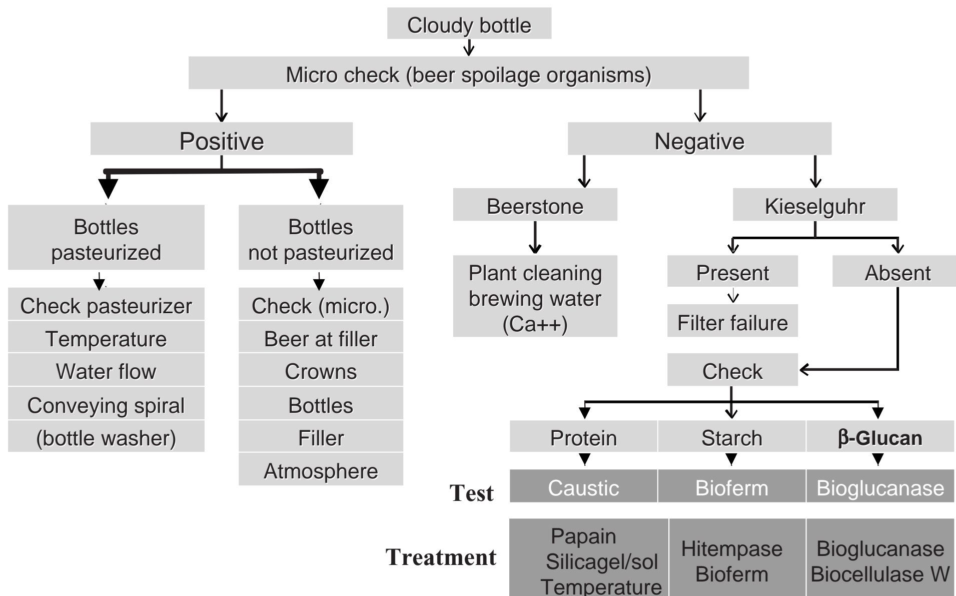


Fig. 8.5 Beer haze and filtration troubleshooting.

Upon oxidation, the polyphenols polymerize and increase in size and they become insoluble at room temperature to form irreversible or permanent haze.

8.6.1 Enzymes for corrective actions (filtration and beer haze)

The traditional method of stabilizing a beer to prevent or slow down beer haze development was to store the beer for prolonged periods at very low temperatures (preferably $<0°C$). In today's competitive brewing environment, this is a costly and time-consuming solution. The current trend is to remove the haze-forming components (proteins and polyphenols). There are a wide variety of processing aids for selectively identifying and removing these components (Fig. 8.5). The most common form of enzyme treatment is to apply the plant-derived enzyme papain. Papain is a proteolytic enzyme extracted from pawpaws, the fruit of the papaya tree. It is applied to prevent colloidal instability problems imparted by protein and therefore extend the shelf life of beer. It works by hydrolyzing protein that would otherwise have formed complexes with polyphenolic materials resulting in chill haze and eventually permanent haze. The enzyme is a mixture of cysteine proteinases and its specificity is broad. It preferentially cleaves at the COOH-terminus of basic amino acids (lysine, arginine). Like with all enzymes the dosage requirement will depend on the quality of the beer and the raw materials used. It can be added during transfer between fermentation and maturation or directly into the maturation tank itself. If the beer is pasteurized at temperatures $>70°C$, the enzyme will be completely destroyed (inactivated). Compared to other stabilization techniques (e.g. PVPP), it is very simple to apply and can be considered a cost-effective solution.

8.6.2 Enhanced maturation

A major flavour component of beer is the vicinal diketone diacetyl. At concentrations >0.1 mg L^{-1} it is universally considered a flavour defect and results in a butterscotch flavour. Diacetyl is a by-product of amino acid metabolism and is formed by the oxidative decarboxylation of α-acetolactate during the exponential growth phase of the yeast during fermentation. Towards the end of fermentation, yeast is capable of reducing diacetyl to acetoin (a flavour component with a much higher flavour threshold). However, natural diacetyl removal in this manner requires healthy yeast, time (storage capacity!) and a high temperature maturation phase. Therefore, a microbial (*Bacillus*) enzyme was developed to convert α-acetolactate into acetoin, before it can be converted into diacetyl. This enzyme is called α-acetolactate-decarboxylase (ALDC). The relevant transformations are depicted in the scheme shown in Fig. 8.6. The benefits for the brewer upon using this enzyme are a reduction in the time-dependent high temperature stand usually employed to reduce diacetyl in the normal fashion. It is an enzymatic method of increasing fermentation capacity, while also giving the brewer an alternative way of controlling final beer diacetyl levels and thus influencing the full flavour profile of the beer.

8.7 THE FUTURE OF ENZYMES IN BREWING

Much of the progress in using enzymes in food processes or in the manufacture of food ingredients has been evolutionary, involving catalytic activities, the ability to deal with a wider range of raw materials, wider variations in processing conditions, pH, temperature and importantly, reduction in the cost of enzymes. Many of these improvements have been

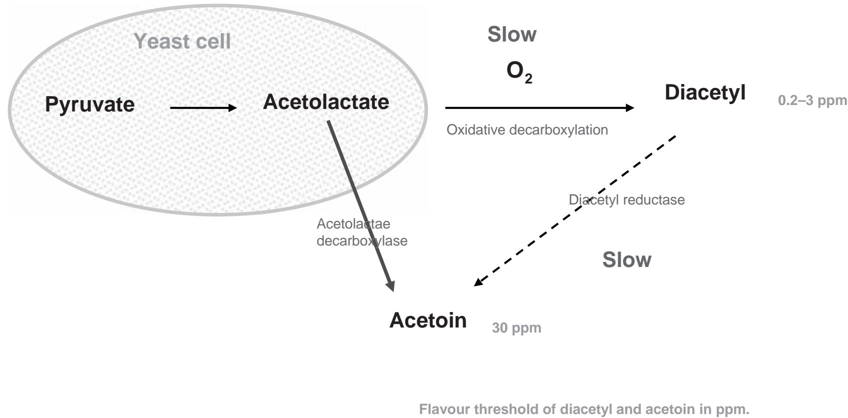


Fig. 8.6 Acetolactate-decarboxylase (ALDC) and diacetyl reduction. (Also see Colour Plate 2.)

brought about by the introduction and use of enzymes from genetically modified microorganisms (GMOs). In addition, there have been new developments that involve new enzyme activities and new applications for enzyme activities. Many of the recent and ongoing developments for use of enzymes in starch hydrolysis are associated with improvements in enzyme activities (α-amylases, glucoamylases and debranching enzymes) and improvements in enzymatic hydrolysis of non-corn cereals such as wheat and barley. One such example where developments in protein engineering have benefited adjunct processing is that of α-amylases and their applications in syrup production. When bacterial α-amylases that were capable of operating under industry-relevant conditions ($>100°C$) were introduced in 1973, it was revolutionary for starch processing. Before then acid hydrolysis of starch was the norm. Acid hydrolysis was environmentally unfriendly and led to significant levels of unwanted by-products. However, two basic problems still existed with enzymatic hydrolysis. First, the pH of the starch slurry had to be adjusted from 4.0 to 6.0. Second, calcium had to be added to stabilize the liquefying bacterial α-amylase. A breakthrough in the mid-1990s was the unveiling of the first three-dimensional structure of a *Bacillus*-derived α-amylase. This meant that through protein engineering new and improved commercial enzyme products could be produced. The next breakthrough for the starch processing industries was the development of very efficient starch hydrolytic enzymes capable of acting below the gelatinization temperature. This has meant that current processes can now be performed in a 'one step' starch hydrolysis process without the need for very high temperature, pH adjustment or calcium addition. Such developments have significantly brought down the cost of starch processing and allow production of more specific liquid adjunct products.

As has been seen in this chapter, the biodiversity of enzymes has provided the brewing industry with a wide range of functionalities. As biotechnology paves the way for making improvements to known enzyme functions as well as opening the door for designing new enzymes with new functionalities, this is likely to increase the possibility of increasing adjunct usage in brewing and for maximizing extractability from poor quality grain sources. Likewise, it will enable the production of superior adjuncts with added benefits specially formulated to the brewers need and may even decrease the cost of production of adjuncts, thereby decreasing the cost of beer production. The GMO issue may continue to be one of controversy for the foreseeable future. The brewing industry has already benefited from several enzymes that

are produced using genetically modified production hosts to reduce the cost or enhance the functionality of the enzyme. With a global shortage of grain, the food industry and indeed the consumer public may be forced to embrace the technological advantages that genetic modification can offer, in terms of disease resistance, climate resistance and technological performance. With increased knowledge of cereal grain structure such as barley endosperm cell walls, future biotechnology applications will focus on delivering to the brewing industry tailor-made enzymes with more specific hydrolyzing capabilities.

Nowadays, many approaches can be taken to control colloidal stability of beer, leading to a product with up to 1-year colloidal stability. However, strategies controlling flavour stability are still scarce. One of the ideas in the past was to apply an oxygen-scavenging enzyme, like glucose-oxidase, into the bottle of beer. Although technically feasible, the idea of having an active enzyme in the final beer was not very appealing. In the meantime, alternative ways have been developed to limit oxygen in the bottle (improved packaging technology). Another general worry to brewers is the oxidative damage that occurs during mashing. It is generally believed that lipoxygenase catalyzed lipid oxidation (and/or auto-oxidation) plays a role in the formation of cardboard off-flavours (trans-2-nonenal) upon ageing. In addition, a technological solution is being developed, by milling and mashing-in under oxygen-limited conditions. Currently, less capital-intensive routes are investigated by adding oxygen-scavenging enzyme systems in the mash. Preliminary work has shown that this approach seems feasible. Suitable oxygen-scavenging enzymes can be selected from the group consisting of glucose-oxidase, hexose-oxidase, sulfhydryl-oxidase, superoxide dismutase, peroxidase, polyphenol-oxidases, such as laccase, and combinations of these enzymes. Particularly the polyphenol-oxidase enzyme has the benefit of not only scavenging oxygen, but at the same time also converting polyphenols into less soluble complexes, which are subsequently removed from the wort upon mash filtration (or lautering). Another benefit of this new enzyme system is that the temperature stability is such that it even remains active for some time after mashing-off (78°C). It thereby also protects the wort lipids from being oxidized, which goes particularly fast at these high temperatures. Ultimately, the enzyme system is destroyed during boiling in the copper.

8.8 CONCLUSION

The years 2006, 2007 and 2008 will best be remembered by brewers across the globe as the catastrophic years of variable malt quality and hop supply problems – unfortunately the outlook for 2009 and beyond looks no better. The overall global cereal supply is currently characterized as poor yielding with low year-to-year stock leads. Perhaps of even more concern is that cereal sources are in higher demand than ever before, not only from the traditional food and beverage industries but also from the bio-fuel industries. Basically, the global food and beverage industry is in a current situation of a higher demand for grain than supply allows, consumption is exceeding production. This has resulted in a high demand for virtually every agricultural crop across the globe. As a result, barley, malt, wheat, maize, rice and sorghum prices have dramatically increased. Likewise, mother nature is not always kind, poor weather conditions can lead to crops of a difficult processing nature. High protein levels, high β-glucan levels and often malt of a quality that can at best be described as variable, have increased difficulties in the brewhouse. Brewers are forced to change processes and increase adjunct proportions in recipe formulations.

The brewing process is a natural biological process and its efficiency relies mainly on the quality of its raw materials. Ways of directly improving the raw material issues can only be controlled outside of the brewery environment and start literally at grassroots, growing the cereal and hop crops. Away from this, within the brewery environment, the only way of controlling the efficiency of the process and the quality of the final product is by development of new equipment technologies and the application of processing aids and ingredients.[11] In essence, optimal application of processing aids and, more specifically, enzymes can give the brewer the freedom to retain and improve their processes, irrespective of starting raw material qualities. Optimal commercial enzyme usage can also mean cost savings.

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REFERENCES

1. Moll, M. (ed.) (1991) *Beers (Including Low-Alcohol and Non-Alcoholic Beers) and Coolers, Definition, Manufacture, Composition*. Intercept Ltd, Andover.
2. Kunze, W. (1999) *Technology, Malting and Brewing*. VLB, Berlin.
3. Bamforth, C.W. (2006) *Brewing – New Technologies*. Woodhead Publishing Limited, Cambridge.
4. Briggs, D.E. (1998) *Malts and Malting*. Chapman and Hall, London.
5. Bamforth, C.W. and Kanauchi, M. (2001) A simple model for the cell wall of the starchy endosperm in barley. *Journal of the Institute of Brewing* **107**, 235–240.
6. Kanauchi, M. and Bamforth, C.W. (2002) Enzymic digestion of walls purified from the starchy endosperm of barley. *Journal of the Institute of Brewing* **108**, 73–77.
7. Kuntz, R.J. and Bamforth, C.W. (2007) Time course for the development of enzymes in barley. *Journal of the Institute of Brewing* **113**, 196–205.
8. Goode, D.L. and Arendt, E.K. (2006) Developments in the supply of adjunct materials for brewing. In: *Brewing – New Technologies* (ed. C.W. Bamforth). Woodhead Publishing Limited, Cambridge, pp. 30–67.
9. Goode, D.L., Wijngaard, H.H. and Arendt, E.K. (2005) Mashing with unmalted barley – impact of malted barley and commercial enzyme (Bacillus sp) additions. *Master Brewers Association of the Americas, Technical Quarterly (MBAA TQ)* **42**, 184–198.
10. Smart, K. (2008) *Brewing Yeast Fermentation Performance*. Blackwell Publishing Professional, Oxford.
11. Goode, D.L. and Lalor, E. (2008) The malt and hop crisis technologies to maximise process ability and cost efficiency. *The Brewer and Distiller International* **4**(3), 37–40.

9 Enzymes in potable alcohol and wine production

Andreas Bruchmann and Céline Fauveau

9.1 ENZYMES FOR POTABLE ALCOHOL PRODUCTION

The conversion of starch containing distillery raw materials is a complex process, which occurs via a few distinct reactions, with the help of different amylolytic enzymes. Enzymes from different raw materials differ in their attributes (properties), although they sometimes carry the same name. The starch-hydrolyzing enzymes which are applicable are shown in the overview in Table 9.1.

9.1.1 Starch-hydrolyzing enzymes

It has been known for a long time that certain enzymes catalyze the conversion of starch into short glucose chains. As early as 1785, J.C. Irvine observed that starch was liquefied with the help of an aqueous extract of germinated barley. In 1814, G.S. Kirchhoff described a substance derived from grain seeds with which starch was converted into sugar. The French chemists Payen and Perzos[1] isolated in 1833 a starch liquefying substance out of germinated barley which they named diastase. In their essay, they described all important characteristics of the diastase and mentioned that malt could be used for the production of dextrins.

Maercker[2] showed in 1878 that malt diastase contains two different enzyme activities, and this was confirmed by Lintner[3] in 1886, a fact which still applies today. Ohlsson[4] in 1926 described both of these important starch-hydrolyzing enzymes as α-(or dextrinogen-) amylase and β-(or saccharogen-) amylase. Later, the term diastase was changed into the correct expression: amylase.

Both enzymes differ in terms of origin, properties and effect and both act in a different way on the starch molecule. Ungerminated barley seed contains β-amylases in an active form described as raw- or own amylase, whereas α-amylase is primarily formed during germination (malting) of the barley.

9.1.1.1 *α-Amylases*

α-Amylase (α-1,4-glucan-glucanhydrolase) is found not only in malt, but also in moulds (for example *Aspergillus oryzae*) and bacteria (for example *Bacillus subtilis*), as well in human and animal saliva (phytalin) and in the pancreas (pankreatin). As endo-enzymes, α-amylases contain a fast liquefaction and a weak saccharification ability. Due to the random cutting of starch glucosidic α-1,4-linkages of the amylose molecule, unbranched dextrins of

Table 9.1 Starch degrading enzymes.

Cereal amylases	Fungal amylases	Bacterial amylases
α-Amylase β-Amylase Border dextrinase R-enzyme α-Glucosidase with isomaltase action	α-Amylase Border dextrinase Glucoamylase	α-Amylase

medium chain length (oligosaccharides) are formed and the thin fluid solution obtained upon α-amylase-catalyzed starch hydrolysis is iodine negative.

At 70–75°C, small amounts of α-amylase will lead to a fast liquefaction of the paste formed from amylopectin. This takes place via the formation of branched pieces (border dextrins) which are composed out of the so-called 'knot points' of amylopectin.

Theoretically, α-amylase can transform linear amylose into 87% maltose and 13% glucose; whereas from the branched amylopectin, 73% maltose, 19% glucose and 8% isomaltose are potentially possible. In practice these maltose values are not be obtained. The reason is that

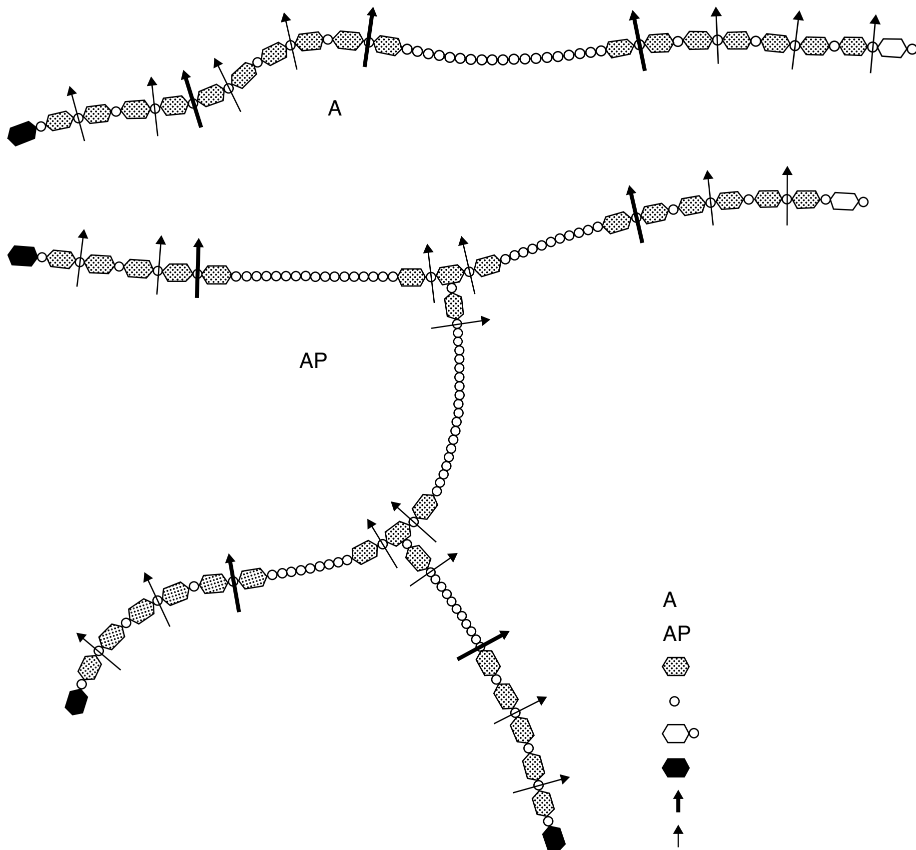

Fig. 9.1 Decomposition of amylose (A) and amylopectine (AP) by α-amylases.[6]

Table 9.2 Activity dependence of α-amylases on temperature and pH.

α-Amylase source	Cereal (malt)	Bacteria	Moulds
pH range	4.5–8.5	4.8–7.5	4.3–6.0
pH-optimum	5.0–5.6	5.3–6.5	4.5–5.5
Temperature range	70–80°C	65–95°C	45–60°C
Optimum temperature	Approx. 75°C	Approx. 70°C	Approx. 55°C

the generated maltose blocks the effect of α-amylase. Only when the maltose fermentation starts, the α-amylase will continue to saccharify virtually all the remaining dextrins.

Kreipe[5] referred to the importance of α-amylases for the saccharification within distillery companies.

It is remarkable that various α-amylases differ substantially in their activity relative to temperature and pH-value. The average values of the most important technical α-amylases are shown in Table 9.2.

Although the pH activity dependences of the various amylases are relatively unimportant, major differences are found in temperature dependences. The temperature optimum of fungal α-amylase is approximately 20°C below that of bacterial α-amylases. This has to be regarded in practical applications. Observations by Underkofler and Hickey[25] showed that bacterial α-amylases produce dextrins faster than fungal amylases.

As demonstrated by Aschengreen,[7] the presence of calcium-ions (Ca^{++}) is required for the adequate effectiveness and stability of microbial α-amylases. This finding led to the addition of defined doses of calcium salts especially in potato processing.

Furthermore, the pH-optimum of the first technical α-amylases was quite high (around 6). Therefore the pH of the mashes had to be raised in most cases. To meet both demands of pH and Ca^{++} requirement, lime hydrate $Ca(OH)_2$ was applied successfully.

Technical bacterial α-amylases that show high activity without the need for adding calcium are nowadays available for distilleries. Furthermore, these enzymes are optimally suited because of their complete effectiveness at pH-values up to 5.0 and at temperatures up to 90°C. They also secure a flawless liquefaction of potato mashes. This can be achieved without the addition of calcium, assuming that the amount of existing calcium present in potatoes is sufficient to ensure the full effect of these α-amylases.

Extensive formation of maltose is a characteristic of fungal α-amylases from *A. oryzae* – and this is obviously beneficial for the fermentation processes in a distillery. With the help of these amylases the fermentation period can be reduced to 34 h, even in potato mashes. Furthermore it appears that upon fermenting wheat mashes, there is a lower tendency to form a solid top layer when fungal α-amylases are used for starch reduction. However, a faultless degradation of starch during the steaming process is an absolute requirement.

9.1.1.2 *β-Amylase*

β-Amylase (α-1,4-glucane-maltohydrolase) is characterized by an extremely slow viscosity reduction and a high capability of sugar formation; that is this enzyme shows a weak liquefying but a strong 'sugaring' effect. Hence it is declared as saccharogen-amylase. Out of the two components of natural starch (amylose and amylopectin), β-amylases degrade the 1,4-glucosidic linear chain of amylose – as exo-enzyme – from the non-reducing end completely. On the other hand, amylopectin is only incompletely degraded into maltose. The

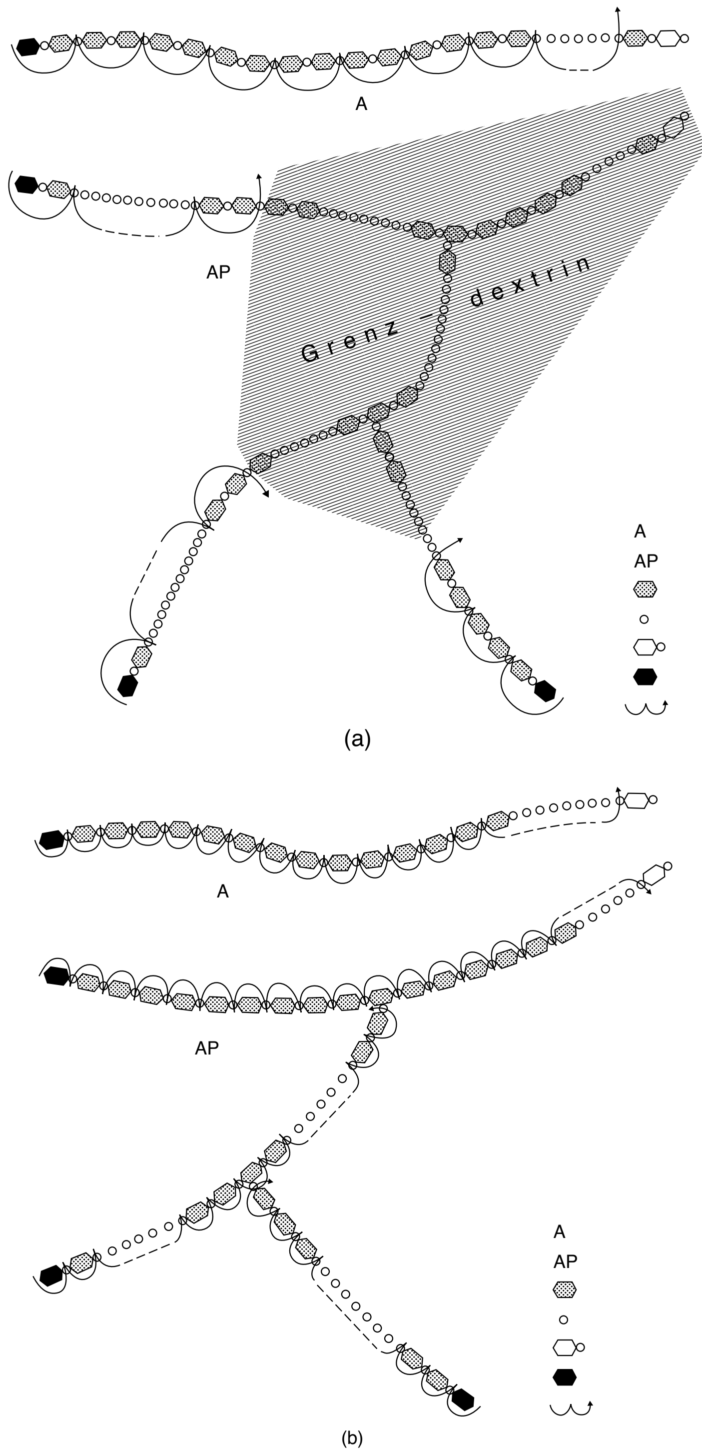

Fig. 9.2 (a) Degradation of amylose (A) and amylopectin (AP) by (exo) β-amylase (adapted from Ref. [6]). (b) Degradation of amylose (A) and amylopectin (AP) by glucoamylase (adapted from Ref. [6]).

reason for this is the 1,6 linkages forming side chains in the amylopectin molecule. Nearly 65% of the theoretically possible amount of maltose is generated, as well as dextrins and small amounts of other sugars. Like α-amylases, β-amylases are blocked by the maltose formed, as long as this is not eliminated by fermentation.

The temperature optimum of β-amylase is below 60°C, and is denatured quickly at temperatures over 60°C. The pH-optimum of β-amylases is around 5.2 and more general between 4.8 and 5.5; the pH-stability is between 4.5 and 7.5. It should be noted that β-amylase is found only in higher plants, like barley, rye and wheat, as 'raw corn amylase', but it is not found in microorganisms.

9.1.1.3 *Border dextrinases*

In addition to α- and β-amylases, border dextrinases (oligo-1,6-glucosidase) are involved in the saccharification of the starch of raw materials used in distilleries. These enzymes are developed from malt, especially oat malt, as well as from moulds and are not able to attack high-molecular weight amylopectin. In contrast, the border dextrinase of malt (pH-optimum 5.1, temperature optimum 40°C) primarily splits border dextrins, thereby developing maltose, maltotriose and some glucose. The acid-sensitive fungal-dextrinase (pH-optimum 6.3) dissolves branched links of lower molecular sugars and degrades, for example the non-fermentable isomaltose into glucose.

9.1.1.4 *R-enzyme*

The R-enzyme (amylopectin-1,6-glucosidase) can only be found in malt and is capable of hydrolyzing α-1,6-bindings in amylopectin, the so-called joints. This splits the giant molecule into smaller degradation products. Border dextrins are not attacked by this enzyme.

9.1.1.5 *Glucoamylase (amyloglucosidase)*

An important enzyme for starch saccharification is glucoamylase (α-1,4-glucan-glucohydrolase), also called amyloglucosidase. This enzyme is found in mould preparations, for example in *Aspergillus niger*. As an exo enzyme, it splits amylose as well as amylopectin from the non-reducing ends into glucose, without being hindered by the branched chains in amylopectin. Dextrines, maltotriose and others sugars are hydrolyzed into glucose as well by this enzyme. While during the processing of starch with malt several enzymes have to interact repeatedly in order to achieve total starch saccharification up to glucose, this can be achieved by using microbial glucoamylase only. However, the formation of glucose by glucoamylase is a much slower process than starch conversion by malt enzymes. The reason may be that only a few chain ends are available for the decomposition. To arrive at the fast starch saccharification desired in practice, the addition of an α-amylase is therefore unavoidable. This enzyme does a major preliminary job for the attack of the glucoamylase by its dextrinizing effect and the multiplication of chain ends.

The optimal conditions for glucoamylase are around 60°C and a pH between 4.0 and 5.2. However, this enzyme also exhibits relatively high activity at pH values of 5.5 and above and at pH 3.5 and below but this does not lead to a requirement to lower the pH during the fermentation process.

9.1.1.6 *α-Glucosidase*

As can be expected, malt also contains an enzyme which forms, in a similar way as microbial glucoamylase, glucose from maltose and isomaltose. It is called α-glucosidase and it has an isomaltose effect.[8] The pH-optimum is at 4.6, the temperature optimum at 45°C.

9.1.1.7 *Transglucosidase*

A transglucosidase, belonging to the main class of transferases, is a microbial enzyme, from for example *A. niger.* This enzyme is able to build up unfermentable dextrins from maltose, as opposed to the starch decomposing enzymes discussed so far. From experience the process of mashing and fermentation in distilleries is not affected yet by this enzyme, since in the presence of yeast during the fermentation, there is no dextrin synthesis from sugar.[9]

9.1.2 Cellulases

Cellulases also have to be mentioned among the important technical enzymes for distilleries. In fact this group of enzymes does not belong to the common distillery enzymes yet, but it can be expected that cellulases will achieve an important position in this area in the future. Exploratory tests have shown that the enzymatic decomposition of cellulose provides a possibility means to increase alcohol recovery.

The interaction of several single enzymes is needed for the enzymatic hydrolysis of cellulose. However, cellulose breakdown is as yet incompletely clarified.[10] Several groups of enzymes involved are defined and must be called 'cellulolytic systems' instead of 'cellulases'.

The C_1-component of these systems does not have a hydrolytic effect but reduces hydrogen bonds which stabilize the crystal structure of the cellulose in and between the β-1,4-glucan chains. This creates additional reaction areas for the hydrolytic acting β-1,4-glucanases. As a third component in a cellulolytic system, β-glucosidases are involved in cellulose breakdown. β-Glucosidases widely differ in their preference for certain chain lengths of the molecules. The final product of enzymatic cellulose hydrolysis is glucose.

Technical enzymes for saccharification of cellulose can be derived from several moulds. The activities of those preparations in relation to native (crystalline) cellulose are, however, relatively low. The optimum effects of these enzyme systems are achieved at pH 4–6 and at 60°C,[10] which is a convenient area for the mashing in distilleries.

9.2 ENZYMES IN WINEMAKING

9.2.1 Introduction

Wine is an alcoholic beverage made from the fermentation of fresh grapes or fresh grape juice. Although other fruits such as apples and berries can be fermented, the resultant 'wines' are normally named after the fruit from which they are produced (e.g. apple wine or elderberry wine) and are generically known as fruit wines. The commercial use of the word 'wine' (and its equivalent in other languages) is protected by law in many jurisdictions. According to the 'Organization Internationale de la Vigne et du Vin' (OIV) 2005,[11] worldwide wine production, excluding grape juices and musts, is estimated to be 282 million hectoliters with Europe accounting for 191 million hectoliters. More and more wine is being produced in

new world winemaking countries; this trend is accompanied by a change in global consumer taste in wine.

Consumers want aromatic fruity wines and in the case of red wines; soft tanins, but above all they want consistency in the profile of the product they buy. This trend has lead to an industrialization of winemaking processes and a more consistent use of recipes and processing aids such as enzymes. The use of pectinases was introduced in winemaking in the 1970s to improve white must clarification. From the 1980s onwards enzymes have been used for colour extraction, filtration and aroma release. Nowadays, enzymes are used not only to improve the efficiency of processes such as pressing and clarification but also to improve wine quality through aroma, mouthfeel and structure.

Although the grape berry as well as the microorganisms performing the transformation of grape juice into wine do perform enzymatic reactions, this chapter only reviews the application of added exogenous industrial enzyme preparations with a special focus on enzymes working on grape-derived substrates.

9.2.2 Grape structure and composition

Although grape berry composition depends on the variety, soil and climatic conditions, there is little variation in the plant cell structure (see chapter on enzymes in fruit and vegetable processing and juice extraction).

The grape berry skin accounts for 6–9% of the total berry weight. Within skin cells are found essential compounds such as anthocyanins (responsible for the red colour of wines), tannins (essential for the structure of red wines) and aroma or their precursors. The thick pecto-cellulosic wall of the skin cells provides rigidity to the berry but when making wine it prevents the diffusion into the must of intracellular components.

The pulp represents 75–85% of the ripe berry weight. It comprises large cells with fine pecto-cellulosic walls offering limited mechanical resistance. In the cell, vacuole is a concentrated solution of organic acids, fermentable sugars, some aroma and precursors. Pectin is located between the cells, in the primary wall and lamella.

9.2.3 Pectin

Pectin is probably one of the most complex macromolecules found in nature. Its content in grapes varies with the variety and maturity.[12] The pecto-cellulosic wall is a complex structure. It is composed of cellulose microfibrilles, linked together by a matrix of xyloglucan, mannan, xylan (generally known as hemicellulose) and pectin, all consolidated by a secondary protein network. Some neutral sugars (galactose and arabinose) make up part of the structure of the lateral pectin chains and form macromolecules with proteins.

9.2.3.1 *Pectin three main components*[13,14]

- Homogalacturonan (HG) is a non-ramified chain of α-1,4-linked galacturonosyl acid residues, partially esterified at C-6 with methanol groups. They are also described as the 'smooth region' of pectin.
- Rhamnogalacturonan I (RG-I). The main chain, comprises an alternating rhamnose and galacturonic acid, bears lateral chains of arabinans and arabinogalactans to form areas known as 'hairy regions'.

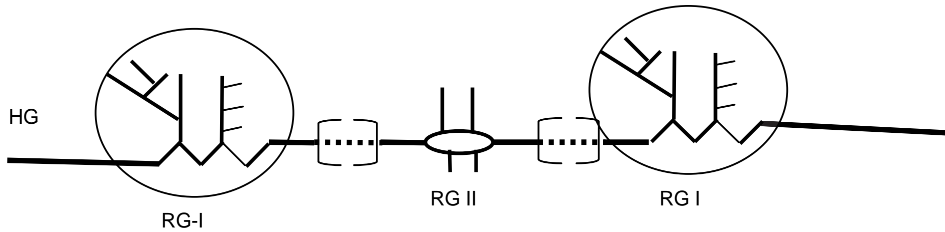

Fig. 9.3 Simplified scheme representing pectin.

- Rhamnogalacturonan II (RG-II) has a very complex structure which cannot be hydrolyzed by enzymes.
- There are several schools of thought on how these three constituents found in the cell walls are organized. It has, however, been shown that they are combined by covalent acidic links to form pectin (Fig. 9.3), with cross links between different pectin chains (ionic, electrostatic and diester borate bridges).

9.2.3.2 *Pectin physical properties; negative effects in winemaking*

Pectin's gel forming and water retention properties prevent the diffusion of phenolic compounds and aromas into the must during the pre-fermentation and fermentation stages.

The high viscosity of pectin solubilized after crushing, hinders juice extraction, clarification and filtration.

9.2.4 Polyphenols

9.2.4.1 *Types of phenolic compounds found in grape*

Phenolic compounds are responsible for major organoleptic characteristics in wines, particularly colour and structure in red wines. However, wine polyphenol composition is still poorly understood; most studies have focused on classes of molecules that can be separated and assayed with high performance liquid chromatography (HPLC) and have neglected polymers that are not as easily determined. Furthermore, polyphenols are highly reactive compounds and good substrates for various enzymes, including polyphenoloxidases, peroxidases, glycosidases and esterases. They undergo numerous enzymatic and chemical reactions during storage and processing, and the structures of the resulting products are still poorly understood.[15]

Chemical reactions of polyphenols are particularly important in wine because they are responsible for the colour and taste changes that occur during aging. Grape polyphenols, namely, anthocyanins, flavonols, hydroxycinnamic acids and flavanols, including catechins and proanthocyanidins, represented only approximately one-half of the polyphenol content of a 2-year-old red wine polyphenol extract. The other one-half consisted of unknown phenolic species derived from grape polyphenol reactions during winemaking and aging.[5] Genuine anthocyanins determined with HPLC contributed 50% of the red colour intensity measured spectrophotometrically after dilution with 1% HCl; therefore, the other one-half could be attributed to anthocyanin-derived pigments.

9.2.4.2 *Polyphenol properties*

Polyphenols exhibit a wide range of properties, depending on their particular structures. They include yellow, orange, red and blue pigments, as well as various compounds involved in flavour. The major flavours associated with polyphenols are bitterness and astringency. Other major polyphenol characteristics include their radical-scavenging capacity, which is involved in antioxidant properties, and their ability to interact with proteins. The latter is responsible for astringency perception (resulting from interactions of tannins with salivary proteins), for formation of haze and precipitates in beverages.

9.2.4.3 *Grape polyphenol localization*

The polyphenols are distributed throughout the grape as follows:[16]

Grape seeds contain 60% of the grape polyphenols. This group is mainly constituted of proanthocyanidins (monomeric flavan-3-ols, and their oligomers and polymers). The lipid layer surrounding the seed prevents their extraction in maceration (with or without enzymes).

Grape skin contains 30% of extractable grape polyphenols, mainly anthocyanins and resveratrol. Resveratrol is an antioxidant found in grapes and red wine, mulberries and peanuts. It has been shown to have positive anti-aging benefits and has also demonstrated benefits in diabetes, heart health, obesity and some cancers. For example, red wine of the pinot noir variety contains 3.1 mg of resveratrol per litre. Grape pulp contains less than 10% of total extractable grape polyphenols.

9.2.5 Wine varietal aromas and their precursors in grapes

In order to produce wines matching consumer expectations, that is with well-defined intense aroma, winemakers must put everything in place to extract aroma and their precursors. Most white varieties, aside from Muscats, do not contain odorous aroma but do contain heavy hydrophilic aroma precursors. These precursors are converted into odorous aroma through a series of chemical and enzymatic reactions naturally occurring during the winemaking process. Once released, these molecules are responsible for the wine varietal aroma. There are several families of molecules. Only the two main varietal aroma families are detailed below but many more do exist including carotenoïd derivatives, dimethyl sulphide (DMS) whose role in wine aroma is still being investigated.

9.2.5.1 *Glycosylated compounds*

Odourless aromatic terpenols precursors are present in the grape skin in a form linked to sugars,[17] their composition and content varies according to the grape variety. In *Vitis vinifera*, monoterpenes precursors are bound to di-glycosides, glucose and another carbohydrate residues such as arabinose, rhamnose or apiose. In varieties such as muscat or riesling, the linalol, nerol and geraniol glycosylated precursors are the most abundant, their 'sugar' part comprising ramnose-glucose for rutinosides, arabinose-glucose for arabinosides and apiose-glucose for apiosides. Sequential hydrolysis of these sugars releases the highly odorous terpenols. When released from their linked sugar, these compounds are responsible for a wide range of aromatic notes from honey to fruity or flowery.

9.2.5.2 *Thiols*

Thiols are sulphur compounds responsible for the typical notes in white wines produced from such grape varieties as Sauvignon, Chenin, Petit and Gros Manseng and Colombard, as well as some red varieties. These thiols are present in the grape skin and/or pulp in the form of odourless precursors bound to an amino acid, cysteine. During grape maturation, the appearance of the 4MMP (4-mercapto-4-methylpentan2-one) precursor precedes that of the 3MH (3-mercaptohexan-1-ol). This explains why, for a given vineyard, Sauvignon wine will express a stronger box tree nuance when the grapes are harvested earlier, while it becomes more fruity with a later harvest.[18]

There are several techniques that, when used alone or in combination, increase and guide the aromatic intensity and profile of the wine. It is possible to intervene by facilitating:

- diffusion of precursors in grape must (cys-4MMP, cys-3MH). This step can be enhanced by the use of a specific maceration enzyme;
- the release of volatile thiols from their precursors (4MMP, 3MH). This reaction occurs during alcoholic fermentation and is performed by specific strains of *Saccharomyces cerevisiae*;
- thiol (A3MH) conversion. This reaction occurs during alcoholic fermentation and is performed by specific strains of *S. cerevisiae*;
- aroma conservation in finished wines.

9.2.6 Legal aspects of the use of enzymes in winemaking

9.2.6.1 *Organizations*

Enzyme preparations used in winemaking must comply with the specifications recommended by Joint FAO/WHO Expert Committee on Food Additives (JECFA) and by Food Chemical Codex (FCC) for food enzymes. The OIV takes decisions on analysis methods and on enological applications and treatments. Local organizations such as the Food and Drugs Administration (FDA) in the United States of America, the Direction Générale de la Concurrence, de la Consommation et de la Répression des Fraudes (DGCCRF) in France or the Food Standards Australia New Zealand (FSANZ) control that laws are being respected and/or ensure people's safety.

OIV

'Organization Internationale de la Vigne et du Vin' (OIV) which replaced the 'International Vine and Wine Office' was established by the Agreement of 3 April 2001. The OIV is an intergovernmental organization of a scientific and technical nature in the fields of vines, wine, wine-based beverages, table grapes, raisins and other vine-based products.[11] The OIV counts 44 member states.

The OIV seeks to foster an environment that is conducive to scientific and technical innovation, the dissemination of the results thereof and the development of the international vitivinicultural sector. It will promote, through its recommendations, international standards and guidelines, harmonization and the sharing of information and sound science-based knowledge, in order to enhance productivity, product safety and quality, and the conditions for producing and marketing vine and wine products.

International enological CODEX
The International Oenological Codex compiles the description of the principle chemical products used to make and store wine. Identification characteristics and purity degree of these products are described in addition to the minimum required efficiency. The definition or a formula synonym of each product is provided. The conditions for their use, the instructions and limits for their usage are set by the '*International Code of Oenological Practices*'. The authorization for their usage is subject to domestic laws.[11]

International code of oenological practices
This technical legal reference document is focused on the standardization of products in the vitivinicultural sector to be used as a base to establish national or supra-national regulations.[11]

JECFA
JECFA is an international scientific expert committee that is administered jointly by the Food and Agriculture Organization of the United Nations and the World Health Organization. It has been meeting since 1956, initially to evaluate the safety of food additives. Its work now also includes the evaluation of contaminants, naturally occurring toxicants and residues of veterinary drugs in food.[19]

The Food and Drugs Administration
FDA is the US Government agency that oversees most medical products, foods and cosmetics.[20]

French General Directorate for Competition Policy, Consumer affairs and Fraud control
The General Directorate for Competition Policy, Consumer affairs and Fraud control (DGCCRF) has a market regulatory role to ensure market balance and transparency. It also ensures consumer economic protection through information against abusive practices and assures consumer physical security and health.[21]

9.2.6.2 *Enzymatic activities currently authorized in winemaking*

The recommendations of the OIV, the European and French Regulations wrap around one another with degrees of precision and ever increasing legal restrictions,[22] while the new *International Oenological Codex* and the *International Code of Oenological Practices* both recognize the use of enzymes where their effectiveness has been proven (resolution 14/2003), and for a whole series of applications (resolutions 11–18/2004).

The OIV resolution, oeno 11–18/2004[11] recognizes the importance in the winemaking process of the following enzymatic activities: pectin lyase, pectin methyl-esterase, polygalacturonase, glycosidase, hemicellulase, cellulase and β-glucanases. The OIV and *International Code of Oenological Practices* authorize Lysozyme (resolution oeno 15/2001) and the use of urease for treatment of wines to reduce the risk of ethyl carbamate formation.

European and French legislation are rather more restrictive and are based on the principle of a positive list. The activities authorized in winemaking are covered by the European Union article 1493/1999. The EC Regulation 1493/1999 only authorizes pectinases from *A. niger*, β-glucanase produced by *Trichoderma harzianum*, urease from *Lactobacillus fermentum* and lysozyme.

9.2.6.3 *Traceability*

From 1 January 2005 the European Regulation EC 178/2002 on food safety has been applicable. Under European regulations, anyone operating in viticulture and/or winemaking is fully liable for the safety of their end product. Those operating in the food industry have to prove they have made every effort in this respect. Traceability is being required at every production step. Those operating at any stage 'from the vineyard to the glass', must work together to ensure complete transparency in the field of food safety especially in respect of allergies.

Following a period of highly publicized food crises (bovine spongiform encephalopathy (BSE), food allergies, etc.) the wine distribution channels are demanding a number of guarantees from their suppliers through the implementation of control systems. Purchasing centres exercise direct pressure on the nature and traceability of source products and products used during the course of winemaking. Their requirements are often more demanding than the current regulation, with highly restrictive responsibilities in respect of resources.

Thus, wine producers have to respond to the requirements of both regulatory and the market controls. In turn, they have to make similar demands on their own suppliers (grape producers, manufacturers of oenological products, dry materials, etc.). Producers of oenological products have a joint responsibility with the wine producers for ensuring the safety of wine.[22]

9.2.6.4 *Labelling*

Industrial enological enzymes fall under the processing aids category and therefore to this date do not require to be labelled on the wine bottle.

According to the FDA, 'processing aids can be substances that are added to a food for their technical or functional effect in the processing but are present in the finished food at insignificant levels and do not have any technical or functional effect in that food.'[20]

Lysozyme, as it is extracted from egg white, falls under the allergen-labelling directive. Starting 31 May 2009, any wine treated with egg white-derived products, albumin or lysozyme, will have to bear on their label the statement: '*contains egg*'.

9.2.7 GMO transparency

Speaking of GMO in respect of winemaking enzymes generates confusion. Genetic modification techniques only apply to the productive microorganism and not to enzymes: enzymes are proteins, not living organisms. But, they can be produced by classical microorganisms or microorganisms that have been obtained by genetic modification. Thus, we can speak of an enzyme as being produced by a genetically modified microorganism as opposed to being produced by a classical microorganism.

Only a targeted question on the conditions for obtaining the productive microorganism strain for the enzyme will provide reliable information on whether genetic engineering was used.

Details of genetic engineering techniques (see Chapter 2) and their advantages in oenological use are not discussed in this chapter. However, such techniques should only be used where full transparency is exercised in respect of clients and when their use is generally agreed upon in the market and amongst consumers.[22]

9.2.8 Production of winemaking enzymes

9.2.8.1 *Enzymes produced through fermentation*

To produce the enzymes used in winemaking, selected strains are cultivated in fermentors under aerobic conditions. *A. niger* for the production of pectinases and β-glycosidases, *T. harzianum* in the case of β-glucanases and *L. fermentum* in the case of urease.

A well-defined composition of the growing medium induces optimal production of the desired enzymatic activities. For example, a growing medium rich in pectin induces the microorganisms to secrete pectinases (pectolytic enzymes) into the media.[23] After fermentation, the pectinases and enzymatic side activities are isolated by centrifuge, ultra filtration and concentration. During these stages microorganisms are completely eliminated from the end product.

9.2.8.2 *Other types of production for enzymes used in winemaking*

Lysozyme is a naturally occurring protein discovered by Fleming in 1922. Hen egg white (HEW) lysozyme is a well-known compound that has been used in pharmaceutical compositions and food since the 1950s. The product is separated from HEW with an ion exchange resin then purified and dehydrated.

9.2.9 Winemaking enzymes composition and formulation

9.2.9.1 *Principal activity*

The main activities in winemaking enzyme formulations are derived from the pectinase family. They include pectin lyase (PL), pectin methyl-esterase (PME) and polygalacturonase (PG). PL-type activity, known as de-polymerizing, cuts the pectin chain between two methylated galacturonic acids, while the PG prefers a non-methylated substrate. PME activity does not depolymerize the pectin chain but releases a methanol molecule from galacturonic esterified acids. It eases the action of PG.

Pectinases, by breaking down pectin, do provide a number of obvious technical advantages, such as accelerating the pre-fermentation stages, increasing free run juice yield, enhancing clarification and pressing, leading to an overall improvement in the grape must quality with increased aroma and polyphenols concentrations.

Pectinase activity units

Today, each enzyme producer uses their own pectinase activity measuring method and unit. These units mainly constitute a way for enzyme producers to compare different products from their own production and are used for product standardization and quality control procedures. Different analytical protocols have been developed and are standardized with different pectin sources showing degrees of methylation different from grape pectin. These measures do not provide an indication of the enzyme efficiency in winemaking. The efficiency of an enzymatic preparation in winemaking is strongly related to the presence of side activities, as the main pectinase activity alone does not ensure all benefits. For this reason, enzymes' efficiency in winemaking ought to be checked through application trials during the development phase of the product.

It is not possible to compare products from different origins based on the activity level provided by the supplier on the product label or specification sheet.

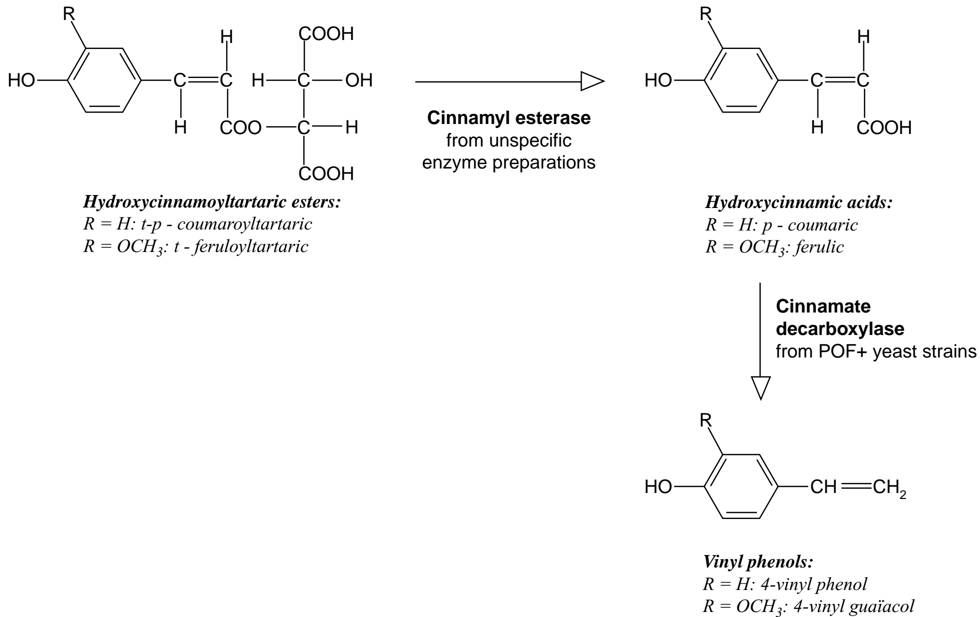

Fig. 9.4 Vinyl phenol formation in white wines.[24]

9.2.9.2 *Side activities*

The nature and complex composition of the growth substrate used to produce the enzymes induce the production of a wide pool of enzymatic activities. The main (principal) activity in the obtained enzymatic product is accompanied by numerous secondary (side) activities that play roles of varying degrees of importance, some being essential other neutral or detrimental in specific winemaking applications. By using *A. niger* strains specifically selected for enological purposes, DSM Food Specialties enzyme preparations are formulated to naturally maintain any unwanted activities to a negligible level. Some side activities might, in certain types of wines, have an undesirable effect and a positive effect in other. Although these secondary activities are tolerated under the legislation, these enzymes can in certain cases spoil wine quality.

- Hemicellulase and cellulase activities are normally present in varying amounts in pectinase preparations. These activities sought for in red grapes maceration in order to extract the maximum skin cells content are better avoided in white grapes maceration to limit grape skin dilaceration which could cause pressing problems.
- Cinnamyl esterase activity in white wines contributes to the hydrolysis of coumaric and ferulic acids, which, after decarboxylation by a POF+ (phenyl off flavour) yeast strain, lead to the formation of vinyl-4-phenol and vinyl-4-guaiacol. These compounds give unpleasant poster paint and nail polish smells.[24] In red wines, vinyl phenols react with polyphenols to form colour-stabilizing compounds. The details of this reaction are developed in a specific section of this chapter. Analysis shows that the level of cinnamyl-esterase activity in DSM NFCE enzymes (naturellement faible cinnamyl esterase) is amongst the lowest of any preparation purified or not available on the market. Using DSM

NFCE enzymes in white winemaking limits the formation of volatile phenols maintaining their concentration under the perception threshold.
- Anthocyanase activity in red wines could cause a colour loss as it releases anthocyanins from their bound sugar; this hydrolysis results in an unstable anthocyanidin form.

9.2.9.3 *Formulation*

Enological enzymes can be formulated in liquid or micro granulated form.

- In the micro granulate form enzyme preparations offer good storage stability. The activity level of these products is stable when stored under the recommended conditions of humidity and temperature. At room temperature, their shelf life varies from 24 to 36 months. This form has no risk of being contaminated even after opening this with no preservatives use.
- Liquid enzymes should be stored at cold temperatures. The shelf life of these products when stored under recommended conditions is between 12 and 24 months. Their microbiological stability is more difficult to guarantee and their formulation often requires the use of preservatives. For example, sorbate salts and potassium chloride are authorized preserving agents used in liquid enzyme preparations. Another stabilizing agent commonly used in liquid enzymes is glycerol, although this compound does not negatively affect wine quality, its addition in the winemaking process is not allowed in most wine-producing countries.

REFERENCES

1. Payen, A. and Perzos, J.F. (1833) Mémoire sur la diastase, les principaux produits de ses réactions et leurs applications aux arts industriels. *Annales de Chimie et de Physique* **53**, 73–92.
2. Maercker, M. (1878) Action of diastase on starch. *Journal of the Chemical Society* **34**, 969–970.
3. Lintner, C.J. (1886) Study over diastase. *Journal für Praktische Chemie* **34**, 378–394.
4. Ohlsson, E. (1926) The two components of malt diastase. *Comptes rendus des travaux du Laboratoire Carlsberg* **16**(7), 1–68.
5. Kreipe, H. (1967) alpha-Amylase in saccharification of starch-containing distillery raw materials. *Brantweinwirtschaft* **107**(5), 110–111.
6. Bruchmann, E.-E. (1976) *Angewandte Biochemie*. Eugen Ulmer, Stuttgart, Germany, pp. 101, 103, 105.
7. Aschengreen, N.H. (1969) Laborversuche mit pH-Aenderungen und Kalziumzusatz zur Kartoffelmaische. *Branntweinwirtschaft* **109**(3), 45–48.
8. Jorgensen, O.B. (1963) Barley malt α-glucosidase. II. Studies on the substrate specificity. *Acta Chemica Scandinavica* **17**, 2471–2478.
9. Okazaki, H. (1958) In: *Proceedings of the International Symposium on Enzyme Chemistry, Tokyo and Kyoto 1957*. Organized by Science Council of Japan under the auspices of International Union of Biochemistry. Pergamon press, London, Vol. **2**, p. 494.
10. Bruchmann, E.E. (1978) Lactones, reductones, and enzymic saccharification of cellulose. *Chemiker-Zeitung* **102**(11), 387–389.
    Bruchmann, E.E., Graf, H., Saad, A.A. and Schrenk, D. (1978) Preparation of highly active cellulase preparations and optimization of enzymic cellulose hydrolysis. *Chemiker-Zeitung* **102**(4), 154–155.
    Bruchmann, E.E., Kirsch, B. and Lauster, M. (1975) Production of highly active cellulase preparations and optimization of enzymic cellulose hydrolysis. *Chemiker-Zeitung* **99**(3), 157–158.
11. Organisation Internationale des Vins' (OIV) (2005) www.OIV.org
12. Mourgues, J. (1983) *Doctoral-Engineering Thesis*. University Paul Sabatier, Toulouse.
13. Voragen, A.G.J., Schols, H.A. and Visser, R.G.F. (eds) (2003) *Advances in Pectin and Pectinase Research*. Kluwer Academic Publishers, Dordrecht, pp. 47–59.

14. Vidal, S., Williams, P., O'Neil, M.A. and Pellerin, P. (2001) Polysaccharides from grape berry cell walls. Part I: tissue distribution and structural characterization of the pectic polysaccharides. *Carbohydrate Polymers* **45**(4), 315–323.
15. Cheynier, V. (2004) Polyphenols in foods are more complex than often thought. In: *Proceedings of the 1st International Conference on Polyphenols and Health*. Vichy, France, 18–21 November.
16. DSM press release.
17. Gunata, Y.Z., Bayonove, C.L., Baumes, R.L. and Cordonnier, R.E. (1985) The aroma of grapes. Localisation and evolution of free and bound fractions of some grape aroma components c.v. Muscat during first development and maturation. *Journal of the Science of Food and Agriculture* **36**(9), 857–862.
18. Fauveau, C. (2009) *Popular Premium Aromatic White Wines – An Equation with Several Variables – Enzyme-Yeast Synergy for Wines Derived from Varietals with Thiol Precursors*. DSM Food Specialties, Montpellier.
19. www.who.int/ipcs/food/jecfa/en/
20. www.FDA.org
21. http://www.minefi.gouv.fr/DGCCRF/
22. Pellerin, P., Bajard-Sparrow, C., Fauveau, C. and Strozyck, F. (2005) Legislation, obligation de tracabilite et securite alimentaire: les reponses d'un producteur d'enzymes. *Revue Française d'Oenologie* **214**, 35–37.
23. Bajard-Sparrow, C., Fauveau, C., Grassin, C. and Pellerin, P. (2006) Enzymes pour l'œnologie. Mode de production, mode d'action et impact sur la transformation du raisin en vin. *Revue des Œnologues* **121**, 29–32.
24. DSM Food Specialties communication – foul smell, www.DSM-oenology.com
25. Underkofler, L.A. and Hickey, R.J. (1954) *Industrial Fermentations*, Vol. **1**. Chemical publishing Co. Inc., New York, p. 62.

10 Enzymes in fish processing

Soottawat Benjakul, Sappasith Klomklao and Benjamin K. Simpson

10.1 INTRODUCTION

Enzyme technology is currently being applied in the fish processing industry to increase fish meat yield and to facilitate the processing as well as to improve the quality of finished products. Enzymes are capable of accelerating desirable reactions, from which a number of advantages can be obtained. Additionally, the enzymatic reactions employed are mild and do not cause an adverse effect, such as the loss of nutritive values of products. Reactions can be manoeuvred or regulated easily without undesirable effects. Various enzymes can be used in seafood industry to increase processing efficacy. However, the maximization of desirable endogenous enzymes or lowering the enzymes causing a negative impact on the seafood quality is the promising means to obtain seafood products with prime quality. Enzymes, especially industrial enzymes, can be used as fish processing aids. However, the industry has to afford for the high cost of those enzymes. Therefore, the recovery of fish enzymes for further uses has been paid attention to in order to gain the potential processing aids cost effectively. Furthermore, the unique characteristics of those enzymes can be exploited.

Different enzymes have been used in seafood processing as the aid to improve yield and quality. Modes of action of different enzymes vary and the processes involved should be carefully considered so as to maximize the benefit of the enzymatic reactions employed.

10.2 PROTEASES

Proteases play an essential role in the growth and survival of all living organisms. The hydrolysis of peptide bonds catalyzed by proteases is a common reaction in nature. Proteases from plants, animals and microorganisms are polyfunctional enzymes catalyzing the hydrolytic degradation of proteins.[1]

Proteases can be classified on the basis of their similarity to well-characterized proteases as trypsin, chymotrypsin, chymosin or cathepsin-like. They may be classified on the basis of their pH sensitivities as acid, neutral or alkaline proteases. They are also characterized by common names and trade names, preferential specificity and response to inhibitor specificity. In the EC system for enzyme nomenclature, all proteases (peptide hydrolases) belong to subclass 3.4, which is further divided into 3.4.11–19 (the exopeptidases) and 3.4.21–24 (the endopeptidases or proteinases).[2] Endopeptidases cleave the polypeptide chain at particularly susceptible peptide bonds distributed along the chain, whereas exopeptidases hydrolyze one amino acid from *N* terminus (amino peptidases) or from *C* terminus (carboxypeptidases).

Exopeptidases, especially aminopeptidases, are ubiquitous, but less readily available as commercial products, since many of them are intracellular or membrane bound. Based on the nature of the catalytic site, proteinases are further classified into four categories: acid or aspartate proteinases, serine proteinases, thiol or cysteine proteinases or metalloproteinases.[3] The enzymes in the different classes are differentiated by various criteria, such as the nature of the groups in their catalytic sites, their substrate specificity, their response to inhibitors or by their activity/stability under acid or alkaline conditions.[2]

10.2.1 Applications of proteases

Proteases are by far the most studied enzymes for industrial bioprocessing. For the fishery industry, proteases are used as processing aids for many products. These include recovery of pigment and flavouring compounds, production of fish protein hydrolyzates, viscosity reduction, skin removal and roe processing.[4–6]

10.2.1.1 *Carotenoprotein extraction*

Various methods have been devised to recover carotenoid or carotenoprotein from crustacean wastes as a potential source of red/orange pigments for use in feed of farmed fish and shellfish. Extraction of shell waste with oil[7] reduces ash and chitin levels and achieves a good recovery of pigment. However, this method suffers the disadvantage of yielding a product devoid of protein, thereby decreasing the stability of the carotenoid to oxidation and failing to recover a valuable nutrient. Since about one-third of the dry matter in crustacean shell waste is protein, an enzymatic process has been developed to extract and recover the protein along with the carotenoid in its native carotenoprotein from crustacean waste. Proteolytic enzymes were used to recover carotenoprotein from shrimp[8] and crab.[9] About 80% of the protein and 90% of the astaxanthin pigment from shrimp processing waste can be recovered as an aqueous dispersion after trypsin hydrolysis. Cano-Lopez *et al.*[10] reported that using Atlantic cod trypsin from pyloric ceca in conjunction with a chelating agent (EDTA) in the extraction medium increased the efficacy in recovering both protein and pigment from crustacean wastes. This method has facilitated the recovery of as much as 80% of astaxanthin and protein from shrimp processing wastes as carotenoprotein complex. Ya *et al.*[11] recovered carotenoprotein from lobster waste by using trypsin from bovine pancreas. The product obtained was found to contain higher protein and pigment content than those of untreated lobster waste and was devoid of chitin and ash. Recently, Klomklao *et al.*[12] also recovered carotenoprotein from black tiger shrimp waste by using trypsin from bluefish pyloric ceca. The product contained higher protein and pigment content than those of untreated black tiger shrimp waste and had low contents of chitin and ash (see Table 10.1). A lower yield of protein (60–70%) and pigment (35–50%) is recovered in the carotenoprotein fraction when protease with broad specificity is used rather than trypsin. Chakrabarti[13] isolated carotenoprotein from tropical brown shrimp shell waste by enzymatic process including trypsin, papain and pepsin. Trypsin yielded the maximum recovery of carotenoid (55%) when hydrolysis was conducted for 4 h at room temperature. Pepsin and papain showed about 50% recovery during the same period. The yield of protein paste isolated with trypsin was highest. Protein-associated astaxanthin is more resistant to oxidation[8] and is deposited in the skin and flesh of rainbow trout more efficiently[14] than free pigment. Use of this process by industry is promising due to a less costly source of trypsin than from the currently available process.

Table 10.1 Proximate composition of black tiger shrimp shell and carotenoprotein recovered with or without bluefish trypsin (reprinted from Ref. [12]).

Compositions (% dry weight basis)	Shrimp shell	Carotenoprotein[a]	
		Control	Bluefish trypsin-aided
Crude protein	30.88 ± 0.76	59.95 ± 0.02	70.20 ± 0.11
Crude fat	3.93 ± 0.79	14.91 ± 0.15	19.76 ± 0.25
Ash	29.98 ± 0.75	17.89 ± 1.12	6.57 ± 0.18
Chitin	32.89 ± 1.55	5.40 ± 0.01	1.50 ± 0.10

[a] Values are mean ± standard deviation from triplicate determinations.

10.2.1.2 *Fish sauce*

Fish sauce is a traditional fermented fish product, which is used as an important source of protein in Southeast Asia. Fish sauce is a clear brown liquid with a salty taste and mild fishy flavour. The product is basically made from a mixture of fish and salt with a weight ratio of 3:1. This mixture is allowed to ferment for more than 6 months at 30–35°C for complete hydrolysis and flavour development.[15] During fermentation, endogenous enzymes slowly degrade the fish tissue in the presence of 20–30% salt to form a clear liquid with a high content of free amino acids and excellent flavour. The fermentation, which proceeds until the desired product is obtained, is time consuming, requires a large storage capacity and is therefore costly. To reduce this capital investment, it is desirable to accelerate the fermentation process. Addition of exogenous proteases to the fermentation can accelerate the process. However, flavour characteristics of the finished product are normally inferior to the traditional product. Plant enzymes were the first to be used. Papain from unripe papaya, bromelain from pineapple stems and ficin from figs have all been used. These enzymes are cysteine proteases, most active under weak acid conditions. Fish sauce recovery was obtained after 2–3 weeks of the fermentation process. Best results were obtained using bromelain preparations, but the characteristic flavour of the finished product was inferior to the traditional one. This accelerated method is used today in commercial fish sauce production in Thailand.[16] The enzymes recovered from fish have also been successfully used as seafood processing aids including the acceleration of fish sauce fermentation. Chaveesuk *et al.*[17] reported that supplementation with trypsin and chymotrypsin significantly increased protein hydrolysis of fish sauce. Fish sauce prepared from herring with enzyme supplementation contained significantly more total nitrogen, soluble protein, free amino acid content and total amino acid content, compared to fish sauce without added enzyme.[17] By supplementing minced capelin with 5–10% enzyme-rich cod pyloric ceca, a good recovery of fish sauce protein (60%) was obtained after 6 months of storage.[18] Additionally, Klomklao *et al.*[15] reported that fish sauce prepared from sardine with spleen supplementation contained greater contents of total nitrogen (see Fig. 10.1), amino nitrogen, formaldehyde nitrogen and ammonia nitrogen than did those without spleen addition throughout the fermentation. Therefore, the addition of spleen can accelerate the liquefaction of sardine for fish sauce production.

10.2.1.3 *Seafood flavouring*

Seafood flavours are in high demand for use in products like artificial crab and fish sausage. Proteolytic enzymes can aid the extraction of flavour compounds from shells and other

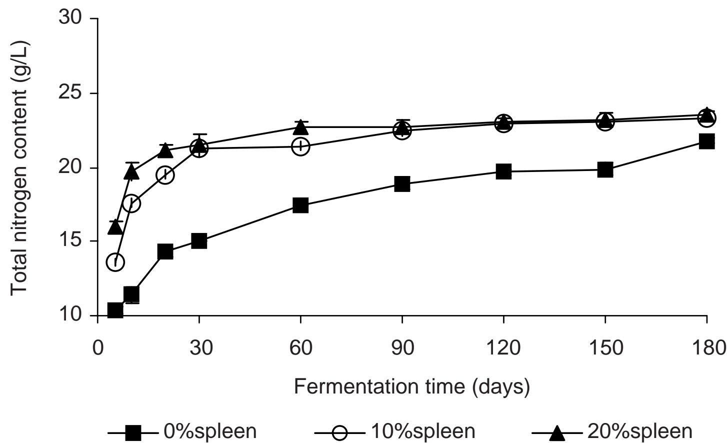

Fig. 10.1 Total nitrogen content of fish sauce samples produced from sardine to which different levels of skipjack tuna spleen are added; blends are fermented for 180 days. (Reprinted from Ref. [15], Copyright (2006), with permission from Elsevier.)

materials. The taste and volatile components of shrimp heads were recovered by enzymatic digestion using Corolase N and koji (a source of protease from *Aspergillus oryzae*) or bacterial strains with high proteolysis rates followed by concentration and spray drying.[19,20] The product contained 9–12% free amino acids, mainly taurine, arginine, glycine and proline and inosine monophosphate as the major nucleotide. Shrimp flavouring can be used as an additive in surimi-based products and cereal-based extrusion products like shrimp chips. Proteases are used in this process, although other types of enzymes may also be involved, to liquefy the material which allows separation of bones and shells and facilitates concentration to 50–60% dry matter. The degree of protein hydrolysis is used to optimize the process with regard to yield and off-flavour formation. Enzymes have also been used to recover flavour from oysters.[20]

10.2.1.4 *Deskinning*

The common method of removing skin from fish fillets is a purely mechanical action, whereby an automated machine in effect tears the skin off the flesh. The ease of deskinning varies greatly between fish species. Some species, such as the starry ray (*Raja radiata*), are so difficult to deskin that automated mechanical deskinning is virtually impossible. Therefore manual deskinning must be performed, but manual deskinning is a labour-intensive task and hence the production costs become prohibitive, with the result that these species tend to be underutilized.[21]

A number of inventions using enzymes to remove the skin from fish, which are difficult to deskin by mechanical means, have been described. Stefansson and Steingrimsdottir[22] described the enzymatic deskinning of *Raja radiate* wings. The process involved partial denaturation of the skin collagen using a gentle heat treatment followed by immersion in an enzyme solution for several hours at low temperature (0–10°C). The dissolved skin could then be rinsed off the skate wings. The enzyme solution contained unspecified proteases and carbohydrases. The presence of carbohydrases, although not essential, enhanced the skin-solubilization process, presumably by loosening the collagenous layer, thereby facilitating

increased access of the proteases to the denatured collagen.[22] Deskinning of tuna (*Thunnus* spp. and related genera) was achieved by preheating with steam to 60°C, followed by digestion of the pre-treated skin with a complex mixture of proteases and carbohydrases at ∼50°C.[23] Deskinning of herring (*Clupea harengus*) with cod pepsin was reported.[24] Raa[25] reported that squid is deskinned and tenderized using papain in a weak salt solution. An enzymatic process was also developed to loosen shrimp shells from muscle tissue prior to peeling.[25]

10.2.1.5 *Collagen extraction*

Fish collagen can be obtained from skin, bone, scale and cartilage of various fish species.[26–29] Collagen is commonly extracted using an acid-solubilization process, in which non-collagenous matter, pigment and lipids are first removed by alkali solution. Pre-treated materials are used for collagen extraction using an acid process. Acetic acid is widely used for collagen extraction. The resulting collagen is usually referred to as 'acid soluble collagen'. However, the acid process gives a low yield and the use of pepsin in combination with acid extraction is implemented to increase the yield of collagen (see Table 10.2). Covalent cross-links at the telopeptide region of collagen molecules are not readily solubilized by acid. However, those cross-links can be cleaved by pepsins without damaging the integrity of triple helix of collagen[30] (see Fig. 10.2). Nagai *et al.*[31] reported that the yield of pepsin-solubilized collagen was higher (44.7%) than acid-solubilized collagen (10.7%). Nagai and Suzuki[32] found that the collagen extracted from the outer skin of the paper nautilus was hardly solubilized in 0.5 M acetic acid. The insoluble matter was easily digested by 10% pepsin (w/v), and a large amount of collagen was obtained with 50% yield (pepsin-solubilized collagen). Collagen from the outer skin of cuttlefish (*Sepia lycidas*) was extracted by Nagai *et al.*[33] The initial extraction of the cuttlefish outer skin in acetic acid yielded only 2% of collagen (dry weight basis). With a subsequent digestion of the residue with 10% pepsin (w/v), a solubilized collagen was obtained with a yield of 35% (dry weight basis). Pepsin-solubilized collagen was extracted from the skin of grass carp (*Ctenopharyngodon idella*) with a yield of 35% (dry weight basis).[34]

Fish pepsin is another promising enzyme for collagen extraction. It can be extracted or recovered from fish processing by-products, especially fish stomach. Furthermore, the cost of pepsin can be reduced and the visceral by-products can be fully utilized. Nalinanon *et al.*[30] studied the use of fish pepsin as tool to increase the yield of collagen extracted from fish skin. Addition of bigeye snapper pepsin at a level of 20 kUnits g^{-1} skin resulted in the increased content of collagen extracted from bigeye snapper skin. The yields of collagen from bigeye snapper skin extracted for 48 h with acid and with bigeye snapper pepsin were 5.31% and 18.7% (dry basis), respectively. With pre-swelling in acid for 24 h, collagen extracted with bigeye snapper pepsin at a level of 20 kUnits g^{-1} skin for 48 h, the yield increased to 19.8%, which was greater than that of collagen extracted using porcine pepsin at the same level (13.0%) (see Table 10.2).[30] All collagens contained β, $\alpha 1$ and $\alpha 2$ chains as the major constituents.

Tuna pepsin has been shown to be an effective aid for collagen extraction from fish skin. Nalinanon *et al.*[35] used the pepsin from the stomach of albacore tuna, skipjack tuna and tonggol tuna for collagen extraction from the skin of threadfin bream. The yield of collagen increased by 1.84–2.32 folds and albacore pepsin showed the comparable extraction efficacy to porcine pepsin. Tuna pepsin-solubilized collagen from threadfin bream had a similar protein pattern to acid-solubilized collagen, which can be classified as type I collagen.

Table 10.2 Pepsins used for collagen extraction.

Raw materials	Pepsin	Concentration of pepsin used	Digestion time	ASC yield (%)	PSC yield (%)	References
Threadfin bream skin	Albacore tuna	10 U g^{-1} skin	12 h	22.45	74.48	35
	Skipjack				63.81	
	Tongol				71.95	
	Porcine				75.92	
Yellowfin tuna dorsal skin	ND	0.98% (w/v)	23.5 h	–	27.1	36
Bigeye snapper skin	Bigeye snapper	20 kUnits g^{-1} skin	48 h	5.31	18.7	30
Grass carp skin	Porcine	1% (w/v)	24 h	–	46.6	34
Black drum skin	ND	0.1% (w/v)	3 days	2.3	15.8	37
Sheephead seabream skin	ND	0.1% (w/v)	3 days	2.6	29.3	
Brownstripe red snapper skin	Porcine	10% (w/v)	48 h	9	4.7	27
Deep-sea redfish skin	Porcine	0.1% (w/v)	48 h	47.5	92.2	38
Sardine scale	Porcine	10% (w/w)	24 h	–	50.9	
Red sea bream scale	Porcine	10% (w/w)	24 h	–	37.5	28
Japanese sea bass scale	Porcine	10% (w/w)	24 h	–	41	
Grass carp scale	ND	1% (w/v)	48 h	–	25.64	39
Bullfrog skin	ND	0.1 g/40 g skin	8 h	–	12.6	40
Octopus *Callistopus arakawai* arm	Porcine	10% (w/v)	48 h	10.4	62.9	31
Cuttlefish outer skin	Porcine	10% (w/v)	48 h	2	35	33
Paper nautilus outer skin	Porcine	10% (w/v)	2 days	5.2	50	32
Rhizostomous jellyfish mesogloea	Porcine	10% (w/v)	48 h	–	35.2	41

However, degradation of α and β components took place when pepsin from skipjack tuna was used (see Fig. 10.3). Albacore pepsin showed comparable extraction efficiency to porcine pepsin and had no adverse effect on the integrity of resulting collagen. Thus, albacore pepsin could be used for collagen extraction from threadfin bream skin.[35]

10.2.1.6 *Protein hydrolyzate*

Fish protein hydrolyzate can be prepared with the addition of protease to increase the endogenous hydrolytic activity and shorten the hydrolysis time. Enzymatic hydrolysis possesses a greater advantage when compared with chemical hydrolysis or autolysis caused by the endogenous enzymes in fish. Hydrolyzates can be obtained by maximization of endogenous

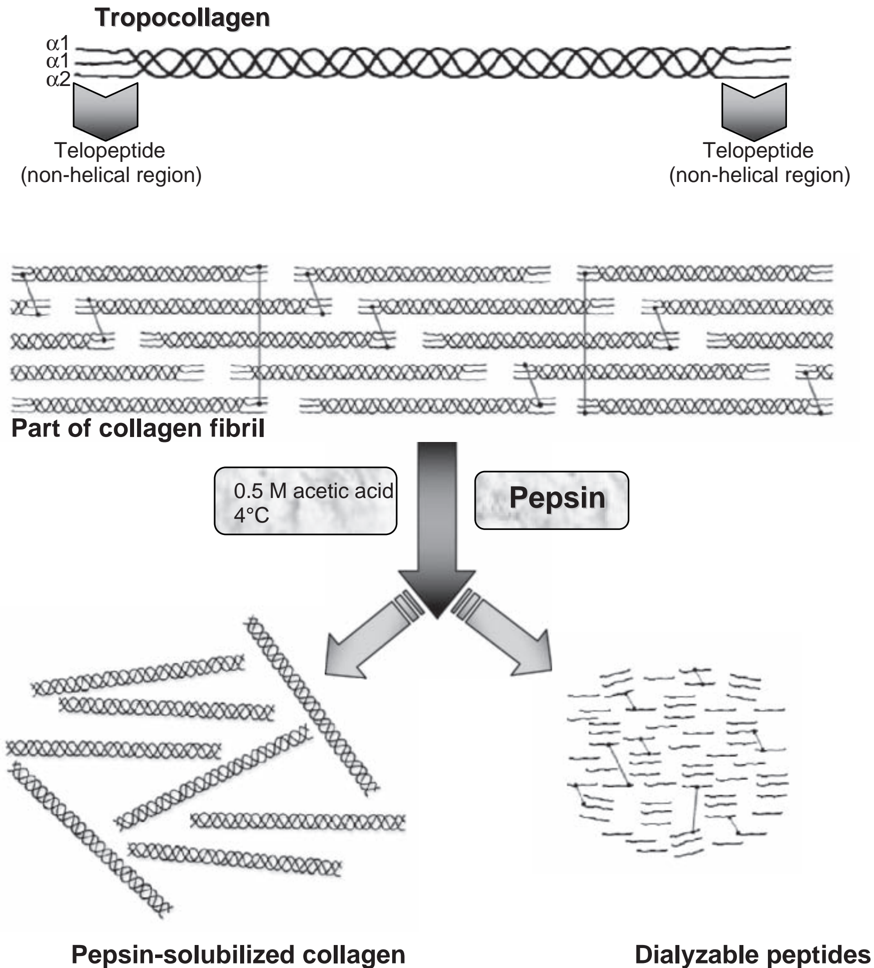

Fig. 10.2 Role of pepsin in the cleavage of telopeptide of collagen fibrils.

proteases, but the process takes an extended time. Fish protein hydrolyzate from Pacific hake containing high endogenous proteolytic activity from *Kudoa paniformis* parasitic infection could be prepared by autolysis at 52°C at pH 5.5 for 1 h.[42] To expedite the hydrolysis process, commercial proteases as well as other proteases have been used widely to hydrolyze proteins, particularly those obtained as by-products or from underutilized species. Some commercial proteases used for the production of fish protein hydrolyzate include Alcalase, Neutrase, Flavourzyme, Protamex, pepsin, chymotrypsin, trypsin, papain, Pronase E, collagenase, etc.[43–51] (see Table 10.3).

In general, different proteases exhibited varying characteristics, including optimal pH and temperatures. Therefore, to maximize hydrolysis, it should be conducted under the appropriate conditions. Additionally, the degree of hydrolysis (DH) can be manoeuvred to obtain a hydrolyzate with desirable properties. Once the required DH is obtained for the hydrolysis, inactivation of protease is required. Otherwise, the DH cannot be controlled and subsequent undesirable effects, particularly the formation of bitterness or the loss in

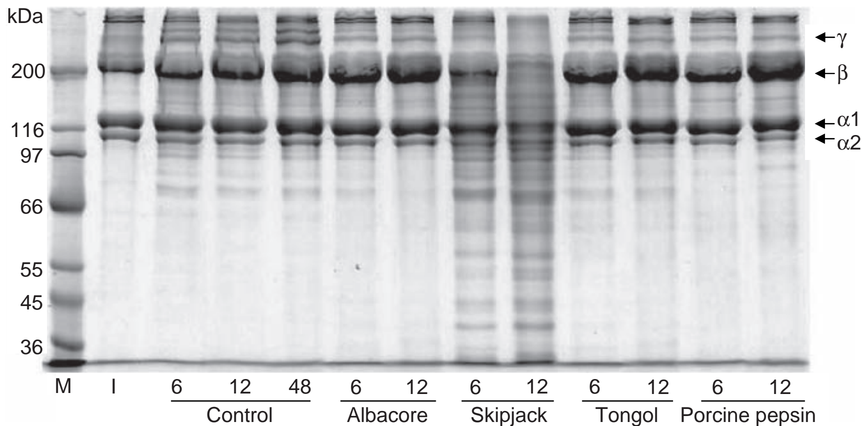

Fig. 10.3 SDS–PAGE patterns of collagen extracted from threadfin bream skin with the aid of pepsin from the stomach of different tuna species and porcine pepsin (10 U g^{-1} defatted skin) for different extracting times. Numbers denote extraction time (h). M and I denote MW protein markers and collagen type I, respectively. (Reprinted from Ref. [35].)

functional properties, can occur. pH adjustment or heat treatments are common practices to terminate the residual proteolytic activity. A flowchart for the production of fish protein hydrolyzate is illustrated in Fig. 10.4.

DH of fish protein is governed by many factors involving the fish species, defatting process, enzymes used, enzyme/substrate ratio, etc. Rapid hydrolysis of trevally mince with and without defatting was observed within the first 3 min in the presence of Alcalase or Flavourzyme, followed by a slower rate of hydrolysis of up to 20 min (see Fig. 10.5).[49] However, the defatted mince was less susceptible to hydrolysis by both enzymes. Proteins which were denatured by the defatting process could not be hydrolyzed effectively by added enzymes. Denatured protein possessed poor wettability, thereby reducing the dispersibility and accessibility of enzyme to the substrate.[69] Moreover, high temperature during the defatting process could inactivate the endogenous enzyme in fish muscle. Typical hydrolysis curves similar to those shown in Fig. 10.5 have been reported for the enzymatic hydrolysis of sardine,[53] capelin,[56] Pacific whiting solid waste,[43] herring and herring by-product,[55,57] salmon.[58]

DH is an important factor determining the characteristics, functional properties and bioactivity of protein hydrolyzates.[47,49] The solubility generally increases as the DH increases. The smaller peptides have more polar residues, which are able to form hydrogen bonds with water and thus increase solubility.[49,58] Protein hydrolyzates are surface active materials and promote oil-in-water emulsion due to their hydrophilic and hydrophobic groups.[58] Exceptional emulsifying activity and stability of protein hydrolyzates are obtained with limited DH.[70] Peptides with a long chain length or more hydrophobic peptides are able to stabilize emulsions.[47,71] On the other hand, excessive hydrolysis produces peptides with shorter chain length and higher hydrophobicity, which exhibit poorer emulsifying properties.[49–70] Small peptides migrate rapidly and adsorb at the interface, but exhibit less efficacy in decreasing the interface tension because they cannot unfold and reorient at the interface to stabilize the emulsion.[49] For foaming ability, excessive hydrolysis results in lower foaming ability. Smaller peptides are not capable of maintaining the foam.[56] High molecular weight peptides mainly contribute to foam stability of protein hydrolyzates. Hydrophobicity of unfolded

Table 10.3 Proteases used for production of fish protein hydrolyzate.

Protease	Raw materials	Bioactivity/functional properties/applications	References
Alcalase	Yellowfin sole (*Limanda aspera*) frame protein	Antioxidative properties	52
		Emulsifying properties	53
	Sardine (*Sardina pilchardus*) meat	Microbial culture	54
	Yellowfin tuna (*Thunnus albacares*) stomach	Emulsifying and foaming properties	55
	Herring (*Clupea harengus*)	Functional properties, antioxidant, yield improvement	56
			57
	Herring (*Clupea harengus*) by-products		
	Capelin (*Mallotus villosus*)	Functional properties and antioxidative properties	43
			58
	Pacific whiting (*Merluccisu productus*) wastes	Functional properties	59
	Salmon by-products	Functional properties	60
	Atlantic cod viscera	Solubility	61
	Sardinnella (*Sardinella aurita*) head and viscer	Functional properties and antioxidative properties	49,50
			47,48
	Pacific whiting (*Merluccius productus*) muscle	Functional properties	62
	Yellow stripe trevally (*Selaroides leptolepis*) muscle	Functional properties and antioxidative properties	63
			64
	Round scad (*Decapteris maruadsi*) muscle	Functional properties and antioxidative properties	46
			44
Flavourzyme	Silver carp (*Hypophthalmichthys molitrix*) protein	Solubility and antioxidative properties	49,50
		Antioxidative properties	47,48
	Tilapia protein isolate	Antioxidative properties	62
	Tuna backbone protein	Antioxidative properties	63
Neutrase	Hoki frame protein	ACE inhibitory activity	43
	Alaska pollack (*Theragra chalcogramma*) skin gelatin	Functional properties and antioxidative properties	56
			59
	Yellow stripe trevally (*Selaroides leptolepis*) muscle	Functional properties and antioxidative properties	63
			64
	Round scad (*Decapteris maruadsi*) muscle	Solubility and antioxidative properties	46
Trypsin	Silver carp (*Hypophthalmichthys molitrix*) protein	Antioxidative properties	64
		Functional properties	46
α-Chymotrypsin	Tilapia protein isolate	Functional properties, antioxidant, yield improvement	52,65
	Pacific whiting wastes		64
	Capelin (*Mallotus villosus*)	Solubility	46
Pepsin	Atlantic cod viscera	Antioxidative properties	52

Table 10.3 (*Continued*)

Protease	**Raw materials**	**Bioactivity/functional properties/applications**	**References**
	Tilapia protein isolate	Antioxidative properties	45
	Tuna backbone protein	Antioxidative properties	64
	Hoki frame protein	Antioxidative properties	52
Papain	Tuna backbone protein	Antioxidative properties	52
	Hoki frame protein	Antioxidative properties/ ACE inhibitory activity	56
			64
	Yellowfin sole (*Limanda aspera*) frame protein	Antioxidative properties	46
Pronase E	Tuna backbone protein	Antioxidative properties	52
	Hoki frame protein	Antioxidative properties	44
Protease A Amano	Yellowfin sole (*Limanda aspera*) frame protein	ACE inhibitory activity	63
Protease N	Alaska pollack (*Theragra chalcogramma*) frame protein	Antioxidative properties	66
		Antioxidative properties	63
		Antioxidative properties	67
Protease N Amano	Tuna backbone protein	Functional properties, antioxidant, yield improvement	66
Protease XXIII from *Aspergillus oryzae*	Yellowfin sole (*Limanda aspera*) frame protein	Antioxidative properties	
	Yellowfin sole (*Limanda aspera*) frame protein	Antioxidative properties	59
		Antioxidative properties	51
Protease SM98011 from *Bacillus* sp.	Capelin (*Mallotus villosus*)	ACE inhibitory activity	63
	Tuna backbone protein	Antioxidative properties	68
	Hoki frame protein	Antioxidative properties	52
Protamex	Yellowfin sole (*Limanda aspera*) frame protein	Antioxidative properties	
		Antioxidative properties	44
Cryotin F	Alaska pollack (*Theragra chalcogramma*) skin gelatin	ACE inhibitory activity	
Mackeral intestine crude enzyme (MICE)	Tilapia protein isolate		
	Mackerel (*Scomber austriasicus*) meat	Solubility	
Collagenase	Tilapia protein isolate	ACE inhibitory activity	
	Tuna cooking juice	Antioxidative properties	
		Antioxidative properties	
	Shark meat	Antioxidative properties	
	Atlantic cod viscera	ACE inhibitory activity	
	Pacific hake (*Merluccius productus*) fillet		
	Tilapia protein isolate		
	Alaska pollack frame protein		
	Yellowfin sole (*Limanda aspera*) frame protein		
	Alaska pollack (*Theragra chalcogramma*) skin gelatin		

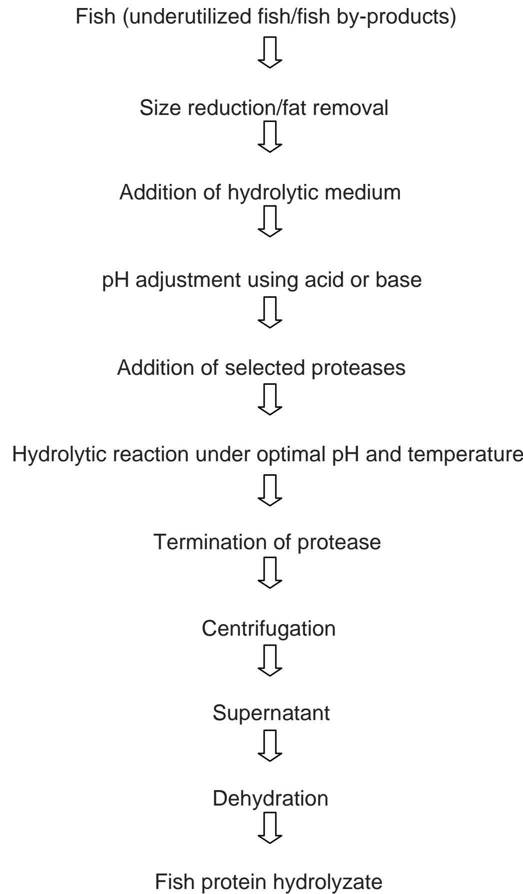

Fig. 10.4 Flowchart for fish protein hydrolyzate by enzymatic process.

proteins is also associated with foaming characteristics.[49] Foam stability depends on the nature of film and reflects the extent of protein–protein interaction within the matrix.[49,71]

Fish protein hydrolyzates may possess a wide range of bioactivities including antioxidative activity and angiotensin converting enzyme (ACE) inhibitory activity.[48,50,72] Additionally, neuroactive peptides and immunoactive peptides have been found in fish protein hydrolyzate.[73,74] Bioactive peptides can be released from the parent proteins by proteolytic enzymes during gastrointestinal digestion or during food processing.[75] Antioxidative peptides can serve as a natural antioxidant to prevent free radical-mediated modification of DNA, proteins, lipids as well small cellular molecules which are associated with a number of pathological processes. These include atherosclerosis, arthritis, diabetes, inflammatory disorders and neurological disorder, etc.[52] ACE inhibitory peptides have a potential as antihypertensive components.[72] In general, ACE plays an important role in the regulation of blood pressure. It is a dipeptidyl-carboxypeptidase which converts the inactive decapeptide, angiotensin I, into a potent vasoconstrictor, the octapeptide angiotensin II.[76]

Bioactivities of peptides generally depend on the source of the parent protein and the sequence of hydrolysis when different enzymes are used. Je *et al.*[64] studied the effect of different types of enzymes (Alcalase, α-chymotrypsin, Neutrase, papain, pepsin and trypsin)

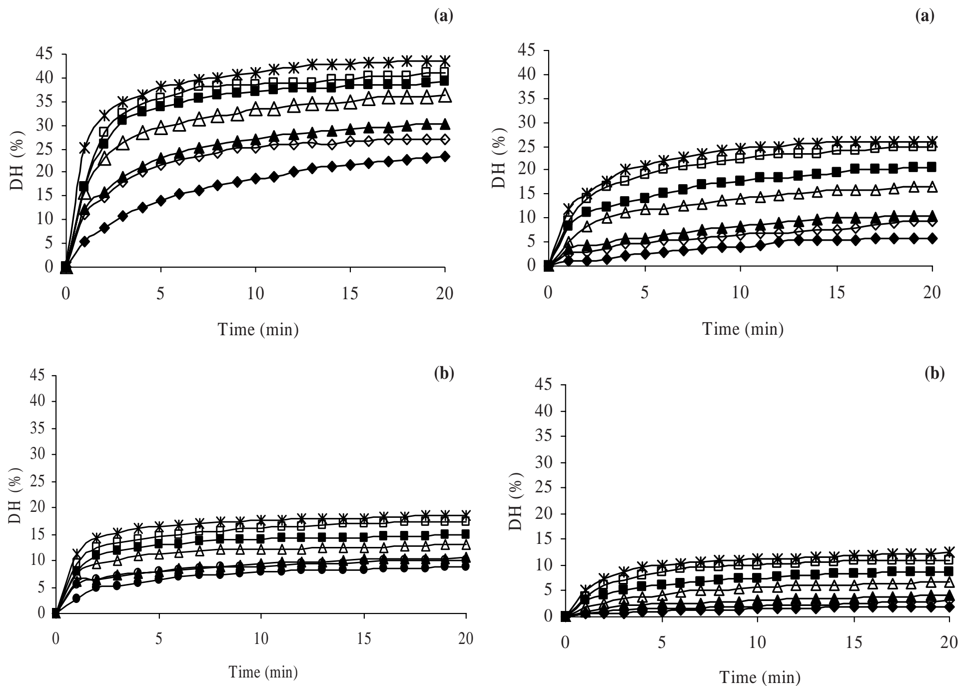

Fig. 10.5 Degree of hydrolysis (DH) of yellow stripe trevally mince (a) and defatted mince (b) during hydrolysis with Alcalase or Flavourzyme at different concentrations (0.25% (♦), 0.5% (◊), 1% (▲), 2.5% (△), 5% (■), 7.5% (□), 10% (*)). (Reprinted from Ref. [49], Copyright (2007), with permission from Elsevier.)

on the antioxidative activity of tuna backbone hydrolyzate. Among all hydrolyzates obtained, peptic hydrolyzate showed the highest antioxidative activity. Klompong *et al.*[50] found that protein hydrolyzate from yellow stripe trevally, prepared using Flavourzyme, had a higher antioxidative activity, compared with a hydrolyzate prepared using Alcalase. At the same DH, protein hydrolyzate from round scad muscle prepared using Flavourzyme exhibited greater 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity and reducing power but a lower Fe^{2+} chelating ability than hydrolyzate produced using Alcalase.[48] ACE inhibitory activity of hydrolyzate from marine protein materials was also governed by the protein source and types of proteases. Hydrolyzate prepared using Protamex and SM98011 protease from *Bacillus* sp. had a lower IC_{50}, when compared with hydrolyzate prepared from other enzymes used.[77] Bioactive peptides have been isolated and characterized as shown in Table 10.4.

10.3 TRANSGLUTAMINASE (TGASE)

10.3.1 Endogenous TGase

TGase, transferase or protein glutamine γ-glutamyltransferase (EC 2.3.1.13), is a catalyst for the acyl transfer reaction between γ-carboxyamide groups of glutamine residues in proteins or peptides and various primary amines.[78] When the ε-amino group of lysine acts as an acyl acceptor, it results in polymerization and inter- or intra-molecular cross-linking of protein

Table 10.4 Active peptides from fish protein hydrolyzates.

Active peptides	Raw materials	Enzyme	Active peptides	IC50	References
Antioxidative	Tuna backbone protein	Pepsin	Val-Lys-Ala-Gly-Phe-Ala-Trp-Thr-Ala-Asn-Gln-Gln-Leu-Ser	NR	64
Peptides	Hoki frame protein	Pepsin	Glu-Ser-Thr-Val-Pro-Glu-Arg-Thr-His-Pro-Asp-Phe-Asn	41.37 μM for DPPH radical	46
ACE inhibitory peptides	Alaska pollack frame protein	Mackerel intestine crude enzyme (MICE)	Leu-Pro-His-Ser-Gly-Tyr	17.77 μM for hydroxyl radical	
	Yellowfin sole frame protein	MICE-pepsin	Arg-Pro-Asp-Phe-Asp-Leu-Glu-Pro-Pro-Tyr	18.99 μM for peroxyl radical	68
	Shark meat		Cys-Phe	172.10 μM for superoxide radicals	
	Yellowfin sole frame protein	Protease SM98011 from *Bacillus* sp.	Glu-Tyr	NR	52
	Alaska pollack frame protein	α-Chymotrypsin	Phe-Glu	NR	66
	Alaska pollack skin gelatin	Pepsin	Met-Ile-Phe-Pro-Gly-Ala-Gly-Gly-Pro-Glu-Leu	1.96 μM	65
		Alcalase-Pronase E-collagenase	Phe-Gly-Ala-Ser-Thr-Arg-Gly-Ala	2.68 μM	
			Gly-Pro-Leu	1.45 μM	45
			Gly-Pro-Met	28.7 μg mL^{-1} 14.7 μM 2.6 μM 17.13 μM	44

NR: not reported.

via formation of iso-peptide, $\varepsilon - (\gamma -$ glutamyl) lysine linkages.[79] This occurs through the exchange of the ε-amino group of the lysine residue for the ammonia at the carboxyamine group of a glutamine residue in protein molecules (see Fig. 10.6). Formation of covalent cross-links between proteins is the basis of the ability of TGase to modify the physical properties of protein foods.[80] The cross-linking capability is influenced by the amino acid sequence and the charge of amino acids surrounding the susceptible glutamine residue and local secondary structures.[81] Proteolysis of non-reactive protein can convert it to a TGase substrate.[82]

Endogenous TGase in fish muscle varies with species and is most likely associated with the setting phenomenon. Setting or suwari involves network formation of fish proteins, mainly myosin, due to the cross-linking by endogenous TGase in the temperature range of 5–40°C. Fish muscle contains TGase ranging from 0.10–2.41 U g^{-1}.[83] This enzyme induces the cross-linking of muscle proteins and affects the textural properties of fish protein gels.[84–87] Benjakul *et al.*[88] confirmed the role of TGase in setting of the surimi from tropical fish

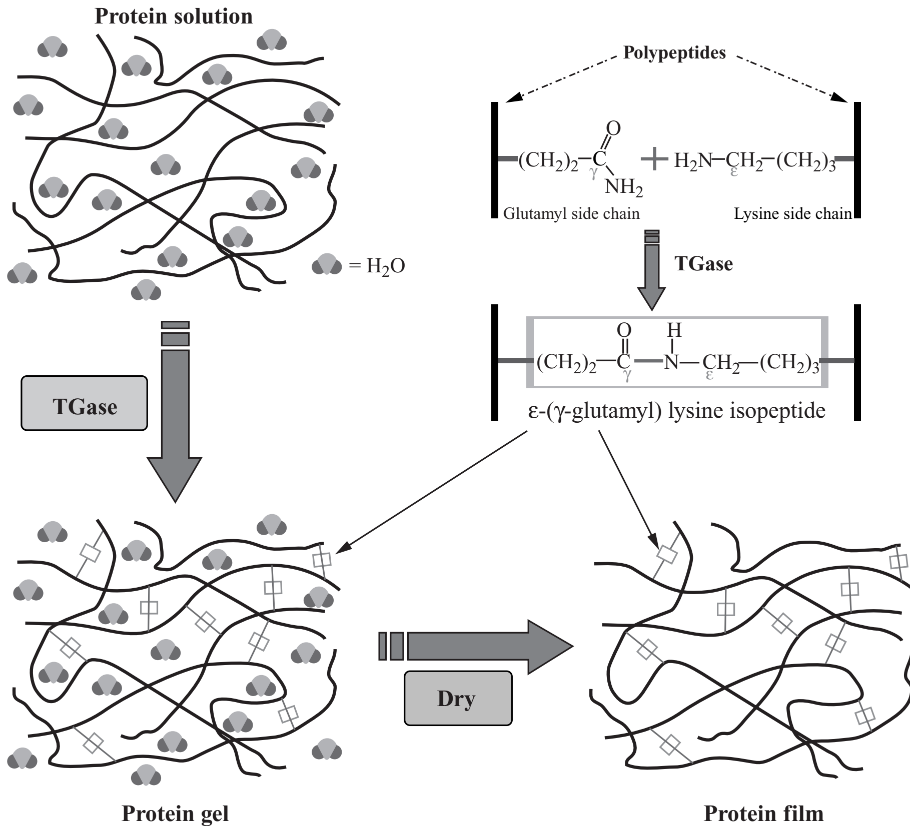

Fig. 10.6 Cross-linking of proteins in gel and film induced by transglutaminase.

including bigeye snapper, threadfin bream, barracuda and bigeye croaker. It induced the formation of non-disulphide covalent bonds, especially in the presence of sufficient calcium ion. The addition of TGase inhibitors including *N*-methylmaleimide (NEM), ammonium chloride and EDTA resulted in the decrease in setting phenomenon.[88]

Endogenous TGase has different molecular weights depending upon fish species. Additionally, the optimal pH and temperature vary with species. Total TGase activity differs depending on the temperature of measurement.[89] Optimum temperatures of TGase vary with species such as threadfin bream (55°C),[90] red sea bream (*Pagrus major*) (55°C),[91] bigeye snapper (*Priacanthus tayenus*) (40°C) and bigeye snapper (*Priacanthus macracanthus*) (25°C).[92] Worratao and Youngsawatdigul[93] reported that optimum temperature and optimum pH of tilapia TGase were 37–50°C and 7.5, respectively. The maximal TGase activity of carp meat (88.5 U g^{-1} sample) was found at 50°C, followed by threadfin bream (28.4 U g^{-1} meat) at 40°C, white croaker (19.7 U g^{-1} meat) at 30°C and red sea bream (17.1 U g^{-1} meat) at 50°C.[89] Therefore, the setting can be maximized based on the pH and temperature profile of individual fish species.

Endogenous TGase is water soluble and can be removed by washing process.[94] Youngsawatdigul *et al*.[90] reported that residual TGase activity of first washed treadfin bream mince was lower than that of mince. Therefore, some TGase is leached out during the washing

Table 10.5 Endogenous transglutaminase activities of surimi from some tropical fish.[95]

Surimi samples	TGase activity (mUnits/g)
Threadfin bream surimi	$14.69 \pm 0.01^{a}c^{b}$
Bigeye snapper surimi	15.95 ± 0.08d
Baracuda surimi	7.97 ± 0.03a
Bigeye snapper surimi	12.40 ± 0.18b

[a]Mean ± SD from triplicate determinations.
[b]Different letters in the same column denote the significant differences ($P < 0.05$).

process for surimi production. Chantarasuwan[95] found different TGase activities in surimi produced from four different tropical fish. Among these bigeye snapper surimi also showed the highest TGase activity (see Table 10.5).

TGase in fish muscle is calcium dependent.[96] Calcium ions are considered to induce the conformational change, in which the exposure of substrate to a cysteine residue in the active site can be favoured.[80] Calcium compounds are commonly added as surimi gel enhancer.[97] During the setting, myosin is denatured, aggregated and simultaneously polymerized by calcium-dependent endogenous TGase. Apart from ε-(γ-glutamyl) lysine isopeptide bonds catalyzed by Ca^{2+}-dependent endogenous TGase, hydrophobic interactions and disulphide linkages could be involved during setting of fish protein.[98] Benjakul and Visessanguan[92] found that the addition of $CaCl_2$ in the sol of surimi from both species of bigeye snapper (*P. tayenus and P. macracanthus*) prior to setting either at 25 or 40°C, followed by heating at 90°C, resulted in an increase in both breaking force and deformation of surimi from both species. The increase was more pronounced with increasing calcium chloride levels (0–100 mmol kg^{-1}). TGase activity from bigeye snapper muscle increased as [$CaCl_2$] increased.[99] Youngsawatdigul *et al.*[90] reported that breaking force of threadfin bream surimi gel, set at 40°C was highest when 0.2% Ca^{2+} was added. Lee and Park[97] found that addition of 0.2% calcium compounds improved shear stress of Pacific whiting surimi whereas lower concentrations (0.05–0.1%) effectively increased the gel texture of Alaska pollack. Optimum $CaCl_2$ concentration required for surimi gel improvement varied with fish species and it was dependent on the initial level of Ca^{2+} in muscle.[97]

10.3.2 Microbial transglutaminase (MTGase)

TGase can be produced by some microorganisms such as *Streptoverticillium mobaraense*,[100] other *Streptoverticillium sp*,[101] *Bacillus subtilis*[102] and *Streptoverticillium ladakanum*.[103] Due to the ease of production and purification, commercialization of MTGase has increased continuously since the market introduction.[104] The isoelectric point of MTGase was approximately 8.9. The molecular weight of MTGase was determined to be 38 000–40 000 D.[104] MTGase has an optimal pH between pH 5 and 8, but it still shows some activity at pH 4 and 9.[78] MTGase is thus considered to be stable over a wide pH range. The optimum temperature for enzymatic activity is 50°C, and MTGase fully retains its activity even after 10 min at 50°C.[72] MTGase from *S. mobaraense* is totally Ca^{2+} independent, while fish endogenous TGase requires Ca^{2+} ion for its activation. MTGase from *S. mobaraense* cross-links myosin heavy chain more rapidly than TGase from carp muscle. Connective tissue appears to be an excellent substrate for MTGase but not for TGase from carp muscle.[105]

10.3.2.1 *Use of MTGase in surimi and fish mince gels*

MTGase is generally mixed with fish paste or surimi sol prior to setting. Jiang *et al.*[106] found that the optimum amounts of MTGase and setting conditions of surimi gel from threadfin bream were 0.3 U g^{-1} surimi either at 30°C for 90 min or at 45°C for 20 min for threadfin bream. For pollack surimi the optimal amount of MTGase is 0.2 U g^{-1} surimi at 30°C for 1 h. Maximal shear stress of surimi gel from striped mullet was obtained when MTGase at 9.3 g kg^{-1} was added and setting was conducted at 37°C for 3.9 h, while the maximal shear strain was gained when MTGase at 5 g kg^{-1} was employed together with setting at 34.5°C for 1 h.[107] In general, inter- and/or intra-molecular cross-links were formed in myosin heavy chain of MTGase containing surimi. Myosin heavy chain is the major myofibrillar protein found in fish muscle and mainly involves in gelation process. Additionally, Thammatinna *et al.*[108] observed that setting at 25°C for 2 h could induce both endogenous and MTGase cross linking activity in Pacific white shrimp gel as evidenced by the increase in breaking force. Setting at 40°C showed lower gel properties, compared with setting at 25°C. The unfolding of muscle protein molecules at higher temperature might favour the aggregation via hydrophobic interaction. As a consequence, the reaction group for cross-linking induced by MTGase could be embedded. Therefore, glutamine or lysine residues were possibly masked and cross-linking mediated by MTGase was impeded.[108] To increase the efficacy of MTGase in gel strengthening, many approaches have been reported. Pressurization improved the mechanical properties of gel made from paste of arrowtooth flounder treated with MTGase and set at 25°C.[109] High pressure processing modified protein structure and allowed MTGase to induce the cross-linking, thus improving texture attributes.

To enhance the gel strength of surimi added with MTGase, a number of ingredients involving protein additives, protease inhibitors as well as hydrocolloids have been used in conjunction. However, some ingredients show a negative effect as evidenced by the lowered gel strength once they are incorporated. Moreno *et al.*[110] reported that MTGase (10 g kg^{-1}) in combination with sodium caseinate (15 g kg^{-1}) in minced hake could increase the mechanical properties of restructured fish muscle processed at low temperature. The use of whey protein concentrate and sodium caseinate in combination of MTGase could improve the mechanical properties of restructure fish products from the filleting waste of silver carp.[111] When chitosan was used in combination with MTGase in the gel of horse mackerel, no synergistic effect was noticeable. However, MTGase addition increased the hardness of the resulting gel regardless of chitosan addition.[112] When low methoxylated pectin was used in conjunction with MTGase in the gel of Mexican flounder mince, the resulting gel had decreased mechanical properties.[113] The disruption effect might be associated with interferences in forming the three-dimensional structures of the gel. The addition of MTGase caused the cross-linking of myosin heavy chain (MHC) and substantially increased the gel strength (from 536.6 to 2012.4 g $\times$ cm). The recombinant cystatin could effectively prevent the MHC degradation and gel softening during the production of mackerel surimi-based products.[114] Combined use of MTGase and recombinant cystatin revealed synergistic effectiveness on improving the quality of mackerel surimi (increase from 435 to 2438 g $\times$ cm). Jiang *et al.*[106] found that the combination of MTGase, reducing agent and proteinase inhibitor seemed to be a better way to improve gel-forming ability of hairtail surimi. The texture degradation caused by endogenous proteinase could be prevented by the inhibitors. The best condition for the improvement of gel forming ability was the combination of 0.35 U MTGase g^{-1}, 0.1%$NaHSO_4$ and 0.01 mM E-64 (trans-epoxysuccinyl-L-leucyl-amido (4-guanidino) butane), a specific cysteine proteinase inhibitor.

MTGase has been used widely in restructured fish products to reduce the salt required due to its ability in protein cross-linking. Usually a decrease in salt level to below 2% has a negative effect on the functional and mechanical properties of meat product. Sufficient amount of salt is required for protein solubilization. Salt at 1.5% is considered to be the minimum level needed for gel development.[115] Tellez-Luis *et al.*[116] found that low salt restructured silver carp products with improved mechanical and functional properties can be successfully prepared using MTGase (3 g kg^{-1}) and 1% NaCl. Additionally, MTGase at 3 g kg^{-1} could be used to improve the mechanical properties of restructured product from a mixture of striped mullet and Mexican flounder (1:1) at both levels of salt (1% and 2%).[117]

MTGase has been proven as a potential gel-enhancing agent, which improves gel strength of surimi produced from low quality lizardfish. Low quality is usually the result of long iced storage time. During handling and storage, fish muscle proteins undergo both denaturation and degradation, leading to poor gel formation.[118] Some fish species with formaldehyde forming ability such as lizardfish are susceptible to protein denaturation. As a consequence, gel formation is generally decreased with increasing storage or handling time. Benjakul *et al.*[119] found that MTGase was a powerful gel enhancer of mince from lizardfish stored in ice for different times. However, the gel enhancing ability was greater in surimi produced from fresh lizardfish (see Fig. 10.7).

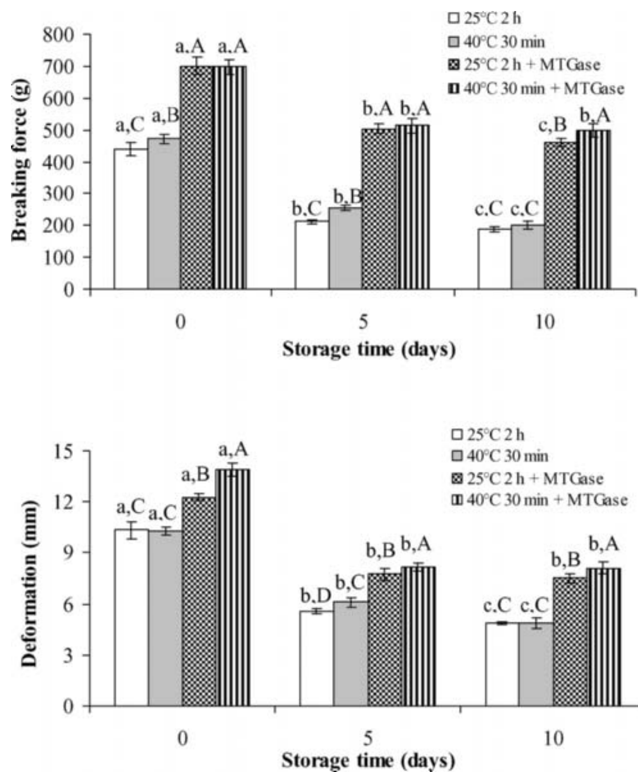

Fig. 10.7 Breaking force and deformation of mince gel from lizardfish stored in ice for different times without and with MTGase addition (0.6 U g^{-1} sample) and set under different conditions. Different letters within the same treatment or different capital letters within the same storage time indicate significant differences ($P < 0.05$). Bars represent standard deviation from five determinations. (Reprinted from Ref. [119].)

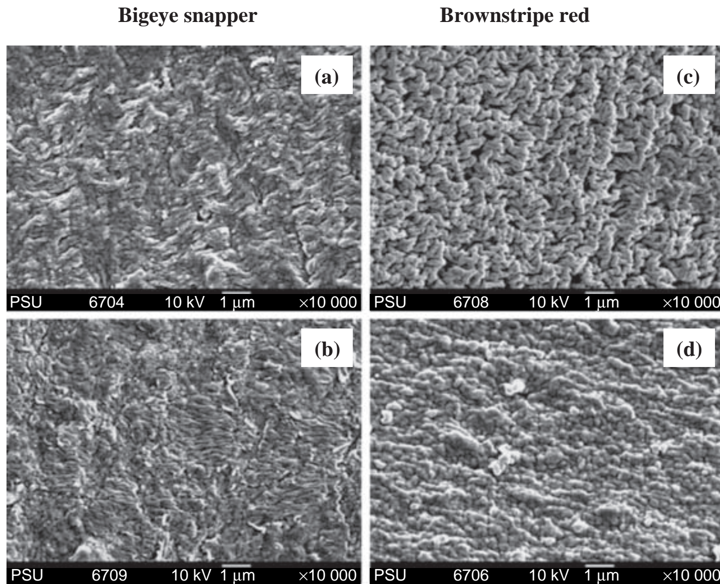

Fig. 10.8 Microstructure of gelatin (magnification: 10 000×). (a) and (b) Gelatin gels of bigeye snapper skin without and with 0.005% (w/v) MTGase, respectively. (c) and (d) Gelatin gels of brownstripe red snapper skin without and with 0.01% (w/v) MTGase, respectively.[120] (Reprinted from Ref. [49], Copyright (2007), with permission from Elsevier.)

10.3.2.2 *Use of MTGase in gelatin gels and films*

Fish gelatin has become increasingly interesting as an alternative for gelatin from land animal sources such as bovine and porcine skin and bone. This is associated with the outbreak of bovine spongiform encephalopathy (BSE) and the foot and mouth disease (FMD) crisis, as well as with aesthetic and religious objections.[120] Gelatin can be extracted from various marine sources. However, collagen and gelatin from fish, especially from cold water fish, have low gelling temperatures and melting points.[121] This limits the use of fish gelatin since it cannot form a gel at room temperature.[122] Also, the bloom strength is generally lower than its mammalian counterpart. Therefore, MTGase have been used to improve the bloom strength of gelatin gel from fish origin via cross-linking of gelatin molecules in the gel matrix. To maximize the bloom strength of gelatin, the appropriate amount of MTGase should be considered. If the enzyme concentration is too low, gel formation cannot occur. The addition of MTGase at levels up to 0.005% and 0.01% increased the bloom strength of gelatin gel from bigeye snapper and brownstripe red snapper skin, respectively. Nevertheless, the bloom strength of skin gelatin gel from both fish species decreased with further increase in MTGase levels. With addition of MTGase, denser aggregates with negligible voids in gel network were noticeable, most likely due to the formation of non-disulphide covalent bonds between adjacent molecules (see Fig. 10.8).

Cross-linking induced by MTGase is also governed by gelatin concentration. Sufficient gelatin concentration renders the better gel than do lower concentrations.[123] MTGase could induce cross-linking of fish gelatin at 4–5°C. MTGase cross-linked gelatin from Baltic cod did not melt in boiling water after 30 min of heating.[123] MTGase showed a higher gel enhancing effect than $MgSO_4$ for gelatin from hake and cod skin.[124] Optimal level for gel

improvement was depending on the source of gelatin and this is most likely associated with different intrinsic properties of protein molecules. The addition of MTGase to gelatin from megrim skin raised the melting point, gel strength and viscosity at 60°C to different degrees, depending on the enzyme concentration and the incubation time. Additionally, thermal inactivation of enzyme had no negative effect on the properties of gelatin.[125]

Biodegradable materials including edible film from biopolymers have gained increasing attention. Gelatin is a possible material for film formation but so far gelatin from skins of cold adapted fish has not been applied for biodegradable food packages. Cod skin gelatin is completely soluble in water and it is not suitable for making film for coating and packaging.[126] Yi *et al*.[127] reported that the use of MTGase could increase cross-linking, tensile strength and melting temperature of gelatin-based film. MTGase reaction did not have any impact on the water vapour permeability of resulting film. To improve the mechanical properties and to reduce the solubility of gelatin-based film, chitosan is blended and MTGase is used as the cross-linker.[126] Fish gelatin from gelatin-chitosan films was almost completely hydrolyzed by trypsin, regardless of MTGase incorporation.[128] Therefore, MTGase can be applied as the potential cross-linker of fish protein, in which the desirable characteristics or properties can be obtained.

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REFERENCES

1. Garcia-Carreno, F.C. and Hernandez-Cortes, P. (2000) Use of protease inhibitors in seafood products. In: *Seafood Enzymes: Utilization and Influence on Postharvest Seafood Quality* (eds N.F. Haard and B.K. Simpson). Marcel Dekker, New York, pp. 531–540.
2. Nissen, J.A. (1993) Proteases. In: *Enzymes in Food Processing* (eds T. Nagodawithana and G. Reed). Academic Press, Inc., New York, pp. 159–203.
3. Simpson, B.K. (2000) Digestive proteinases from marine animals. In: *Seafood Enzymes: Utilization and Influence on Postharvest Seafood Quality* (eds N.F. Haard and B.K. Simpson). Marcel Dekker, New York, pp. 531–540.
4. Haard, N.F. (1990) Enzymes from myosystems. *Journal of Muscle Foods* **1**, 293–338.
5. Wasserman, B.P. (1990) Evolution of enzymes technology: progress and prospects. *Food Technology* **44**(4), 118.
6. Simpson, B.K. and Haard, H.F. (1987) Cold-adapted enzymes from fish. In: *Food Biotechnology* (ed. D. Knorr). Marcel Dekker, New York, pp. 495–528.
7. Chen, H.M. and Meyers, S.P. (1982) Extraction of astaxanthin pigment from crawfish waste using a soy oil process. *Journal of Food Science* **47**, 892–896, 900.
8. Simpson, B.K. and Haard, H.F. (1985) The use of proteolytic enzymes to extract carotenoproteins from shrimp wastes. *Journal of Applied Biochemistry* **7**, 212–222.
9. Manu-Tawiah, W. and Haard, N.F. (1987) Recovery of carotenoprotein from the exoskeleton of snow crab, *Chionoecetes opilio*. *Canadian Institute of Food Science and Technology* **20**, 31–35.
10. Cano-Lopez, A., Simpson, B.K. and Haard, N.F. (1987) Extraction of carotenoprotein from shrimp processing wastes with the aid of trypsin from Atlantic cod. *Journal of Food Science* **52**, 503–506.
11. Ya, T., Simpson, B.K., Ramaswamy, H., Yaylayan, V., Smith, J.P. and Hudon, C. (1991) Carotenoproteins from lobster waste as a potential feed supplement for cultured salmonids. *Food Biotechnology* **5**, 87–93.

12. Klomklao, S., Benjakul, S., Visessanguan, W., Kishimura, H. and Simpson, B.K. (2009) Extraction of carotenoprotein from black tiger shrimp shell with the aid of bluefish trypsin. *Journal of Food Biochemistry* **33**, 201–217.
13. Chakrabarti, R. (2002) Carotenoprotein from tropical brown shrimp shell waste by enzymatic process. *Food Biotechnology* **16**, 81–90.
14. Long, A. and Haard, N.F. (1988) The effect of carotenoid protein association on pigmentation and growth rates of rainbow trout, *Salmo gairdneri*. In: *Proceedings of the Aquaculture International Congress*. Vancouver, B.C., pp. 99–101.
15. Klomklao, S., Benjakul, S., Visessanguan, W., Kishimura, H. and Simpson, B.K. (2006) Effects of the addition of spleen of skipjack tuna (*Katsuwonus pelamis*) on the liquefaction and characteristics of fish sauce made from sardine (*Sardinella gibbosa*). *Food Chemistry* **98**, 440–452.
16. Gildberg, A. (1993) Enzymic processing of marine raw materials. *Process Biochemistry* **28**, 1–15.
17. Chaveesuk, R., Smith, J.P. and Simpson, B.K. (1993) Production of fish sauce and acceleration of sauce fermentation using proteolytic enzymes. *Journal of Aquatic Food Product Technology* **2**(3), 59–77.
18. Gildberg, A. (2001) Utilization of male Arctic capelin and Atlantic cod intestines for fish sauce production-evaluation of fermentation conditions. *Bioresource Technology* **76**, 119–123.
19. Pan, B.S. (1990) Recovery of shrimp waste for flavourant. In: *Advance in Fisheries Technology and Biotechnology for Increased Profitability* (eds M.N. Voigt and J.R. Botta). Technomic Publishing Co., Inc., Lancaster, PA, pp. 437–452.
20. Haard, N.F. (1992) A review of protolytic enzymes from marine organisms and their application in the food industry. *Journal of Aquatic Food Product Technology* **1**(1), 17–35.
21. Vihelmsson, O. (1997) The isolate of enzyme biotechnology in the fish processing industry. *Trends in Food Science and Technology* **8**, 266–270.
22. Stefansson, G. and Steingrimsdottir, U. (1990) Application of enzymes for fish processing in Iceland – present and future aspects. In: *Advance in Fisheries Technology and Biotechnology for Increased Profit* (eds M.N. Voigt and J.R. Botta). Technomic Publishing Co., Inc., Lancaster, PA, pp. 237–250.
23. Fehmerling, G.B. (1973) Separation of edible tissue from edible flesh of marine creatures. United States Patent US 3729324.
24. Haard, N.F. (1994) Protein hydrolysis in seafoods. In: *Seafood Chemistry Processing Techonology and Quality* (eds F. Shahidi and J.R. Botta). Chapman & Hall, New York, pp. 10–33.
25. Raa, J. (1990) Biotechnology in aquaculture and the fish processing industry: a success story in Norway. In: *Advance in Fisheries Technology and Biotechnology for Increased Profit* (eds M.N. Voigt and J.R. Botta). Technomic Publishing Co., Inc., Lancaster, PA, pp. 509–524.
26. Jongjareonrak, A., Benjakul, S., Visessanguan, W. and Tanaka, M. (2005a) Isolation and characterization of collagen from bigeye snapper (*Priacanthus macracanthus*) skin. *Journal of the Science of Food and Agriculture* **85**, 1203–1210.
27. Jongjareonrak, A., Benjakul, S., Visessanguan, W., Nagai, T. and Tanaka, M. (2005b) Isolation and characterisation of acid and pepsin-solubilised collagens from the skin of Brownstripe red snapper (*Lutjanus vitta*). *Food Chemistry* **93**, 475–484.
28. Nagai, T., Izumi, M. and Ishii, M. (2004) Fish scale collagen. Preparation and partial characterization. *International Journal of Food Science and Technology* **39**, 239–244.
29. Mizuta, S., Hwang, J.H. and Yoshinaka, R. (2003) Molecular species of collagen in pectoral fin cartilage of skate (*Raja kenojei*). *Food Chemistry* **80**, 1–7.
30. Nalinanon, S., Benjakul, S., Visessanguan, W. and Kishimura, H. (2007) Use of pepsin for collagen extraction from the skin of bigeye snapper (*Priacanthus tayenus*). *Food Chemistry* **104**, 593–601.
31. Nagai, T., Nagamori, K., Yamashita, E. and Suzuki, N. (2002) Collagen of octopus *Callistoctopus arakawai* arm. *International Journal of Food Science and Technology* **37**, 285–289.
32. Nagai, T. and Suzuki, N. (2002) Preparation and partial characterization of collagen from paper nautilus (*Argonauta argo*, Linnaeus) outer skin. *Food Chemistry* **76**, 149–153.
33. Nagai, T., Yamashita, E., Taniguchi, K., Kanamori, N. and Suzuki, N. (2001) Isolation and characterisation of collagen from the outer skin waste material of cuttlefish (*Sepia lycidas*). *Food Chemistry* **72**, 425–429.
34. Zhang, Y., Liu, W., Li, G., Shi, B., Miao, Y. and Wu, X. (2007) Isolation and partial characterization of pepsin-soluble collagen from the skin of grass carp (*Ctenopharyngodon idella*). *Food Chemistry* **103**, 906–912.

35. Nalinanon, S., Benjakul, S., Visessanguan, W. and Kishimura, H. (2008) Tuna pepsin: characteristics and its use for collagen extraction from the skin of threadfin bream (*Nemipterus spp.*). *Journal of Food Science* **73**, 413–419.
36. Woo, J.W., Yu, S.J., Cho, S.M., Lee, Y.B. and Kim, S.B. (2008) Extraction optimization and properties of collagen from yellowfin tuna (*Thunnus albacares*) dorsal skin. *Food Hydrocolloids* **22**, 879–887.
37. Ogawa, M., Moody, M.W., Portier, R.J., Bell, J., Schexnayder, M.A. and Losso, J.N. (2003) Biochemical properties of black drum and sheepshead seabream skin collagen. *Journal of Agricultural and Food Chemistry* **51**, 8088–8092.
38. Wang, L., An, X., Xin, Z., Zhao, L. and Hu, Q. (2007) Isolation and characterization of collagen from the skin of deep-sea redfish (*Sebastes mentella*). *Journal of Food Science* **72**, 450–455.
39. Li, C.M., Zhong, Z.H., Wan, Q.H., Zhao, H., Gu, H.F. and Xiong, S.B. (2006) Preparation and thermal stability of collagen from scales of grass carp (*Ctenopharyngodon idellus*). *European Food Research and Technology* **222**(3–4), 236–241.
40. Li, H., Liu, B.L., Gao, L.Z. and Chen, H.L. (2004) Studies on bullfrog skin collagen. *Food Chemistry* **84**, 65–69.
41. Nagai, T., Worawattanamateekul, W., Suzuki, N., Nakamura, T., Ito, T., Fujiki, K., Nakao, M. and Yano, T. (2000) Isolation and characterization of collagen from rhizostomous jellyfish (*Rhopilema asamushi*). *Food Chemistry* **70**, 205–208.
42. Samaranayaka, A.G.P. and Li-Chan, E.C.Y. (2008) Autolysis-assisted production of fish protein hydrolysates with antioxidant properties from Pacific hake (*Merluccius productus*). *Food Chemistry* **107**, 768–776.
43. Benjakul, S. and Morrissey, M.T. (1997) Protein hydrolysates from Pacific whiting solid wastes. *Journal of Agricultural and Food Chemistry* **45**, 3423–3430.
44. Byun, H.G. and Kim, S.K. (2001) Purification and characterization of angiotensin I converting enzyme (ACE) inhibitory peptides from Alaska pollack (*Theragra chalcogramma*) skin. *Process Biochemistry* **36**, 1155–1162.
45. Je, J.Y., Park, P.J., Kwon, J.Y. and Kim, S.K. (2004) A novel angiotensin I converting enzyme inhibitory peptide from Alaska pollack (*Theragra chalcogramma*) frame protein hydrolysate. *Journal of Agricultural and Food Chemistry* **52**, 7842–7845.
46. Kim, S.Y., Je, J.Y. and Kim, S.K. (2007) Purification and characterization of antioxidative peptide from hoki (*Johnius belengerii*) frame protein by gastrointestinal digestion. *Biochemistry* **18**, 31–38.
47. Thiansilakul, Y., Benjakul, S. and Shahidi, F. (2007a). Compositions, functional properties and antioxidative activity of protein hydrolysates prepared from round scad (*Decapterus maruadsi*). *Food Chemistry* **103**, 1385–1394.
48. Thiansilakul, Y., Benjakul, S. and Shahidi, F. (2007b). Antioxidative activity of protein hydorlysate from round scad muscle using Alcalase and Flavourzyme. *Journal of Food Biochemistry* **31**, 266–287.
49. Klompong, V., Benjakul, S., Kantachote, D. and Shahidi, F. (2007) Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally (*Selaroides leptolepis*) as influenced by the degree of hydrolysis and enzyme type. *Food Chemistry* **102**, 1317–1327.
50. Klompong, V., Benjakul, S., Kantachote, D. and Shahidi, F. (2008) Comparative study on antioxidative activity of yellow stripe trevally protein hydrolysate produced from Alcalase and Flavourzyme. *International Journal of Food Science and Technology* **43**, 1019–1026.
51. Cinq-Mars, C.D., Hu, C., Kitts, D.D. and Li-Chan, E.C.Y. (2008) Investigations into inhibitor type and mode, simulated gastrointestinal digestion, and cell transport of the angiotensin I-converting enzyme-inhibitory peptides in Pacific hake (*Merluccius productus*) fillet hydrolysate. *Journal of Agricultural and Food Chemistry* **56**, 410–419.
52. Jun, S.Y., Park, P.J. and Jung, W.K. (2004) Purification and characterization of an antioxidative peptide from enzymatic hydrolysate of yellowfin sole (*Limanda aspera*) frame protein. *European Food Research and Technology* **219**, 20–26.
53. Quaglia, G.B. and Orban, E. (1987) Enzymic solubilisation of proteins of sardine (*Sardina pilchardus*) by commercial proteases. *Journal of the Science of Food and Agriculture* **38**, 263–269.
54. Guerard, F., Dufosse, L., DeLaBroise, D. and Binet, A. (2001) Enzymatic hydrolysis of proteins from yellowfin tuna (*Thunnus albacares*) wastes using Alcalase. *Journal of Molecular Catalysis* **11B**, 1051–1059.
55. Liceaga-Gesualdo, A.M. and Li-Chan, E.C. (1999) Functional properties of fish protein hydrolysate from herring (*Clupea harengus*). *Journal of Food Science* **64**, 1000–1004.

56. Shahidi, F., Han, X.Q. and Synowiecki, J. (1995) Production and characteristics of protein hydrolysates from capelin (*Mallotus villosus*). *Food Chemistry* **53**, 285–293.
57. Sathivel, S., Bechtel, P.J., Babbitt, J., Smiley, S., Crapo, C., Reppond, K. and Prinyawiwatkul, W. (2003) Biochemical and functional properties of herring (*Clupea harengus*) byproduct hydrolysates. *Journal of Food Science* **68**, 2196–2200.
58. Gbogouri, G.A., Linder, M., Fanni, J. and Parmentier, M. (2004) Influence of hydrolysis degree on the functional properties of salmon byproduct hydrolysates. *Journal of Food Science* **69**, 615–622.
59. Aspmo, S.I., Horn, S.J. and Eijsink, V.G.H. (2005) Enzymatic hydrolysis of Atlantic cod (*Gadus morhua* L.) viscera. *Process Biochemistry* **40**, 1957–1966.
60. Souissi, N., Bougatef, A., Triki-Ellouz, Y. and Nasri, M. (2007) Biochemical and functional properties of sardinella (*Sardinella aurita*) by-product hydrolysates. *Food Technology and Biotechnology* **45**, 187–194.
61. Pacheco-Aguilar, R., Mazorra-Manzano, M.A. and Ramirz-Suarez, J.C. (2008) Functional properties of fish protein hydrolysates from Pacific whiting (*Merluccius productus*) muscle produced by a commercial protease. *Food Chemistry* **109**, 782–789.
62. Dong, S., Zeng, M., Wang, D., Liu, Z., Zhao, Y. and Yang, H. (2008) Antioxidant and biochemical properties of protein hydrolysates prepared from silver carp (*Hypophthalmichthys molitrix*). *Food Chemistry* **107**, 1485–1493.
63. Raghavan, S. and Kristinsson, H.G. (2008) Antioxidative efficacy of alkali-treated tilapia protein hydrolysates: a comparative study of five enzymes. *Journal of Agricultural and Food Chemistry* **56**, 1434–1441.
64. Je, J.Y., Qian, Z.J., Byun, H.G. and Kim, S.K. (2007) Purification and characterization of an antioxidative peptide obtained from tuna backbone protein by enzymatic hydrolysis. *Process Biochemistry* **42**, 840–846.
65. Jung, W.K., Mendis, E., Je, J.Y., Park, P.J., Byeng, W.S., Hyoung, C.K., Yang, K.C. and Kim, S.K. (2006) Angiotensin I-converting enzyme inhibitory peptide from yellowfin sole (*Limanda aspera*) frame protein and its antihypertensive effect in spontaneously hypertensive rats. *Food Chemistry* **94**, 26–32.
66. Wu, H.C., Chen, H.M. and Shiau, C.Y. (2003) Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (*Scomber austriasicus*). *Food Research International* **36**, 949–957.
67. Jao, C.L. and Ko, W.C. (2002) 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging by protein hydrolysates from tuna cooking juice. *Fisheries Science* **68**, 430–435.
68. Je, J.Y., Park, P.J. and Kim, S.K. (2005) Antioxidative activity of a peptide isolated from Alaska pollack (*Theragra chalcogramma*) frame protein hydrolysate. *Food Research International* **38**, 45–50.
69. Hoyle, N. and Merritt, J.H. (1994) Quality of fish protein hydrolysates from herring (*Clupea harengus*). *Journal of Food Science* **59**, 76–79.
70. Kristinsson, H.G. and Rasco, B.A. (2000) Fish protein hydrolysates: production, biochemical and functional properties. *Critical Reviews in Food Science and Nutrition* **40**, 43–81.
71. Multilangi, W.A.M., Panyam, D. and Kilara, A. (1996) Functional properties of hydrolysates from proteolysis of heat-denatured whey protein isolate. *Journal of Food Science* **61**, 270–274.
72. Vercruysse, L., vanCamp, J. and Smagghe, G. (2005). ACE inhibitory peptides derived from enzymatic hydrolysates of animal muscle protein: a review. *Journal of Agricultural and Food Chemistry* **53**, 8106–8115.
73. Gildberg, A., Bogwald, J., Johansen, A. and Stenberg, E. (1996) Isolation of acid peptide fractions from a fish protein hydrolysate with strong stimulatory effect on Atlantic salmon (*Salmon salar*) head kidney leucocytes. *Comparative Biochemistry and Physiology* **114B**, 97–101.
74. Bernet, F., Montel, V., Noel, B. and Dupouy, J.P. (2000) Diazepam-like effects of a fish protein hydrolysate (Gabolysat PC60) on stress responsiveness of the rat pituitary-adrenal system and sympathoadrenal activity. *Psychopharmacology* **149**, 34–40.
75. Meisel, H. (1997) Biochemical properties of bioactive peptides derived from milk proteins: potential nutraceuticals for food and pharmaceutical applications. *Livestock Production Science* **50**, 125–138.
76. Coates, D. (2003) The angiotensin converting enzyme (ACE). *International Journal of Biochemistry & Cell Biology* **35**, 769–773.
77. He, H.L., Chen, X.L., Wu, H., Sun, C.Y., Zhang, Y.Z. and Zhou, B.C. (2007) High throughput and rapid screening of marine protein hydrolysate enriched in peptides with angiotensin-I-converting enzyme inhibitory activity by capillary electrophoresis. *Bioresource Technology* **98**, 3499–3505.

78. Motoki, M. and Seguro, K. (1998) Transglutaminase and its used for food processing. *Trends in Food Science and Technology* **9**, 204–210.
79. de Jong, G.A.H. and Koppelman, S.J. (2002) Transglutaminase catalyzed reactions: impact on food applications. *Journal of Food Science* **67**, 2798–2806.
80. Ashie, I.N.A. and Laneir, T.C. (2000) Transglutaminase in seafood processing. In: *Seafood Enzymes Utilization and Influence on Postharvest Seafood Quality* (eds N.F. Haard and B.K. Simpson). Marcel Dekker, New York, pp. 147–166.
81. Folk, J.E. (1980) Transglutaminase. *Annual Review of Biochemistry* **17**, 517–531.
82. Greenberg, C.S., Birckichler, P.J. and Rice, R.H. (1991) Transglutaminase: multifunctional cross-linking enzymes that stabilize tissue. *FASEB Journal* **5**, 3071–3077.
83. Araki, H. and Seki, N. (1993) Comparison of reactivity of transglutaminase to various fish actomyosins. *Nippon Suisan Gakkaishi* **95**, 711–716.
84. Kimura, I., Sugimoto, M., Toyoda, K., Seki, N. and Fujita, T. (1991) A study on the cross-linking reaction of myosin in kamaboko 'suwari' gels. *Nippon Suisan Gakkaishi* **57**, 1386–1396.
85. Sakamoto, S., Kumazawa, Y. Kawajiri, H. and Motoki, M. (1995) $\varepsilon - (\gamma$-glutamyl) lysine crosslink distribution in foods as determined by improved method. *Journal of Food Science* **60**, 1412–1416.
86. Benjakul, S., Chantarasuwan, C. and Visessanguan, W. (2003) Effect of medium temperature setting on gelling characteristics of surimi from some tropical fish. *Food Chemistry* **82**, 657–574.
87. Benjakul, S., Visessanguan, W. and Chantarasuwan, C. (2004a). Effect of high-temperature setting on gelling characteristic of surimi from some tropical fish. *International Journal of Food Science and Technology* **39**, 671–680.
88. Benjakul, S., Visessanguan, W. and Pecharat, S. (2004b). Suwari gel properties as affected by transglutaminase activator and inhibitors. *Food Chemistry* **85**, 91–99.
89. Tsukamasa, Y., Miyake, Y., Ando, M. and Makinodan, Y. (2002) Total activity of transglutaminase at various temperatures in several fish meats. *Fisheries Science* **68**, 929–933.
90. Youngsawatdigul, J., Worratao, A. and Park, J.W. (2002) Effect of endogenous transglutaminase on threadfin bream surimi gelation. *Journal of Food Science* **67**, 3258–3263.
91. Yasueda, H., Kumazawa, Y. and Motoki, M. (1994) Purification and characterization of tissue-type transglutaminase from red sea bream (*Pagrus major*). *Bioscience Biotechnology Biochemistry* **58**, 2041–2045.
92. Benjakul, S. and Visessanguan, W. (2003) Transglutaminase-mediated setting in bigeye snapper surimi. *Food Research International* **36**, 253–266.
93. Worratao, A. and Youngsawatdigul, J. (2005) Purification and characterization of transglutaminase from tropical tilapia (*Oreochromis niloticus*). *Food Chemistry* **93**, 651–658.
94. Nowsad, A., Katho, E., Konoh, S. and Niwa, E. (1994) Setting of surimi paste in which transglutaminase is inactivated by ρ-chloromercuribenzoate. *Fisheries Science* **60**, 189–191.
95. Chantarasuwan, C. (2001) Role of endogenous transglutaminase in setting of surimi from some tropical fish. MSc Thesis, Prince of Songkla University, Thailand.
96. Ho, M.L., Leu, S.Z., Hsieh, J.F. and Jiang, S.T. (2000) Technical approach to simplify the purification method and characterization of microbial transglutaminase produced from *Streptoverticillium ladakanum*. *Journal of Food Science* **65**, 76–80.
97. Lee, N.G. and Park, J.W. (1998) Calcium compounds to improve gel functionality of Pacific whiting and Alaska pollack surimi. *Journal of Food Science* **63**, 969–974.
98. Hemung, B. and Yongsawatdigul, J. (2005) Ca^{2+} affects physicochemical and conformational changes of threadfin bream myosin and actin in a setting model. *Journal of Food Science* **70**, 455–460.
99. Benjakul, S., Visessanguan, W. and Chantarasuwan, C. (2004c). Cross-linking activity of sarcoplasmic fraction from bigeye snapper (*Priacanthus tayenus*) muscle. *Lebensmittel-Wissenschaft und-Technologie* **37**, 79–85.
100. Gerber, U., Jucknischke, U., Putzein, S. and Fuchsbauer, H.L. (1994) A rapid and simple method of purification of transglutaminase from *Streptovercillium mabaraense*. *Journal of Biochemistry* **299**, 825–829.
101. Ando, H., Adachi, M., Umead, K.K., Matsuura, A., Nonaka, M., Uchio, R., Tanaka, H. and Motoki, M. (1989) Purification and characteristics of novel transglutaminase derived from microorganisms. *Agricultural and Biological Chemistry* **53**, 2613–2617.
102. Ramanujam, M.V. and Hangeman, J.H. (1990) Intracellular transglutaminase (EC 2.3.2.13) in a prokaryote: evidence from vegetative and sporulating cells of *Bacillus subtilis*. *FASEB Journal* **4**, 2321–2328.

103. Tsai, G.J., Lin, S.M., and Jiang, S.T. (1996) Transglutaminase from *Streptoverticillum ladakanum* and application to minced fish product. *Journal of Food Science* **61**, 1234–1240.
104. Kanaji, T., Ozaki, H., Takao, T., Kawajiri, H., Ide, H., Motoki, M. and Shimonoshi, Y. (1993) Primary structure of microbial transglutaminase from *Streptoverticillium sp.* strain s-8112. *Journal of Biological Chemistry* **268**, 11565–11572.
105. Nakahara, C., Nozawa, H. and Seki, N. (1999) A comparison of cross-linking of fish myofibrillar proteins by endogenous and microbial transglutaminases. *Fisheries Science* **65**, 138–144.
106. Jiang, S.T., Hsieh, J.E., Ho, M.L. and Chung, Y.C. (2000) Combination effect of microbial transglutaminase, reducing agent and proteinase inhibitor on the quality of hair tail surimi. *Journal of Food Science* **65**, 241–245.
107. Ramirez, J.A., Rodriguez-Sosa, R., Morales, O.G. and Vazquez, M. (2000) Surimi gels from striped mullet (*Mugil cephalus*) employing microbial transglutaminase. *Food Chemistry* **70**, 443–449.
108. Thammatinna, A., Benjakul, S., Visessanguan, W. and Tanaka, M. (2007) Gelling properties of Pacific white shrimp (*Penaeus vannamei*) meat as influence by setting condition and microbial transglutaminase. *LWT – Food Science and Technology* **40**, 1489–1497.
109. Uresti, R.M., Velazgguez, G., Vazquez, M., Ramirez, J. and Torres, A.J. (2006) Effect of combining microbial transglutaminase and high pressure treatment on the mechanical properties of heat – induce gels prepared from arrowtooth founder (*Atheresthes stomias*). *Food Chemistry* **94**, 202–209.
110. Moreno, H.M., Carballo, J. and Borderias, A.J. (2008) Influence of alginate and microbial transglutaminase as binding ingredients on restructured fish muscle processed at low temperature. *Journal of the Science of Food and Agriculture* **88**(9), 1529–1537.
111. Uresti, R.M., Tellez-Luis, S.J., Ramirez, J.A. and Vazquez, M. (2004) Use of dairy proteins and microbial transglutaminase to obtain low-salt fish products from filleting waste from silver carp (*Hypophthalmichthys molitrix*). *Food Chemistry* **86**, 257–262.
112. Gomez-Guillen, M.C., Montero, P., Solas, M.T. and Perez-Mateos, M. (2005) Effect of chitosan and microbial transglutaminase on the gel forming ability of horse mackerel (*Trachurus* spp.) muscle under high pressure. *Food Research and Technology* **38**, 103–110.
113. Uresti, R.M., Ramirez, J.A., Lopez-Arias, N. and Vazquez, M. (2003) Negative effect of combining microbial transglutaminase with low methoxyl pectin on the mechanical properties and colour attributes of fish gels. *Food Chemistry* **80**, 551–556.
114. Hsieh, J.R., Tsai, G.J. and Jiang, S.T. (2002) Microbial transglutaminase and recombinant cystatin effects on improving the quality of mackerel surimi. *Journal of Food Science* **67**, 3120–3125.
115. Gomez-Guillen, C., Solas, T. and Montero, P. (1997) Influence of added salt and non-muscle proteins on the rheology and ultrastructure of gels made from minced flesh of sardine (*Sardina pilchardus*). *Food Chemistry* **58**, 193–202.
116. Tellez-Luis, S., Uresti, R.M., Ramirez, J.A. and Vazquez, M. (2002) Low-salt restructured fish products using microbial transglutaminase as binding agent. *Journal of the Science of Food and Agriculture* **82**, 953–959.
117. Ramirez, J.A., del Angel, A., Uresti, R.M., Velazquez, G. and Vazquez, M. (2007) Low-salt restructured fish products using low-value fish species from the Gulf of Mexico. *International Journal of Food Science and Technology* **42**, 1039–1045.
118. Benjakul, S., Visessanguan, W., Ishizaki, S. and Tanaka, M. (2002) Gel-forming properties of surimi produced from bigeye snapper, *Priacanthus tayenus* and *Priacanthis macracanthus*, stored in ice. *Journal of the Science of Food and Agriculture* **82**, 1442–1451.
119. Benjakul, S., Phatcharat, S., Tammatinna, A. and Visessanguan, W. (2008) Improvement of gelling properties of lizardfish mince as influenced by microbial transglutaminase and fish freshness. *Journal of Food Science* **73**(6), 239–246.
120. Jongjareonrak, A., Benjakul, S., Visessanguan, W. and Tanaka, M. (2006) Skin gelatin from bigeye snapper and brownstripe red snapper: chemical compositions and effect of microbial transglutaminase on gel properties. *Food Hydrocolloids* **20**, 1216–1222.
121. Regenstein, J.M. and Zhou, P. (2007) Collagen and gelatin from marine by-products. In: *Maximising the Value of Marine By-Product* (ed. F. Shahidi). CRC Press LLC, Boca Raton, FL, 279–303
122. Norland, R.E. (1990) Fish gelatin. In: *Advances in Fisheries Technology and Biotechnology for Increased Profitability* (eds M.N. Voight and J.K. Botta). Technomic Publishing Co., Lancaster, PA, pp. 325–333.

123. Kolodziejska, I., Kaczorowski, K., Piotrowska, B. and Sadowska, M. (2004) Modification of the properties of gelatin from skins of Baltic cod (*Gadus morhua*) with transglutaminase. *Food Chemistry* **86**, 203–209.
124. Fernandez-Diaz, M.D., Montero, P. and Gomez-Guillen, M.C. (2001) Gel properties of collagens from skins of cod (*Gadus morhua*) and hake (*Merluccius merluccius*) and their modification by the coenhancers magnesium sulphate, glycerol and transglutaminase. *Food Chemistry* **74**, 161–167.
125. Gomez-Guillen, M.C., Sarabia, A.I., Solas, M.T. and Montero, P. (2001) Effect of microbial transglutaminase on the functional properties of megrim (*Lepidorhombus boscii*) skin gelatin. *Journal of the Science of Food and Agriculture* **81**, 665–673.
126. Koladziejska, I. and Piotrowska, B. (2007) The water vapour permeability, mechanical properties and solubility of fish gelatin-chitosan films modified with transglutaminase or 1-thyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and plasticized with glycerol. *Food Chemistry* **103**, 295–300.
127. Yi, J.B., Kim, Y.T., Bae, H.J., Whiteside, W.S. and Park, H.J. (2006) Influence of transglutaminase-induced cross-linking on properties of fish gelatin films. *Journal of Food Science* **71**, 376–383.
128. Sztuka, K. and Kolodziejska, I. (2008) Effect of transglutaminase and EDC on biodegradation of fish gelatin and gelatin-chitosan films. *European Food Research and Technology* **226**, 1127–1133.

11 Enzymes in fruit and vegetable processing and juice extraction

Catherine Grassin and Yves Coutel

11.1 INTRODUCTION

Some 1.3 billion metric tonnes (bnmt) of vegetables and 417 million metric tonnes (mmt) of fruit were produced globally in 2005.[1] China ranks as the number one producer for almost every type of fruit and vegetable. It is by far the world's largest apple grower, and produced an estimated 23 mmt in 2007/2008, when world production was forecasted to reach 41 mmt.[2] Global citrus production for 2007/2008 was forecast at nearly 71 mmt, of which China produced 17.6 mmt.[3] Global fruit juice sales hit the 54 bn litre (bnL) mark in 2007 and are forecast to rise 15% by 2011 to 62 bnL. Asia is expected to be the top juice market by 2009/2010, consuming more than 14 bnL.[4] Western Europeans currently consume an average of 31 L of juice a year per capita. Juice consumption is growing fast in Eastern Europe (18 L are consumed annually per capita in Russia), as well as in India and China. In the US, consumption peaked at 40 L per capita in 2006, with particular growth evident in concentrated juice and niche juice sectors.[5]

The first commercial use of pectinases in juice processing dates back to the 1930s when used solely for apple juice clarification. Pectinases decreased juice viscosity by hydrolyzing soluble pectin and resulted in faster clear apple juice production. Pectinases were added to apple mash in the 1980s, facilitating a dramatic rise in juice production and making juice concentration possible through water evaporation, leading to perfect stable concentrates. Volume for storage was reduced by a ratio of one to five, juice to concentrate. Since then, other fruits such as berries, tropical fruits and citrus have been processed using enzymes.

In recent years, European and American fruit-processing industries have come under increasing pressure from Asia. Mounting competition from the Far East has forced Western processing companies to amalgamate, use new and more cost-effective technology, launch new beverage products targeting different market sectors and reduce waste by adding value to by-products. These four trends are addressed below:

- Consolidation within the fruit-processing industry has occurred through mergers and acquisitions. In Europe, a handful of large organizations have replaced the thousands of juice processors that operated in the 1980s. The result is a more organized, vertically integrated industry.
- The use of more cost-effective technology, better equipment – such as presses or separators with new hardware for continuous automatic control – and new enzymes have resulted in higher juice yield, faster processing and higher plant throughput.

- New beverage products, in particular different fruit and vegetable blends like carrot/pineapple/peach or blueberry/purple-carrot/grape/beet are now commercially available and marketed for their health benefits. They are rich in antioxidants, vitamins and fibre. New fruits, such as açai, acerolla, cashew apple or pitanga, are now processed and used in these blends for their attractive colour and aroma. Fruit juice processing therefore requires more sophisticated tools than simple mechanical pressing or physical extraction. Enzyme suppliers offer fruit juice producers tailored enzyme preparations that take into account fruit composition and final fruit product specifications: clear juice, cloudy juice, puree, smoothy. Application of specific purified enzymes can also result in the selective extraction of components such as aroma, colour and phenolic compounds facilitating the production of different end products and fruit derivatives.
- Waste reduction and sustainable production are now important considerations for juice processors. After initial juice extraction, leaching with enzymes allows sugar to be extracted from the fruit pomace a second time. This sugar can be used for the manufacture of natural fruit sweeteners or ethanol production. Enzymes improve the quality and stability of fruit products, and boost factory productivity. Combined with new equipment and processing technologies, industrial enzymes allow processors to add value to raw materials for food and thus reduce waste, facilitating sustainable production.

11.2 FRUIT COMPOSITION

Fruits are composed of peel, flesh tissue and seeds or stones. Flesh cells are held together by a rigid cell wall (up to many micrometers thick) that gives them a very defined shape and protects them from internal pressure and external shocks.[6] In the primary wall, the major polysaccharides are pectin, hemicelluloses and cellulose.[7,8]

11.2.1 Pectin

Pectin forms a family of complex polysaccharides that contain 1,4-linked α-D-galacturonic acid. To date, they have been classified into three groups: homogalacturonan, rhamnogalacturonans and substituted galacturonans.

- Homogalacturonan (HG) is a homopolymer of [(1 $\rightarrow$ 4)-α-D-galactosyluronic acid] residues, known for its ability to form gels. The primary cell wall HG can be methyl-esterified at the galacturonic carboxylic group, C6 position, and acetyl-esterified at the C2 or C3 position. Methyl and acetyl esterification degrees and molecular weight of pectin are specific to fruit species (see Table 11.1).
- Rhamnogalacturonan I (RGI) has a backbone of as many as 100 repeats of the disaccharide [1 $\rightarrow$ 2)-α-L-rhamnosyl-(1 $\rightarrow$ 4)-α-D-galactosyluronic acid]. It can be substituted by arabinan or arabinogalactan. Side chains of neutral sugars are covalently attached to RGI to the O-4 of rhamnose residue.
- Arabinans are mostly (1 $\rightarrow$ 5)-α-L-arabinosyl residues forming linear chains, but arabinosyl units can be connected and more or less ramnified with (1 $\rightarrow$ 5), (1 $\rightarrow$ 3) or (1 $\rightarrow$ 2)-α-linkages.
- Arabinogalactan (AG) type I with a (1 $\rightarrow$ 4)-β-D-galactan backbone and AG type II with a (1 $\rightarrow$ 3),(1 $\rightarrow$ 6)-β-D-galactan backbone, both AG have (1 $\rightarrow$ 3)α-L-arabinosyl residues.

Table 11.1 Fruit composition.

	Pectin% of fresh weight	Methylation %	Endogenous enzymes	pH	Titratable acidity % as tartaric	Citric % of acids	Malic%	Other acids	Solids %	Fibres %
Apple	0.7–0.8	75–92	PE PG	3.3–3.9	0.5–1.4	–	>90	Quinic 5%	15	2
Apricot	–	–	–	3.3–3.8	1.1–1.3	25	75	Quinic	15	2
Banana	0.5–0.7	50–60	PE PG	4.5–5.2	0.3–0.4	20	70	Oxalic 10%	25–30	3
Blackberry	0.7–0.9	–	–	3.8–4.5	0.9–1.3	50	50	–	15–18	7
Blackcurrant	1.1	50–80	PE	2.8–3.0	3.0–4.0	90	10	Oxalic	20–23	8
Cherry	0.2–0.3	40	PE	3.3–3.8	0.4–0.6	10	90	–	14–16	2
Cranberry	1.0	–	–	2.3–2.5	–	–	–	–	–	–
Grape	0.1–0.4	50–65	PE PG	2.8–3.2	0.4–1.3	–	20	Tartaric	20	1.5
Grapefruit	1.3–1.6	–	PE	3.0–3.7	2.0	95	5	–	9–10	1
Lemon	2.0	65–70	–	2.0–2.6	4.0–4.5	95	5	Quinic	13–15	5
Mango	0.3–0.4	78–85	PE PG	3.4–4.6	0.2–1.2 cit.	–	–	–	–	–
Orange	0.6–0.9	65–70	PE	3.3–4.2	0.8–1.1	90	10	–	14	2
Peach	0.3–0.4	60–80	PE PG CEL PPO	3.3–4.0	0.5–0.8	25	75	–	11–13	2
Pear	0.7–0.9	50–70	PE PG	4.0–4.6	0.2–0.4	–	>90	–	15–17	2
Pineapple	0.04–0.1	22–40	PG	3.2–4.0	0.8–1.3	80	20	–	15	1
Plum	0.7–0.9	70–75	PPO	3.6–4.3	1.4–1.7	–	>95	Quinic	15	1.5
Raspberry	0.4–0.5	20	–	3.2–3.9	1.4–1.6	75	25	–	15–20	7
Strawberry	0.4–0.5	20–60	PE CEL PPO	3.0–3.9	0.6–1.5	90	10	Succinic	10	2

PE: pectinmethylesterase; PG: polygalacturonase; CEL: cellulase; PPO: polyphenoloxidase; (–) unknown.

- Rhamnogalacturonan II (RGII) is a low molecular weight about 4.8 KDalton complex polysaccharide composed of 12 different sugars, with a homogalacturonan backbone composed of about nine $1 \rightarrow 4$-α-D-galactosyluronic acid residues. Four different complex side chains are attached to O-2 or O-3 of the galacturonic acid.[9]

11.2.2 Hemicellulose

Two main hemicelluloses of primary cell walls are xyloglucan and arabinoxylan. Minor components such as glucomannan or galactoglucomannan have also been identified. Hemicelluloses bind tightly via hydrogen bonds to the surface of cellulose cross-linking microfibrils creating a cellulose–hemicellulose network. Interconnections with the pectic polysaccharides are of primary importance to the integrity of the pectin network. In 1993–1994, Vincken and Voragen demonstrated that xyloglucan is a key structure of the apple cell wall, which helps break down cell-wall-embedded cellulose if it is first hydrolyzed.[10] The apple xyloglucan fraction makes up about 24% of the total amount of sugar, and the cellulose–xyloglucan complex accounts for approximatively 57% of the apple cell wall matrix.

11.2.3 Cellulose

Cellulose is particularly abundant in secondary cell walls. This is a [$1 \rightarrow 4$-β-D-glucan] polysaccharide. Cellulose microfibrils, which give shape and tensile strength to the primary cell wall, are coated with hemicelluloses that bind tightly to their surface.[11] It is not necessary to break down this cellulose network for juice extraction when using classic fruit maceration processes. In fact, if it were destroyed, the fruit mash would soften and lose its pressability and drainability.

11.2.4 Starch

Starch is present as an energy reserve in unripe apples and pears, as well as many other fruits and vegetables. It is not present in berries, however. Starch is synthesized in amyloplasts and is present in granulated form in fruit cells. This polysaccharide is composed of amylose and amylopectin. Amylose, the minor component, is a linear $1 \rightarrow 4$-α-D-glucan. Amylopectin contains α-$1 \rightarrow 6$ and α-$1 \rightarrow 4$ glucose linkages. Its degree of polymerization is far greater than amylose. Apple starch jellifies around 75°C. It is only sensitive to α-amylase and glucoamylase hydrolysis after swelling by jellification.

Cell wall composition is similar in all species of fruit, but neutral sugar type can vary according to fruit species. The proportion of different components such as polysaccharides, polyphenols or proteins depends also on agronomic and climatic conditions, fruit ripeness and conditions and duration of fruit storage. The most important components, pectin characteristics and average composition are described in Table 11.1.

Fruits are processed for their nutritional properties and pasteurization extends their shelf life. However, extracting juice is frequently difficult and yields are often very low. The main obstacle is pectin. Because this hydrocolloid has a high affinity for water, it starts to dissolve in the juice, increasing viscosity as soon as the fruit is crushed. Pectin can also form a gel when sugar concentration increases at the juice evaporation stage. The type of enzyme needed to hydrolyze a fruit's pectin can be identified once the fruit's pectin composition is

examined. Pectin hydrolysis with enzymes facilitates easier juice extraction and results in higher yields.

11.3 PECTIN DEGRADING ENZYMES

Aspergillus niger is the main microorganism used for large-scale production of pectinases for the fruit industry. Wild strains of this fungus secrete large amounts of different enzymes to break down the substrate on which they grow into nutrients for their own metabolism. This characteristic is harnessed for industrial enzyme production. *A. niger* is also a suitable host for homologous and heterologous gene expression. Using classic strain improvement and defined genetic modification methods, enzyme producers have successfully cultivated pure enzymes from *A. niger*, as well as boosted its pectinase production. In June 2000, DSM discovered new enzymes in *A. niger* by sequencing its complete genome. The complete 35.9 megabase genome of *A. niger* was sequenced.[12] Over 14 000 open reading frames were identified and functionally classified using international databases as a reference. From a total of 845 EC identified enzymes, 97 pectinase genes were annotated, of which about 60 were new. The names and gene code of *A. niger* pectinases are outlined in Table 11.2.

Table 11.2 *Aspergillus niger* pectinases names, E.C. numbers and gene codes.

Name	E.C. number	Gene codes
Homogalacturonan degrading enzymes		
Endo polygalacturonase	3.2.1.15	pga A B C D E F I II X
Exo polygalacturonases	3.2.1.67	pgx A B C D E
Pectin lyase	4.2.2.10	pel A B C D E II
Pectate lyase	4.2.2.2	ply A
Accessory enzymes		
Pectin methylesterase	3.1.1.11	pme A B C
Pectin acetylesterase		pae A B C D
Rhamnogalacturonan degrading enzymes		
Rhamnogalacturonan hydrolase		rgh A B C D E F
Rhamnogalacturonan lyase		rgl A B D
Xylogalacturonan hydrolase		xgh A C D
Endo xylanase	3.2.1.32	xln A C D E F
Accessory Enzymes		
Rhamnogalacturonan acetylesterase		rgae A
Arabinofuranosidase α	3.2.1.55	abf A B C D
Endo-arabinase	3.2.1.99	abn A B C D E F
Galactanase β 1,4	3.2.1.89	gal A B
Arabinogalactanase β 1,3	3.2.1.90	agn
Galactosidase β	3.2.1.23	lac A B C D E F G H
Galactosidase α	3.2.1.22	agl A B C D E F
Feruloyl acid esterase		fae A B C D E F G H
Rhamnosidase β	3.2.1.43	rha A B C D E F G H
Fucosidase α	3.2.1.51	fuc A
Xylosidase α	3.2.1.37	xal A
Xylosidase arabinosidase		xar A B
Glucuronidase β	3.2.1.31	gus A B
Arabinoxylanase		axh A
Mannanase	3.2.1.101	man A
Mannosidase β	3.2.1.25	mnd A

Table 11.3 Biochemical properties of main pectinases from *Aspergillus niger* for fruit processing.

Enzyme	Mode of action	pH range	Temp °C range	Preferred substrate	Functionality
Endo-pectinlyase	Eliminative cleavage of (1,4)-α-D-galacturonan methyl ester in oligosaccharides with 4-deoxy-6-*O*-methyl-α-D-galact-4-enuronosyl groups at their non-reducing ends	4.0–6.0	30–45°C	Fully methyl-esterified oligogalacturonides	Pectin viscosity decrease
Endo-polygalacturonase	Random hydrolysis of 1,4-α-D-galactosiduronic linkages in pectate and other galacturonans hydrolysis of *O*-glycosyl bond	3.5–6.0	40–45°C	α-D-galacturonosyl-(1,4)-*O*-α-D-galacturonate + H_2O	Pectin viscosity decrease
Exo-polygalacturonase	Catalyzing the degradation of D-galacturonans by terminal action pattern	3.0–5.2	30–60°C	(1,4-α-D-galacturonide) 2 + H_2O	Release of galacturonic acid
Pectinmethylesterase	Hydrolysis of carboxylic ester	3.5–5.5	40°C	Methyl-esterfied oligogalacturonides + H_2O	Release of methanol
Arabinofuranosidase	Hydrolysis of terminal non-reducing α-L-arabinofuranoside residues in α-L-arabinosides	2.0–5.5	50–60°C	1,2–1,3-α-L-arabinosides	Improved access to abn and agn
Endo-arabinase	Endohydrolysis of 1,5-α-arabinofuranosidic linkages in 1,5-arabinans	4.5–4.8	40–45°C	1,5-α-L-arabinan + H_2O	Prevents araban haze formation in apple and pear concentrate

Commercial pectinases for the fruit juice industry come from selected strains of *A. niger*. After strain growth in a defined culture medium, exo-cellular enzymes are purified and concentrated.

Pectinases are defined and classified on the basis of their reaction to pectin and are grouped into three main categories: lyases, hydrolases and esterases. The biochemical properties of the main *A. niger* pectinases used in fruit processing are summarized in Table 11.3.

Figure 11.1 shows a schematic pectin composition.[13] Enzyme positioning on their substrate named with gene codes is described in Table 11.2.

11.4 COMMERCIAL PECTINASES

11.4.1 Production

In juice processing it is not necessary to completely break down the fruit-flesh cell wall to extract fruit juice. The main goal is to decrease the pectin's viscosity within the mash or

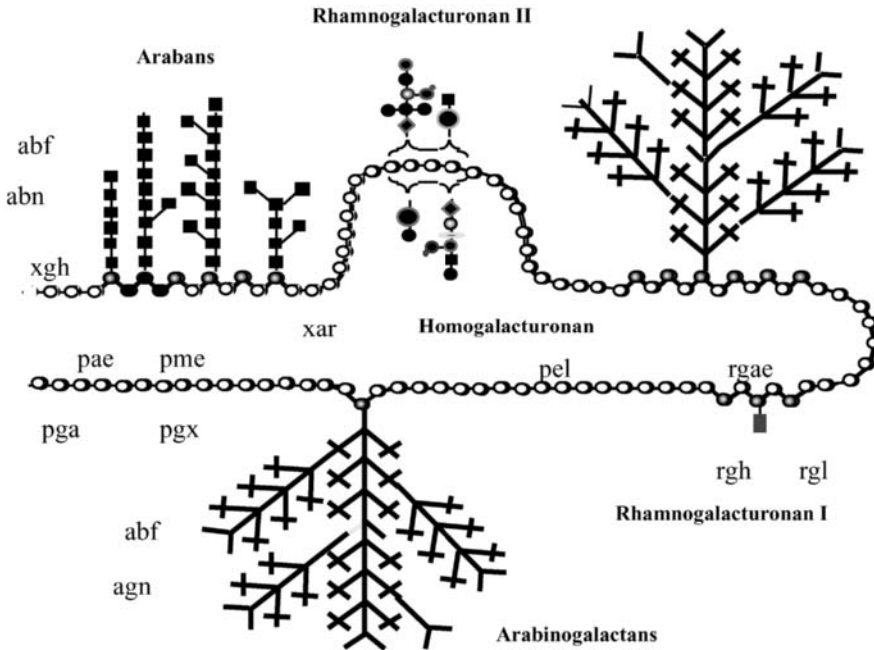


Fig. 11.1 Pectin composition and pectin degrading enzymes (adapted from Ref. [13].)

the juice to speed up the process. Pectin is a polysaccharide present in every fruit that gels after crushing. It holds the juice within the mash, reduces pressability, decreases juice yield, slows down juice clarification and makes water evaporation for concentration impossible. By chance, enzymes from *A. niger* are globally efficient at natural fruit pH, from 2.5 to 5.5. In addition, fruits and vegetables are processed in cool conditions to maintain freshness (processing temperatures, in general, never exceed 50°C). These factors are conducive to pectinase use in juice processing. Classic *A. niger* strains produce pectinases as a core activity, as well as numerous other enzymes such as hemicellulases or glycosidases as side activities. All have a specific function and role to play in fruit processing. Juice processors expect enzymes to be efficient, so it is imperative that the enzyme producer controls and standardizes pectinase activity in every batch. Pectinases are produced from a pure culture of one *A. niger* strain, selected on the basis of its productivity. Although the fungus species is *A. niger*, strains differ and are the property of the enzyme producer. Strains are grown in solid media (solid state culture) or liquid mediums (submerged fermentation). Enzymes produced during the culture are strain- and medium-dependent, with variable and controllable concentration, range and ratio of activities. This is why the enzyme range differs from one commercial product to another, even if sold with the same name of pectinase or amylase. Enzyme suppliers must therefore provide the user with complete information on: product reliability, specifications, conformity with legislation, whether or not genetically modified microorganisms were used and safety in use.[14]

11.4.2 Specifications

An enzyme specification sheet and certificate of analysis describe product composition, stabilizing systems, chemical properties, microbiological properties, stability and status (whether

or not from genetically modified microorganisms). One difficulty for enzyme regulators and users is that enzyme activity is 'company-dependent'. The unit definition for pectinase, for example is not standardized, and analytical methods are not often disclosed by the supplier or cannot be compared. Standardization would make it easier for regulators and users to compare enzyme activity. But this is almost impossible for technical reasons, as in the example of the synergetic effect of pectinases PL, PME and PG on pectin breakdown. For the time being, the comparison of commercial pectinases is relative and made on the basis of dosage/price ratio of enzymes used per kilogram of fruit for same technological efficiency.

11.4.3 Legislation

Pectinases for the fruit industry bear the GRAS status (generally recognized as safe) and must comply with general specifications for food enzymes as laid down by JECFA (1981, revised in 2006),[15] Food Chemical Codex (5th edition 2004),[16] Europe Guideline Scientific Committee on Food (SCF) 1991[17] and French Enzyme legislation 2006.[18]

Enzymes permitted as food additives in the EU are lysozyme (in wine) and invertase (in confectionery). France and Denmark have national legislation governing enzymes used as processing aids. On EU level, horizontal legislation was discussed in parliament in June 2007. Vertical EU legislation on fruit juice follows a positive list of processing aids including 'pectinase, amylase, protease'. A processing aid like pectinase is defined as a substance that has a function during processing and can be present but without function in the end product. A processing aid is not labelled. This is the case for pectinases or amylases derived from classic or genetically modified strains of microorganisms.

11.4.4 Genetically modified microorganisms

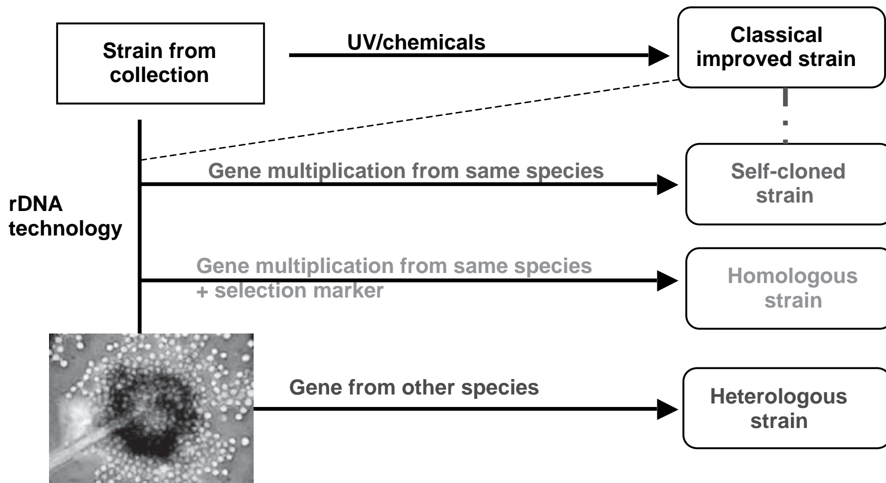
Enzymes derived from genetically modified microorganisms (GMMs) are increasingly found in the fruit industry, especially in apple processing. *Aspergillus* strains are genetically modified to produce pure enzymes without side activities. Genetic modification techniques are applied only to the reproductive strain and not to the enzymes: enzymes are thus not genetically modified, but are derived from GMMs. If no DNA from another species is introduced in this process, it is called homologous recombination, which is comparable to classic genetic techniques (e.g. random mutation with UV or chemicals). If foreign DNA is introduced in the receptor strain, the process is called heterologous recombination. Only homologous and self-cloned modified strains are authorized in the fruit industry.

Self-cloning is the term used when the selection marker is removed from the DNA. GMM technology guarantees that enzyme proteins are identical to those produced from classic strains. DNA molecules are absent from final products (see Fig. 11.2).

Commercial pectinases can have very different compositions. Several pure enzymes from GMMs can be used separately or blended together; or enzymes from GMMs can be added to enrich classic products for certain activities.

The legal situation is complex. Self-cloned microorganisms, as defined in the EU Directive 98/81/EC, are not considered genetically modified, according to:

- Deutsches Gentechnikgesetz implementing Directive 90/129/EC (Germany).
- Österreichischem Gentechnikgesetz BGBL 510/1994 (Austria).
- Verordnung über den Umgang mit Organismen in der Umwelt SR 814.911 (Switzerland).



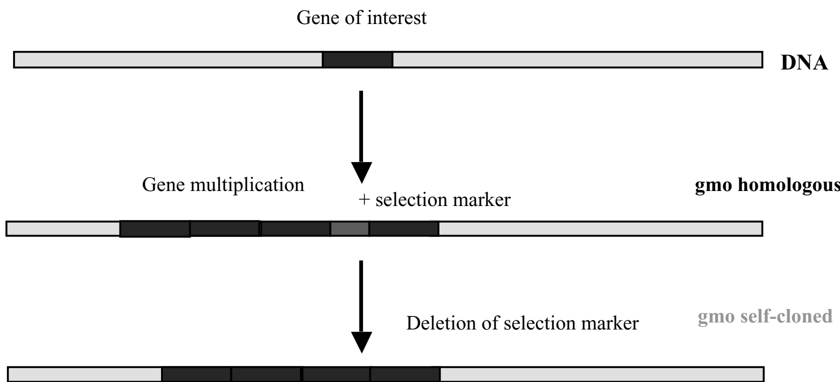


Fig. 11.2 Construction of industrial *Aspergillus niger* strains and different techniques.

- In Australia and the US, no additional approval is needed where classic strains are already accepted.
- In all other countries in Europe, enzymes from self-cloned microorganisms are considered enzymes from GMMs.

11.5 ENZYMES FOR FRUIT PROCESSING

Processors wishing to produce clear concentrated fruit juices must overcome many challenges, including handling different kinds of fruits in the same factory, and managing fluctuating quantities of fruit with variable compositions and textures depending on their ripening stage. Dealing with these factors requires efficient and reliable tools, such as equipment and

enzymes. Enzymes are processing aids used worldwide for fruit processing, particularly for the production of clear fruit juice and concentrate. They offer numerous advantages:

- **Economic**: When added to fruit after crushing, pectinases quickly decrease the viscosity of the mash by pectin hydrolysis. They facilitate juice extraction from pulpy fruit, increase press loading and increase juice yield. Residual waste, such as apple pomace, is reduced and is drier. Enzymes increase the overall productivity of the processing plant. Once added to the juice, pectinases and amylases hydrolyze residual pectin and starch. Their use is vital for fast juice clarification, filtration, pasteurization and concentration. As a result, the juice remains stable, long term, without additives or preservatives, storage volumes are reduced and shipping weight reduced.
- **Quality**: Fast juice processing with enzymes lowers the risk of microbial spoilage, reduces oxidation and improves juice and concentrate shelf life. Pectin hydrolysis of fruit cell walls weakens cells and vacuoles and thereby maximizes extraction of their components such as the red colour from berries (anthocyanins), aromas and antioxidants of phenolic type – known for their positive effect on human health, particularly heart disease prevention.
- **Sustainability**: The use of enzymes has a positive effect on sustainable production. They lower energy consumption (electricity, steam and water), reduce waste flow by maximizing fruit use and reduce dependency on chemicals used in equipment cleaning products.

11.5.1 Apple processing

Worldwide apple production rose from 59.1 mmt in 2000 to 63.5 mmt in 2004.[1] The USDA calculated 2007/2008 global production at around 42 mmt, with China accounting for 56% of the total output (23 mmt). China is fast approaching the EU as the world's largest apple exporter in volume terms with a 19% share market share compared to the EU's 22% in 2007/2008. But its exports' value ranks third behind the EU and market leader, the US.[2] In 2006/2007, 1.42 mmt of apple juice concentrate (AJC) was exported globally, representing 11% of total apple juice production. Volumes almost quadrupled between 2000 and 2007. Chinese AJC exports in 2006/2007 reached 894 823 tonnes. The same year, EU AJC and single-strength-juice imports reached 1 mmt in 2006.[5] Apple production in countries such as France, Germany and Japan is decreasing, while in China, Poland and Russia it is increasing.

11.5.1.1 *Apple juice concentrate with press*

This classic process is described in Fig. 11.3. Apples are washed, sorted and crushed. At this stage, mash pectinases are added continuously with a metering pump after dilution in approximately 10–20 times the volume of water.

The enzymated mash is then pumped into holding tanks. After 30–60 min at ambient temperature, the mash is pumped into the press. The juice is extracted and immediately pasteurized for aroma recovery and microbial stability. It can be pre-concentrated at this stage from 11° to 18–20° brix. It is cooled to 45–50°C and pectinases are added to the juice. Amylases are added together with pectinases at the start of the processing season when apples contain starch. After about 2 h, the juice is depectinized and no longer contains starch. It is then clarified by cross-flow filtration and finally concentrated at 72° brix by water evaporation, and stored in stainless steel tanks below 10°C.

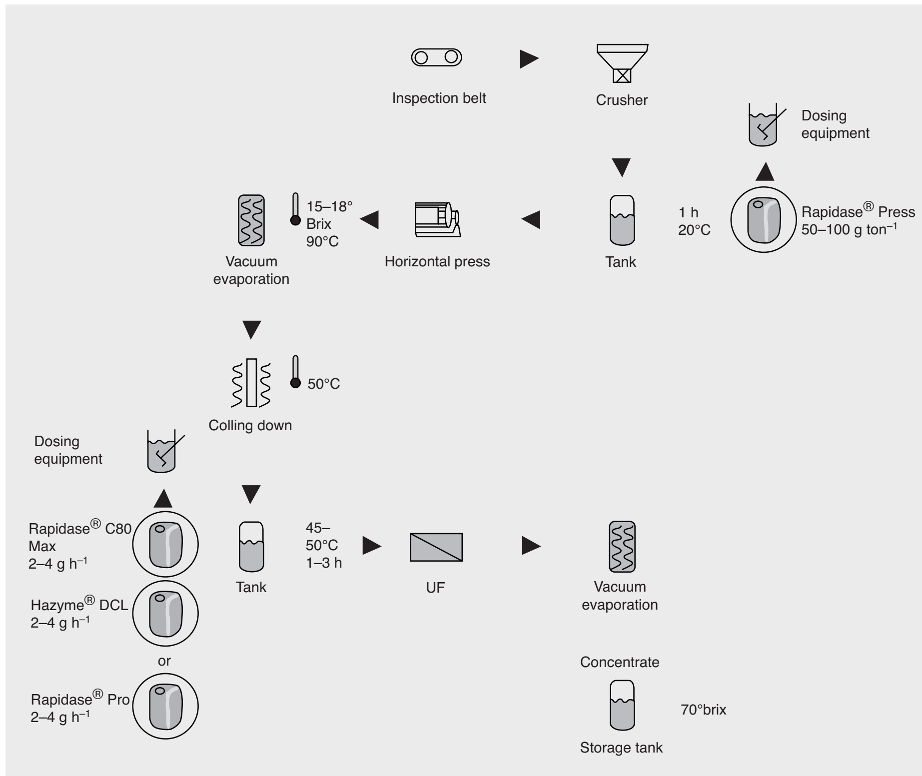


Fig. 11.3 Process of apple juice concentrate with press. *Source*: From ProJuice CDrom, DSM Food Specialties BV2006.

Commercial mash pectinases are Pectinex Mash (Novozym), Peclyve PR (Lyven), Pektozyme® MAXLiq (Danisco) or Rapidase® Press (DSM) with different concentrations and ratios. Adding a broad range of pectinases derived from classic *A. niger* strains results in maceration of apple mash, which means solubilization of protopectin and hemicellulose before juice extraction. For example, certain endo-pectinases, sometimes called 'protopectinases', have more macerating power than others.[19] At high dosage, addition of protopectinases results in a sticky, soft mash that adversely affects pressability. Press throughput decreases because of slower belt press speed, lower press loading and longer cycles for horizontal press. The residual wet pomace can leak from storage bins and is more difficult to dry. For this reason pectinases from self-cloned *A. niger* strains have been developed, such as Rohapect® MA plus (ABEnzymes), Pectinex Yield MASH (Novozymes) or Rapidase® Smart (DSM). Since it is not necessary to completely break down apple pectin, limited and controlled hydrolysis of soluble pectin with non-macerating pectinases is sufficient for obtaining a high juice yield (above 90% with pomace leaching), improving press loading, avoiding ultrafiltration problems and producing dry pomace. The release of galacturonic acid is limited to the minimum, because of the absence of exopolygalacturonase in the pectinase products described above. Cellobiose release in the juice is avoided because there is no cellulolytic side activity in these pectinase products, answering to legislation constraints such as in Germany.

Commercial juice pectinases such as Rohapect DA6L (ABEnzymes), Pectinex (Novozymes), Peclyve CP (Lyven), Pektozyme POWERClear (Danisco) or Rapidase C80Max (DSM) completely hydrolyze the remaining pectin in the juice. Pectinase dosage is adjusted to achieve pectin breakdown in accordance with processing time constraints. Juice depectinization is recorded by performing a laboratory acidic alcohol test.[8] At the start of the juice processing season, green apples contain up to 2 g L^{-1} of starch. When the juice is heated to 75–80°C, the starch is gelatinized. Once the juice is cooled, these molecules can reform into amorphous aggregates giving it a cloudy appearance. It is therefore important to add commercial amylases in order to completely hydrolyze apple starch and prevent retrogradation and cloud formation in the juice. Commercial products such as Rohapect S (ABEnzymes), Amylase AG (Novozymes), Amylyve TC (Lyven), Diazyme™ Power Clear (Danisco) or Hazyme® DCL (DSM) contain α-amylase and amyloglucosidase from *A. niger* with variable concentrations and ratios. The dosage is determined by the iodine test. Juice solids separation, 'fining' and clarification are complete when pectin and starch lab tests are negative. Cross-flow filtration is the most commonly used technique, particularly using ultrafiltration equipment with polysulfone or ceramic membranes with cut-off around 10 up to 20–30 KDa. Insoluble particles and soluble colloidal material of larger molecular dimensions than the pore size are discarded in the retentate. The filtrate, as clear juice, is pasteurized and bottled or concentrated. Ultrafiltration is often the bottleneck in production, when the extraction of cell wall components is too intense (i.e. diffusion process, mash heating, mash enzymatic over-maceration or maceration with hemicellulases), or when clarification enzyme preparations do not contain sufficient amounts of various activities or their dosage is too low. Large polysaccharides like RGII, which are not broken down by pectinases in the previous stage of the process, bind with proteins and polyphenols and are thus responsible for progressive membrane fouling. Commercial enzymes like Rapidase Optiflux (DSM) are sold to prevent this problem. Different enzymes, such as cellulases, amylases or proteases, can be used for membrane cleaning in the cleaning in place (CIP) process, partially replacing the use of chemicals. The process can run longer without the need to stop for cleaning, and the shelf life of the membranes is extended.

11.5.1.2 *Apple juice concentrate with decanter*

Apple juice extraction using a decanter in multi-extraction stages requires enzymatic preparations with a broader range of activities. The process starts with strong crushing and a hot break of the mash; this results in decreased mash viscosity and in the solubilization of apple protopectin.

A high dosage of enzymes combined with extended treatment at 50°C results in dramatically decreased mash viscosity, facilitating easy separation and optimal juice yield using decanters. Rapidase Adex-D (DSM) has been developed specifically for apple juice extraction using decanters. It contains the correct proportion of specific pectinases, arabanases and rhamnogalacturonase activities to break down the fruit's structural compounds in a single extraction stage. The pomace is then leached using water to extract the remaining sugar. The juice is pre-concentrated and clarified with pectinases and amylases prior to ultrafiltration and final concentration.

11.5.1.3 *Cloudy apple juice*

In countries like Germany and Italy, cloudy apple juice is considered a natural product and commands a higher value than clear juice. However, its production is impossible with classic

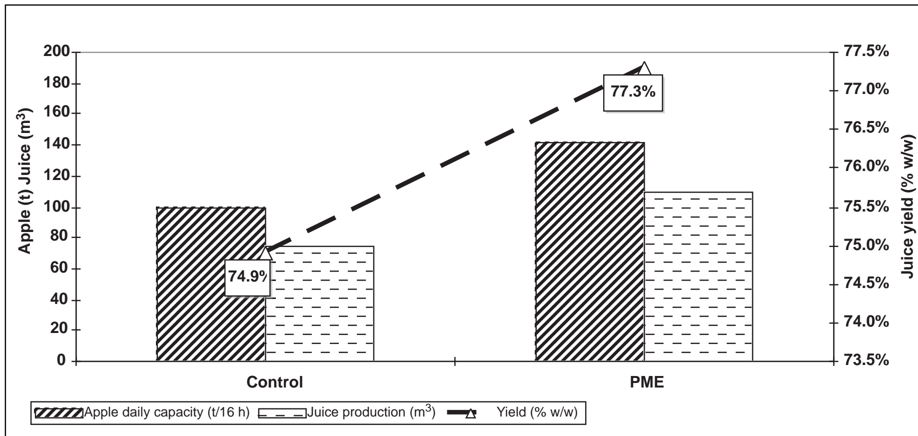


Fig. 11.4 Effect of *Aspergillus niger* pectin methyl esterase on cloudy apple juice extraction using HP BUCHER 5005 xi.

pectinases because of fast juice clarification. Upon using a pure pectinmethylesterase like Rapidase FP Super and Rapidase PEP (DSM) there is no clarification effect but numerous advantages. The demethylation of soluble pectin within the mash results in the formation of insoluble pectate firming the mash. This facilitates juice extraction: yields are subsequently increased by 1–2% and press throughput by about 30–40%, resulting in an overall productivity increase (see Fig. 11.4). The juice's cloudy state is stable since no decrease of pectin molecular weight has occurred (Fig. 11.5). Ascorbic acid can be used in the process to prevent browning.

11.5.1.4 *Pear juice concentrate*

Pear processing is similar to apple processing. Commercial mash and juice pectinases and amylases used for apples work well for pear processing. However, pears are very delicate. They ripen fast, resulting in decreased mash pressability and yield. The presence of stone cells with high cellulose content makes filtration and ultrafiltration difficult. Araban, hemicellulose and cellulose content are higher in pears than apples. In some cases, after partial enzymatic hydrolysis, linear arabans can condense with hydrogen bonds and slowly form an araban haze after juice's concentration. It is therefore important to use pectinases with high exo and endo-arabinases, such as Pearlyve (Lyven) or Pearex® (DSM), to prevent haze forming in the concentrate.

11.5.2 Red berry processing

Red berries, such as blackcurrants, strawberries, aronias (choke berry), cherries, grapes and cranberries have common characteristics. The anthocyanin pigment gives rise to the red colour and has antioxidant properties. Most of these fruits have an appealing flavour. Red berries are therefore considered a valuable raw material for juice processing and can be blended with other fruit or vegetable products since they are often too acidic or too sweet to be consumed as single strength juice. Poland is an important berry producer, harvesting

Fig. 11.5 Cloudy apple juice (left) control (right) process with *Aspergillus niger* pectin methyl esterase. (Also see Colour Plate 3.)

40 000–45 000 tonnes of raspberries and 110 000–115 000 tonnes of sour cherries in 2007. Berry composition is outlined in Table 11.4.[20]

Anthocyanins are the most valuable compounds found in red berries. They are also the most sensitive components, prone to being destroyed at each stage of the process.[21] The presence of sugar helps the anthocyanin maintain its solubility in water. Anthocyanin is anthocyanidin esterified with a sugar molecule at 3 position. Each aglycone has a characteristic colour and spectrum. Sugar moiety differs with fruit species (Table 11.4). If sugars are enzymatically or chemically hydrolyzed, the solubility of the molecule decreases and the red colour tends to stabilize. Berries are very sensitive to mould contamination, especially to *Botrytis cinerea*. Moulds secrete a high laccase (oxidase) activity, as well as a viscous β-glucan. This results in fast juice browning, which hinders juice extraction and fouls up filters. Enzymatic breakdown of *B. cinerea* β-glucan is not permitted by fruit-processing legislation. Heating above 80°C is therefore necessary to complete mechanical cell breakdown, release the juice and the colour and destroy endogenous oxidases. However, high temperatures bring about pectin dissolution in the mash. Pectinase use is therefore necessary to increase juice yield, and release colour and aromas.

Table 11.4 Red berry composition.

Red berry types	Pectin% of fresh weight	pH	Total acidity (% tartaric)	Polyphenols (% tanins)	Anthocyanin type	Linked sugars
Blackberry	0.7–0.9	3.8–4.5	0.9–1.3	0.2–0.35	Cyanidin	GLU RUT
Cherry	0.2–0.3	3.3–3.8	0.4–0.6	0.1	Cyanidin	RUT GLU
Blackcurrant	1.1	2.7–3.1	3.0–4.0	0.35	Cyanidin Delphinidin 800 ppm	GLU RUT
Bilberry	1.2	3.8–4.4	0.9–1.0	0.1–0.2	Delphinidin Pelargonidin Malvidin	GLU GAL ARA
Cranberry	1.0	2.3–2.5	2.7–3.5	0.3	Cyanidin Peonodin Delphinidin 400 ppm	GAL GLU RUT
Elderberry	0.7–0.9	3.2–3.6	0.8–1.3	0.5–0.6	Cyanidin	GLU SAM
Grape	0.1–0.4	2.8–3.2	0.4–1.3	0.1–0.3	Delphinidin Petunidin Malvidin Peonidin	GLU
Raspberry	0.4–0.5	2.5–3.1	1.4–1.7	0.2–0.3	Cyanidin Pelargonidin 400 ppm	SOP GLU RUT
Redcurrant	0.4–0.6	3.2–3.6	2.0–2.5	0.1	Cyanidin	GLU
Strawberry	0.4–0.5	3.0–3.9	0.6–1.5	0.3–0.5	Pelargonidin	GLU

Bound sugars
GLU: glucose; GAL: galactose; ARA: arabinose; RUT: rutinose = rha-glu.
SAM: sambubiose = xyl-glu; SOP: sophorose = glu-glu.

11.5.2.1 *Blackcurrant juice concentrate*

The process is described in Fig. 11.6. Frozen blackcurrant or aronia is ground and heated to 90–92°C for a few seconds then cooled to 50°C. Polyphenoloxidases are quickly destroyed and anthocyanins are released.

High temperature, strong fruit acidity and high phenolic content have an inhibiting effect on exogenous pectinases. Pectinases must therefore be able to withstand these conditions and remain stable throughout the process. Mash pectinases such as Rohapect 10L (ABEnzymes), Pectinex BE Colour (Novozymes), Peclyve FR (Lyven), Klerzyme® 150 or Rapidase Intense (DSM) can be used. After around 1 h held in tanks at 50°C, the juice is extracted by press or decanter. During the hotbreak process, one high dose of pectinase can be enough to completely degrade the pectin and avoid further juice depectinization. Over 85% of the juice is removed after first extraction and over 90% after pomace leaching. The fruit's red colour is successfully extracted and is stable in the juice (Fig. 11.7).

For good red colour stability, it is necessary that the pectinases used in the process have no discolouring side activities, like the enzyme anthocyanase, which is sometimes present as side activity of classic pectinases. After extraction, the juice is pasteurized, clarified by filtration and concentrated.

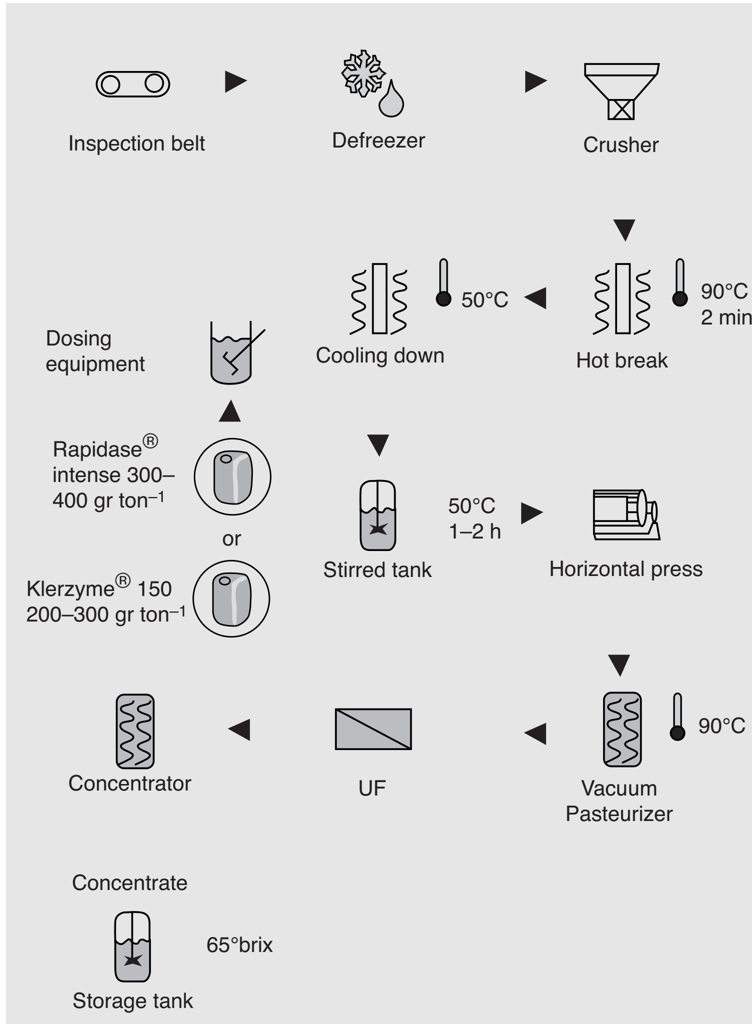


Fig. 11.6 Process of blackcurrant juice concentrate. *Source*: From ProJuice CDrom, DSM Food Specialties BV 2006.

11.5.2.2 *Strawberry juice concentrate*

Heat treatment cannot be applied to soft berries like strawberries or raspberries (unlike blackcurrant and aronia) because it purees them – making it impossible to extract the juice with a press – oxidizes their colour and destroys their aroma. It is therefore necessary to quickly process only good-quality fruits. Strawberries and raspberries are defrosted and processed at ambient temperature (around 20°C). Pectinases are added to the mash, which is held in tanks for about an hour. Then the juice is extracted using a press or decanter. Juice yield varies from 70% to 80%. The juice is pasteurized and aromas are recovered. The juice is then cooled to 30–35°C to be depectinized with Pectinex BE Colour (Novozymes), Peclye FR (Lyven), Rapidase C80Max or Rapidase Intense (DSM) for 1–2 h. Pasteurization must be carried out at a temperature high enough to completely denature the fruit proteins to

Averaged values for duplicate trials	Control	Trial 1 (two stages)	Trial 2 (one stage)
Enzyme dosage (g/t)	0	50	150
Enzymatic maceration time	0	60	60
Temperature	55°C	55°C	55°C
Juice weight (g)	616	632.7	633.8
Density	1.07956	1.08134	1.08279
Dry material extracted	19.685	20.085	20.41
Brix% of the juice after pressing	21	21.7	21.9
Juice yield (base on extract %)	**77**	**80.7**	**82.1**
Acidic Alcohol test	**,++**	**,+**	**,–**
Neutral Alcohol test	**,++**	**,+**	**,–**
Acetone test (% precipitate)	**<5%**	**<1%**	**<1%**
Colour 520 nm (at 0.50% brix)	0.679	0.697	0.746
Colour 420 nm (at 0.50% brix)	0.453	0.473	0.476
Colour 620 nm (at 0.50% brix)	0.095	0.104	0.108
Ratio 520/420 (red note on yellow)	1.50	1.47	1.57
Ratio 620/520 (blue note)	0.14	0.15	0.14
Colour improvement (Red 520 nm for 0.50% brix)	100%	103%	110%
Colour extracted [(g · OD Unit) /0,50% brix]	17567	19139	20709
Colour total extraction improvement [f(brix) + volume increase]	**100%**	**109%**	**118%**

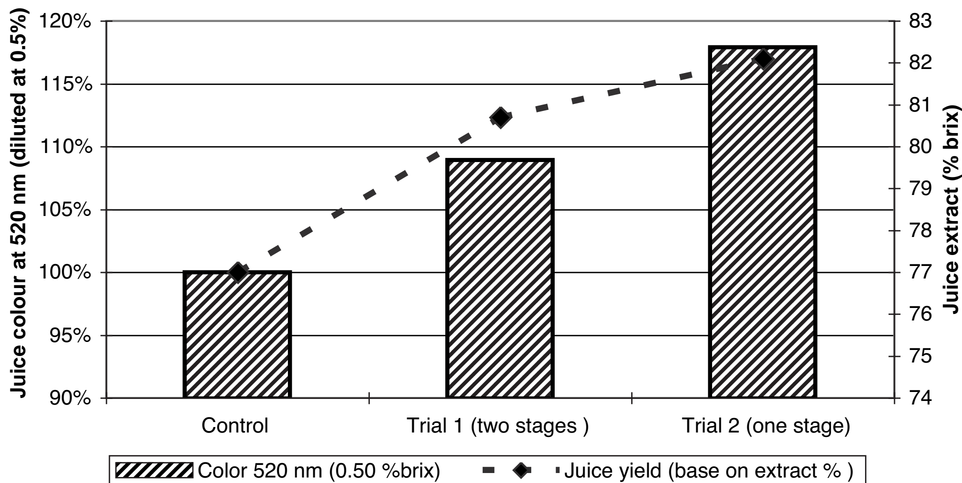


Fig. 11.7 Effect of maceration enzyme on Aronia juice and colour extraction. Trial 1: two enzyme additions with 50 ppm at maceration stage; Trial 2: one enzyme addition process with 150 ppm enzyme only at maceration stage. Relative juice colour based on control as 100% determined by OD at 520 nm of diluted juice at 0.5%. Yield based on total soluble extract.

prevent protein haze formation in the juice and concentrate. The juice is clarified (preferably using cross-flow filtration) to avoid colour loss, then concentrated.

11.5.3 Tropical fruit processing

Mango and banana are the top two tropical fruits grown worldwide, followed by pineapple, papaya and avocado. These five account for approximately 75% of global fresh tropical fruit production. Others like lychees, durian, rambutan, guava and passionfruit are produced in

smaller volumes. Global production of the top five tropical fruits is expected to reach 62 mmt by 2010.[22] Asia-Pacific will remain the key producing region – accounting for over 56% of global production – followed by Latin America and the Caribbean (32%), and Africa (11%). Global mango production is forecast to reach 31 mmt by 2010, accounting for nearly 50% of world tropical fruit production – of which 77% will be produced in the Asia/Pacific region. India currently grows 50% of the world's mango – around 15 mmt. China is forecast to see the greatest production increase, rising from 2.8 mmt to 6.3 mmt. Global pineapple production reached 18 mmt in 2006. Thailand and Brazil are the top producing countries. Papaya output, meanwhile, is expected to reach 12.4 mmt by 2010, with nearly 65% cultivated in Latin America and 30% in Asia Pacific. In Iran, total pomegranate production increased 15% from 700 000 tonnes to 800 000 tonnes in 2007. India, China and Turkey are the next largest pomegranate producers. Some pomegranate juice may contain as much as 2.5 g of phenolic content with antioxidant activity per litre of juice.[23] Mango, guava, papaya, banana and more recently pomegranate are the most commonly processed tropical fruits. Other exotic fruits, such as açai, acerola and prickly pear, are now being processed because of their nutritional value and benefits to human health. Average tropical fruit composition is described in Table 11.5.[24]

The percentage of total global tropical fruit production used in processing is small (no official figures are available). Tropical fruits have a very short shelf life, fragile texture and delicate flavour. Processors must therefore have access to well-organized logistics and excellent storage facilities, as well as efficient and adaptable equipment. This also holds for stonefruits, such as apricot, peach and plums. In general, the fruit is first pureed using fast viscosity reduction (hotbreak and pectinases), then passes through a finisher (screen) before being placed in aseptic storage. The puree can then be transformed into a cloudy juice or clear concentrate.

11.5.3.1 *Process for puree nectar and clear concentrate*

The general process for tropical fruits is described in Fig. 11.8.

Following inspection, ripe fruit is washed (subjected to steam treatment in the case of papaya) and peeled. After destoning, the cut or crushed fruit is blanched for microbial stabilization and for deactivation of endogenous polyphenoloxidase. Ascorbic acid is added at this stage to prevent browning. The pulp is cooled before enzymes are added. Because of the high cellulose and hemicellulose content in tropical fruit, pectinases are combined with hemicellulases (arabinases, xylanases and galactanases are important for mango and guava maceration). Rapidase TF (DSM), Pectinex UltraSP (Novozymes) or Rohapect PTE (AB Enzymes) are recommended for viscosity reduction in pulpy tropical fruit. Once enzyme treatment is complete, the pulp is run through two finisher stages – one with an average 0.7–1 mm screen to remove residual fibres, skin and pip particles; another through 0.5 mm or smaller to produce a homogenous (smooth) puree – before undergoing a final hotbreak process in a tubular heat exchanger. The juice can then be concentrated, canned or de-aerated for aseptic cold storage. Fruit nectars can be produced by diluting the puree, adding sugar syrup, acidification using citric acid to maintain a constant sugar–acid ratio and heating to 100°C for aseptic packaging. Clear juice and concentrate can also be processed from the puree with a second addition of pectinases for complete depectinization. The use of exogenous enzymes helps produce high yields of purees or clear juices with good flavour and colour, and facilitates juice concentration.

Table 11.5 Tropical fruit composition.

Tropical fruit types	Total solids (%)	Soluble solids °brix	Specific components for 100 g	Titrable acidity g/100 g as malic	Aver pH	Total phenolics mg/100 g as gallic	Anthocyanins mg/100 g as cyanidine 3 glycoside	Antioxidant activity (μmol Trolox/g)
Açai	14.6	3.0	Lipids 5.1 g	0.13–0.3	4.5	280–530	40–95	20–40
Acerola	10.1	9.4	VitC 800 mg	1.10	3.2	248.9	25	57.7
Cashew apple	12.4	11.5	Vit C 139 mg Carotenoids 3.32 mg	0.16	4.7	295.4	7.2	16.7
Cajà	10.6	10	Sugars 4.54 g Fibre 0.75 g VitC 23.7 mg Carotenoids 28.3 μg	1.86	2.5	Tanins 300	–	–
Camu-camu	5.8	5.8	VitC 1.96 g	2.0	2.9	623.1	1.7	281.3
Cupuaçu	17.9	11–14	Sugars 9 g	2.17	3.6	253.2	–	–
Mango	17.9	11–14	Sugars 13.5 g Fibres 3.28 g Carotenoids 130–430 μg	–	3.6	–	–	–
Pineapple	10.1	10	Sugars 9.8 g Fibres 1.2 g VitC 17 mg	0.8	3.6	89.1	–	–

Pitanga	9.5	11.5	Sugars 8.26 g Fibres 2.1 g VitC 14 mg Carotenoids 900 µg	1.24	3.3	–	16.2	–
Papaya	12	7	Fibres 1.9 g VitC 62 mg Carotenoids 280 µg	–	5.5	–	–	–
Pomegranate	18	16.1	Fibres 1 g VitC 22 mg Carotenoids 40 µg	0.6	3.0	Until 2.5 g	175–380 mg L^{-1}	TEAC 20.5
Prickly pear	14	7	Fibres 3.5 g VitC 20 mg Carotenoids 60 µg	–	5.8	–	25 mg	–

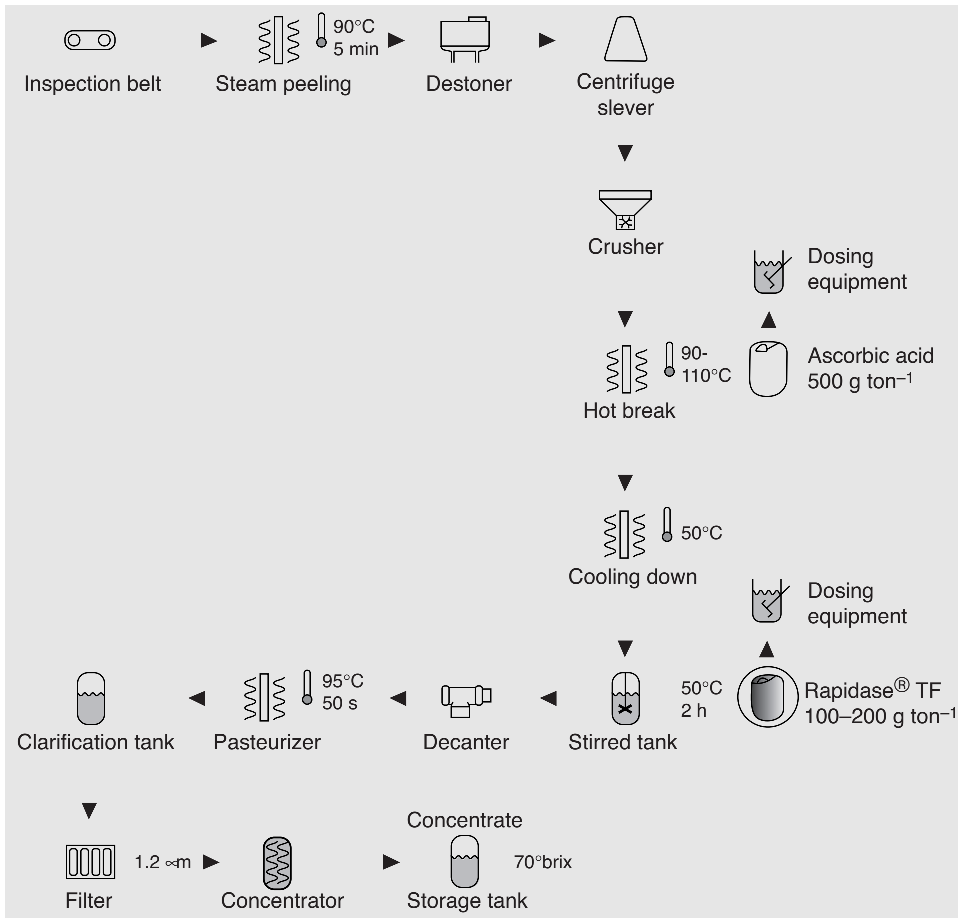


Fig. 11.8 Process for puree nectar and clear concentrate. *Source*: From ProJuice CDrom, DSM Food Specialties BV 2006.

11.5.3.2 *Pineapple mill juice*

In 2006, world pineapple production reached 18 mmt. That year the EU imported 188 052 tonnes of pineapple juice concentrate and 160 907 tonnes of single strength juice. Meanwhile, the US imported 223 139 tonnes of single strength pineapple juice, of which 141 987 tonnes came from the Philippines, 55 404 tonnes from Thailand and 21 088 tonnes from Indonesia (which exported a total of 32 829 tonnes). Thailand exported 182 043 tonnes of pineapple juice concentrate in 2006.[4] Philippines, Thailand and Indonesia are the world's leading pineapple producers. Pineapple juice is typically produced as a by-product of pineapple canning. Cloudy pineapple juice is processed from residual pulp (rejected chunks or flesh scraped from the skin) using a screw press, decanter and finishers to remove raw solids without using enzymes. A clear concentrate can also be processed from residual waste. So-called pineapple mill juice results from pressing surplus juice drained from eradicator meat, fruits that are too small for slicing, residual shell and skins, pineapple cores, trimmings and end cuts. Juices and solid fruit constituents are blended, crushed and pressed with a

screw press to get a 9–10° brix cloudy green juice. The juice is centrifuged to reduce green solid particles to below 5% and pasteurized. Pasteurization results in protein flocculation and juice is then cooled for clarification by static decantation. The resulting juice can be concentrated or used as canning syrup after fining, classic filtration and decantation. The systematic use of ultrafiltration is possible when using enzymatic treatments at the clarification stage after cooling to 50°C for several hours. Enzymes like Peclyve Pineapple (Lyven), Rohapect B1L (ABEnzymes) or Rapidase Pineapple (DSM) are used to process pineapple mill juice concentrate. The presence of natural gum in pineapple juice has been found to decrease ultrafiltration capabilities, to increase pulp suspension and foaming properties of the juice. This gum is a neutral polysaccharide containing 70% sugars that are predominantly galactomannans (2.25 mannose:1 galactose).[25] Because of this gum, the ultrafiltration flux rate quickly drops, becoming a bottleneck in the process (cut-off 10 000 Dalton). Enzymes improve the ultrafiltration flux rate since they contain the right hemicellulases to hydrolyze the gum. Juice viscosity is decreased, making it easier to clarify and concentrate at 62° brix for use as a cover syrup or a fruit sugar source after deionization of colour and aroma removal.

11.5.4 Citrus processing

Combined citrus production in Brazil, USA, China, Mexico and Spain was estimated at 72.8 mmt in 2006, of which 47.1 mmt were oranges, 15 mmt tangerines, 4.3 mmt lemons and 4 mmt grapefruits. That year Brazil produced the most oranges (18.2 mmt), followed by the USA (10.6 mmt), China (4.45 mmt) and Spain (2.7 mmt).[26] Global orange juice concentrate production in 2006 reached 2.3 mmt (at 65° brix). Europe imported 1.4 mmt of orange juice concentrate that year (excluding products from non-concentrate juice), and 92 840 tonnes of grapefruit juice concentrate. Argentina is the world's leading lemon producing country. It exported 46 347 tonnes of lemon juice in 2006.[4] A schematic diagram of orange processing is shown in Fig. 11.9 from Goodrich and Braddock.[27] Citrus juice processing gives rise to numerous by-products, of which pectin and essential oils are the most important.

There are several reasons why commercial pectinases are not used for premium juice processing. Their use is not permitted in certain countries, and classic pectinases containing pectinmethylesterase activity clarify the juice. Enzymes are therefore only used to process by-products. The key applications are described below.

11.5.4.1 *Pulp washing*

Expressed juice from extractors contains pulp and cell membrane residue. This is filtered out when the juice is run through a finisher to reduce pulp content to about 12%. The excess pulp and tissue by-products leaving the primary finishers are then put through a pulp wash system. The pulp wash system comprises a series of secondary finishers with multi-stage counter current washing systems. Around 5–7% of soluble solids are recovered, with a high pectin content. Pectinases Rohapect PTE (ABEnzymes), Citrozym (Novozymes) or Rapidase C80KPO or Rapidase Citrus cloudy (DSM) are added at ambient temperature for viscosity reduction, inducing recovery of sugars and soluble solids with high yield. The pulp wash product can be concentrated at 65° brix. This product can be added to sodas as a natural source of cloudiness. In this process, it is important to control and limit pectin degradation to reduce viscosity without destabilizing the particles that give

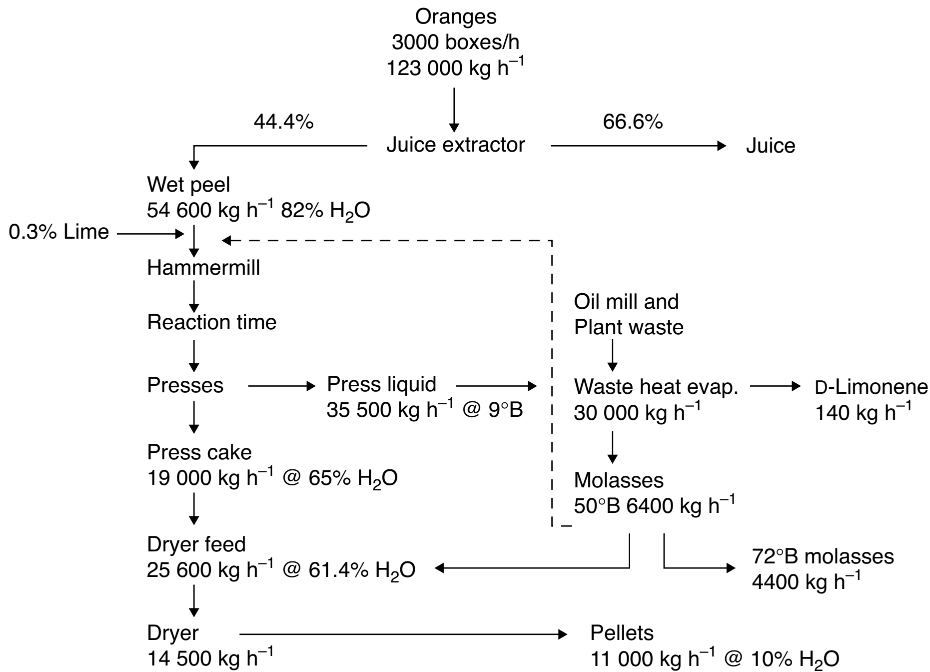


Fig. 11.9 Orange process diagram.

rise to the cloudiness. This can be achieved using classic pectinases with a high ratio endopolygalacturonase to pectinmethylesterase, or with a pure pectinlyase.

11.5.4.2 *Oil recovery*

Citrus peel contains essential oils. These oils are formed in the cells of the peel's so-called oil glands and they are one of the most important by-products of citrus fruit processing. Essential oils are in great demand from the food industry as taste and aroma agents, as well as from manufacturers of scented products. Several methods are available to recover essential oils. Juice and oil can be extracted in one step when FMC extractors are used for processing citrus fruit. In other methods of recovering juice, either the peel of the citrus fruit is pricked before the extraction process and the oil which appears is washed away with water (BROWN), or the outer section of peel (flavedo) is rasped before being mixed with water and then further processed (INDELICATO). The oil–water emulsion obtained in modern fruit processing lines contains approximately 70–90% of the essential oil naturally present in the peel. The so-called peel wash water contains between 2 and 5 kg essential oil as emulsion per 100 kg of raw material processed, depending on the type of fruit and the processing technology used. Depending on the type of fruit, the proportion of oil in the wash water is between 0.5% and 2.0% (vol.). The total volume of oil contained in the peel wash water can be efficiently recovered as clear essential oil with a suitably rated two-stage centrifuge system.[28] The finished emulsion passes through a sand cyclone and is fed to a desludger centrifuge to produce an oil-rich emulsion called cream, which is sent to a polisher that recovers the clear oil. After the desludger stage, the oil is concentrated up to 60–80%. In

the second stage, the polisher removes the remaining water and the very fine solid particles. Enzymes can improve the process speed, yield of oil from the cream and quality of the final product. Pectinases are often needed to improve oil–water separation in the emulsion. By hydrolyzing part of the complex pectin–proteins, oil is released more easily from the aqueous phase. Numerous pectinases preparations are available such as Citrozym CEO (Novozymes), Rohapect DA6L (ABEnzymes), Peclyve Citrus Oil (Lyven), Pektozyme Essential (Danisco) or Rapidase C80KPO or C80 Max (DSM). They are added to the emulsion before the desludger centrifuge. Oil yield recovery can be increased by 10–15% using these enzymes.

11.5.4.3 *Viscosity reduction*

Extracted finished juice is sensitive to microbiological contamination, enzymatic activities and chemical reactions, which all lead to fast deterioration in quality. Problems encountered in unpasteurized juices include cloud loss due to citrus endogenous pectinmethylesterase, gelation of the concentrate, fermentation and spoilage – even if the pH is low (3.0–4.0). These problems are prevented by quick pasteurization to inhibit the enzymes, even though this may cause undesirable browning and flavour changes in the juice. In countries where the use of pectinases is authorized, that is Brazil, citrus juice can be concentrated more easily after its viscosity is reduced. When added after the first finisher, pectinases such as Peclyve Citrus Juice (Lyven) or Rapidase Citrus Cloudy (DSM) speed up concentration without clarification if the holding time is limited and facilitate the production of any citrus juice concentate at 65° brix.

11.5.4.4 *Clear lemon juice concentrate*

Argentina, the world's number one lemon producing country, exported almost 50 000 tonnes of lemon juice in 2006. Lemon juice contains a complex colloidal system of particles that make up the lemon cloud. Lemon cloud is composed of approximately equal amounts of proteins and pectin to flavonoids and phospholipids.[29] Lemon can be processed as cloudy juice; however, demand for clarified lemon juice concentrate has increased in recent years, particularly from manufacturers of lemon-flavoured mineral water. Enzymatic clarification of lemon juice is very difficult because of its extremely low pH (around 2) which inhibits most fungal pectinases. In the past, the addition of a large amount of sulphur dioxide (about 2 g L^{-1}) resulted in juice clarification after 4–16 weeks. Recommended pectinases for clear lemon juice concentrate production are Rohapect 10L (ABEnzymes) or Rapidase Intense (DSM). These products contain pectinases that remain stable in lemon juice. After lemon juice extraction, the juice passes through a finisher and is then clarified. Pectinases are added to the juice after pasteurization and once the juice has cooled to 50°C or lower (4–8°C), oxidation is prevented. However, even if the juice is depectinized, it is not necessarily clarified. At this stage, it is still possible to produce a cloudy concentrate or a clear juice and concentrate. Addition of fining agents, such as high doses of bentonite or silica sol, is necessary for complete clarification. After a few hours, the juice can be centrifuged, filtered and concentrated.

11.5.4.5 *Citrus peeling and canning*

Fruit peeling, segmentation, residual albedo and segment membrane removal are necessary stages to prepare citrus salads using single or mixed fruits in fruit salad. The consumption

of thick skin citrus fruits, such as grapefruits, is enhanced by the commercialization of pre-peeling. This can be done by using mechanical peeling and chemical solubilization of the membrane using sodium hydroxide (I N) at high temperature (90–95°C). However, to achieve high-quality products from citrus fruits with fragile and thick skin albedo, manual methods are still commonly used. Alternatively, enzymatic treatments may be used to replace or facilitate the process, thus drastically decreasing the manpower needed and achieving gentler conditions. Citrus fruit skin is first scored or cut longitudinally. Albedo jellified by fruit blanching, especially useful for thick albedos species and cultivars, is digested by enzymatic treatment using Pectinolytic enzymes such as Peelzym (Novozymes) or Rapidase C80 Max (DSM). This provides easy fruit peeling and removal of albedo parts that usually stick on the surface of segments. This can be completed by residual albedo digestion which facilitates segment separation and cleaning.

Segment membrane removal or segment extraction can be facilitated by hand or by chemical and enzymatic dissolution. Manual extraction gives 'ready to can' cleaned segments; chemical and enzymatic dissolution will break down the membrane which is removed afterwards by water jet. This last step also removes chemicals or enzymes prior to canning and sterilization. In this way no residual enzyme activity will be present which may hamper final product quality.

Sterilization treatment and sometimes citric acid addition decreases the cohesion of vesicles at this stage by partial dissolution of pectin and the calcium chelating pectates, thus providing visible depreciation of segments aspects. Enzymatic demethylation treatment using NOVOSHAPE (Novozymes), Rapidase FP Super or Rapidase PEP (DSM), in combination with calcium and demethylated pectin to form more stable and less soluble pectate acting as adhesive in between the vesicles, preserves the texture and cohesion aspect of citrus segments during sterilization and shelf life.

11.6 FRUIT FIRMING

Consumers want fruit in processed food to look and taste good (i.e. have a firm texture, and a natural colour and flavour). However, most processed fruit, particularly soft fruit like strawberries or raspberries, is damaged through mechanical and thermal treatment, freezing or pasteurization. This has a negative effect on fruit texture, giving a mushy appearance and consistency. Fruit texture is attributed to the structural integrity of the primary cell wall and the middle lamella. Most ripe fruit has highly methylated pectin (HM pectin) above 50%. Adding a pure pectinmethylesterase results in partial pectin demethylation. Pectic acid, so formed, binds with bivalent cations like calcium, forming strong insoluble pectate gels in situ (egg box conformation). The gelling characteristics of the pectin are altered from HM pectin to low methylated pectin (LM pectin): LM pectins do not only form gels with sugar and acid, but also with less soluble solids and calcium ions (unlike HM pectin). LM pectates are also less soluble at high temperatures (often encountered during processing). The result is increased firmness, allowing the fruit to maintain its shape and consistency throughout the manufacturing process.

The DSM FirmFruit concept consists of combined fruit pectin demethylation in situ, with fungal pectin methylesterase enzyme and creation of a strong pectate network with added calcium, which overcomes the negative effects of mechanical and thermal treatment during fruit processing.

Many types of fruit may be processed using this concept, such as strawberries, raspberries, apples and pears, as well as tomatoes or any other pulpy fruit or vegetable, whether they are fresh, frozen or thawed.

DSM recommends a pure pectinmethylesterase Rapidase FP Super produced from a non-genetically modified strain of *A. niger*, or Rapidase PEP produced from a genetically modified strain of *A. niger.* Both are destroyed above 85°C during the final heating stage. Fruit damage and disintegration are minimized thanks to pectate stability throughout the manufacturing process. Yield, taste and appearance are improved.

11.7 VEGETABLE PROCESSING

World vegetable production reached some 1.3 bnmt in 2005. China was the top producer with around 620 mmt, followed by India (105.5 mmt) and the USA (57.1 mmt). In volume terms, potatoes (including sweet potato), tomatoes, watermelon, cabbage and onion are the top five 'vegetables' produced globally.[1] Vegetable juices are most commonly made from tomato, carrot and red beet (though tomato is actually a fruit). V8 Vegetable Juice is one commercial example, made mainly from tomatoes and the juices of seven additional vegetables: beets, celery, carrots, lettuce, parsley, watercress and spinach. Tomato juice makes up 87% of the total beverage. Consumer demand for vegetable juices is increasing and fruit and vegetable juice combinations are becoming more popular. Numerous products came on the market in 2007. These new drinks are cloudy and pulpy. It may be easy to produce tomato juice without enzymes, but this is not the case for carrot, leek or cabbage. In fruit, pectin is the major component to break down in order to facilitate juice extraction. But vegetables also have a high content of dietary ligno-cellulosic fibres, which hinder juice extraction. Vegetable juice processing therefore requires more cellulases in addition to pectinases to reduce viscosity sufficiently for juice extraction using a decanter. Figure 11.10 outlines cloudy and concentrate carrot juice processing.

Peclyve LI (Lyven) or Rapidase Vegetable Juice (DSM) is recommended for vegetable juice extraction: they contain pectinases and cellulases. After blanching (to limit oxidation), crushed vegetables are cooled to 50°C and mixed with enzymes. After 1–2 h, the juice is extracted using a decanter, pasteurized and possibly concentrated. The juice's carotene content is not decreased by enzymes, and the cloud is stable for the duration of the juice's shelf life.

11.8 NEW TRENDS AND CONCLUSION

Fruit and vegetable juices are natural, healthy beverages offering plenty of benefits to the consumer: they contain many important nutrients, improve general well-being, counteract negative aspects of a poor diet, have an appealing colour and flavour, and contain components like antioxidants that prevent coronary disease and delay cell ageing. In line with rising consumer diet and health awareness, the fruit and vegetable juices sector is expected to grow. Average global per capita juice consumption is set to rise from 24 L in 2007 to some 30 L in 2010.

Enzyme producers are constantly making advances in microbiology, genetics, fermentation, enzymology and application research, which they share with end-users. High-quality

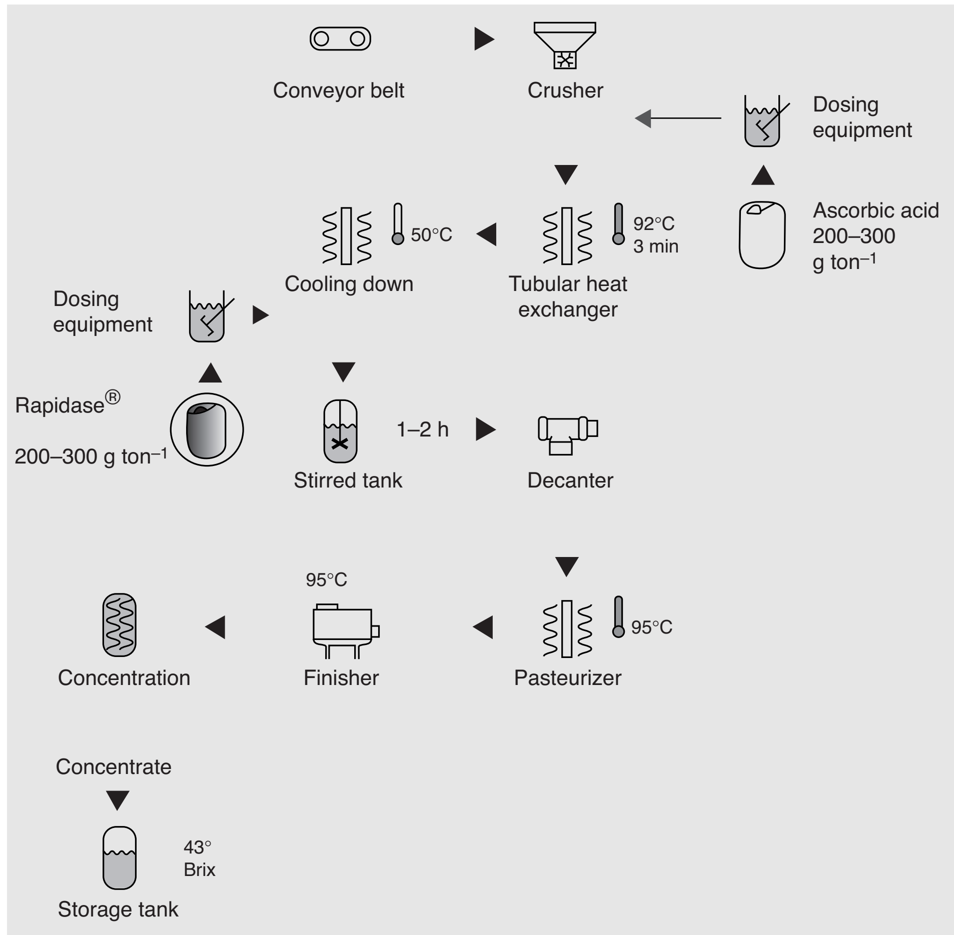


Fig. 11.10 Cloudy carrot juice concentrate. *Source*: From ProJuice Cdrom, DSM Food Specialties BV 2006.

enzymes are crucial processing aids, offering numerous advantages to fruit and vegetable processors – as outlined in this chapter. Enzyme suppliers are committed to selling high-quality enzymes to the fruit industry with reliable specifications and safety guidelines, providing all product information with transparency and assisting fruit processors in promoting fruit juices as healthy and natural products.

REFERENCES

1. United States Agriculture Department, Economic Research Centre (2008) *Vegetables and Melons Outlook/VGS-326*. USDA, Washington.
2. United States Agriculture Department, Foreign Agricultural Service (2008) *Market News: World Apple Situation/Apples*. USDA, Washington.
3. United States Agriculture Department, Foreign Agricultural Service (2008) *Citrus: World Market and Trade*. USDA, Washington.

4. Euromonitor (2006) *The Global Multiple Beverage Marketplace*. Euromonitor International Report.
5. Yanovsky, A. (2007) Sustaining growth in the Russian juice market. Paper given at *Foodnews World Juice Symposium*. Barcelona, 8–10 October.
6. Keegstra, K., Talmadgge, K., Bauer, W. and Albersheim, P. (1973) The structure of plant cell walls, part III. *Plant Physiology* **51**, 188–196.
7. Albersheim, P., Darvill, A.G., O'Neill, M.A., Schols, H.A. and Voragen, A.G.J. (1996) An hypothesis: the same six polysaccharides are components of the primary cell walls of all higher plants. In: *Pectin and Pectinases* (eds J. Visser and A.G.J. Voragen). Elsevier Science B.V., The Netherlands, pp. 47–55.
8. Grassin, C. and Fauquembergue, P. (1996) Fruit juices. In: *Industrial Enzymology*, 2nd edn (eds T. Godfrey and S. West). Macmillan Press Ltd, London, pp. 227–264.
9. http://www.uea.ac.uk/cap/carbohydrate/projects/RGII.htm
10. Vincken, J.P., Beldman, G. and Voragen, A.G.J. (1994) The effect of xyloglucans on the degradation of cell wall embedded cellulose by the combined action of cellobiohydrolase and endoglucanases from Trichoderma viride. *Plant Physiology* **104**(1), 99–107.
11. Carpita, N. and Gibeaut, D. (1993) Structural models of primary cell walls in flowering plants. *The Plant Journal: For Cell and Molecular Biology* **3**, 1–30.
12. http://www.dsm.com/en_US/html/dfs/genomics_aniger.htm
13. Doco, T., Lecas, M., Pellerin, P., Brillouet, J.-M. and Moutounet, M. (1995) Les polysaccharides pectiques de la pulpe et de la pellicule de raisin. *Revue Francaise d'Oenologie* **153**, 16–23.
14. Grassin, C., van Schouwen, D. and Veerkamp, H. (2007) Why invest in high quality enzymes? Economy, quality, sustainability and safety in fruit processing. Paper given at *IFU Congress*. Scheveningen, 17–22 June.
15. ftp://ftp.fao.org/docrep/fao/009/a0675e/a0675e00.pdf
16. Committee on Food Chemicals Codex (2003) *Food Chemicals Codex*, 5th edn. National Academy Press, Washington, DC, p. 999. http://www.usp.org/fcc/
17. http://ec.europa.eu/food/fs/sc/scf/reports_en.html
18. http://www.afssa.fr/
19. Grassin, C. (2004) Rapidase Smart, a new pectinase for apple juice extraction. *Fruit Processing* **3**, 172–176.
20. http://www.nal.usda.gov/fnic/foodcomp/search/
21. Macheix, J.J., Fleuriet, A. and Billot, J. (1990) Phenolic composition of individual fruit. In: *Fruit Phenolics* (eds J.J. Macheix, A. Fleuriet and J. Billot). CRC Press Inc., Boca Raton, FL, pp. 105–148.
22. http://www.fao.org/docrep/006/y5143e/y5143e1a.htm
23. Gil, M., Tomás-Barberán, F.A., Hess-Pierce, B., Holcroft, D.M. and Kader, A.A. (2000) Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. *Journal of Agricultural and Food Chemistry* **48**(10), 4581–4589.
24. Martins da Matta, V. (2008) Tropical fruit in Brazil, Embrapa Food Technology. Personal Communication.
25. Chenchin, K., Yugawa, H. and Yamamoto, H. (1978) Enzymic degumming of pineapple mill juices. *Journal of Food Science* **49**(5), 1327–1329.
26. http://www.ers.usda.gov/publications/vgs/tables/world.pdf
27. http://edis.ifas.ufl.edu/pdffiles/FS/FS10700.pdf
28. http://www.westfalia-separator.com/applications-processes/citrus-essential-oils.php
29. Carter, B. (1993) Lemon and lime juices. In: *Fruit Juice Processing Technology* (eds S. Nagy, C.S. Chen and P.E. Shaw). Agscience, Auburndale, FL, pp. 215–270.

12 Enzymes in meat processing

Raija Lantto, Kristiina Kruus, Eero Puolanne, Kaisu Honkapää, Katariina Roininen and Johanna Buchert

12.1 INTRODUCTION

Consumers' demands for high quality and moderate price meat products have been the driving forces to develop enzymatic methods to add value to lower quality meat piece, thus maximizing the efficiency of carcass utilization and of course improving the market value. In the meat industry, there are two distinct applications in which enzymes can markedly boost the manufacturing process and upgrade meats of poorer quality. These applications are tenderization of too tough meat parts and restructuring of fresh low-value meat pieces and trimmings to higher quality steaks. This chapter reviews those commercially available enzymes, that are already exploited in meat industry. In addition, the potential of several novel enzymes not yet marketed for meat processing use is discussed.

In meat industry and catering predominantly protein-degrading enzymes have been used. Of the protein cross-linking enzymes, transglutaminases (TGase) have been used as texture improvers already for several years. In addition to these, novel enzymes are discovered and their application potential assessed as well as novel applications for already known enzymes are developed. Structure engineering by oxidative enzymes and flavour design by lipases, glutaminases, proteases and peptidases are examples of emerging enzyme technologies in the food sector.

12.2 MEAT AS RAW MATERIAL

The composition of animals varies significantly, depending on species, breed and method of production, mostly feeding. The relative amounts of muscles, bones, fat and edible offal or inedible by-products obtained vary due to slaughtering methods and cultural reasons. As an example, blood or stomach and several other organs are in some countries used as feed ingredients and in some others partly as food. The way meat is consumed also varies greatly throughout the world.

The world meat production has increased tremendously during the last few decades, particularly in the developing countries.[1] The total meat production has risen, for example from 70 million tons in 1961 to 272 million tons in 2006, and the increase is expected to continue to 327 million tons till 2020.

12.2.1 Structure of muscle

The dominant feature of muscle is its fibrous structure that makes the function of a muscle in contraction possible. Due to its structure, muscle is capable of providing tensile strength and transmitting the force needed in contraction. Conversion of muscle to meat (discussed in more detail in Section 12.4.1) is a prerequisite for the development of desired eating qualities. Muscle begins to convert to meat when an animal has been slaughtered. At the death of an animal, a complex set of biochemical and biophysical changes is initiated in muscle. At a later stage, degradation of muscle proteins by the indigenous protease system leads to softening of myofibrillar structure and ultimately to meat tenderization. However, the fibrous structure is maintained, which gives meat its characteristic texture recognized by consumers.

A skeletal muscle is an assembly of bundles of muscle fibres that appears in bundles surrounded by a connective tissue sheath called perimysium. Muscle fibres are narrow, long (commonly several centimetres long with a diameter of 10–100 μm) and multinucleate cells.[2] They are the basic cellular units of living muscle and meat and are surrounded by a connective tissue sheath called endomysium, consisting mainly of collagen. Muscle fibres in turn consist of myofibrils, composed of parallel myofilaments. The functional (contractile) unit of a myofibril, a sarcomere, is an organized assemblage of parallel running actin and myosin filaments interacting with each other during contraction. In meat, these proteins affect functional properties such as texture and water holding.[3]

12.2.2 Chemistry and biochemistry of muscle

Meat consists of muscular tissue (containing also lipids and collagen), fat tissue and connective tissue. The chemical content of muscles is rather stable, but carcass meats are subjected to huge variation caused by animal species, age, anatomical location, nutritional condition, feed, etc. The main nutrition-dependent variable is the fat content and composition. A typical proximate composition of muscle is given in Table 12.1.

The amino acid composition of muscle protein is rather similar in different meat animals (Table 12.2). Collagen comprises about 25% total body protein and in muscular tissues the variation is from 0.2 to several percentages. In some tissues, for example in connective tissue membranes, skin and tendons, practically all proteins are collagen and elastin. Every third amino acid in the collagen molecules is glycine and other common amino acids are proline,

Table 12.1 Chemical composition of typical mammalian muscle (modified from Ref. [4]).

Component	Content (%)
Water	75
Protein	19
Myofibrillar	11.5
Sarcoplasmic	5.5
Connective tissue and organelles	2.0
Lipids	2.5
Carbohydrates and derivatives	1.8
Other	2.3
Minerals	0.7
Non-protein soluble substances	1.6

Table 12.2 Amino acid composition of fresh meats.[6]

Amino acid	Beef (%)	Pork (%)	Lamb (%)
Essential			
Isoleucine	5.1	4.9	4.8
Leucine	8.4	7.5	7.4
Lysine	8.4	7.8	7.6
Methionine	2.3	2.5	2.3
Phenylalanine	4.0	4.1	3.9
Threonine	4.0	5.1	4.9
Tryptophan	1.1	1.4	1.3
Valine	5.7	5.0	5.0
Non-essential			
Alanine	6.4	6.3	6.3
Arginine[a]	6.6	6.4	6.9
Aspartic acid	8.8	8.9	8.5
Glutamic acid[b]	14.4	14.5	14.6
Glycine	7.1	6.1	6.7
Cystine	1.3	1.3	1.3
Histidine[a]	2.9	3.2	2.7
Proline	5.4	4.6	4.8
Serine	3.8	4.0	3.9
Tyrosine	3.2	3.0	3.2

[a] Essential for infants.
[b] Glutamic acid and glutamine together.

hydroxyproline and alanine. Tryptophan is virtually non-existing, which lowers the nutritive value of collagens.[5]

12.2.3 Conversion of muscle to meat

Hundreds of different enzymes act in concert in a living muscle fibre enabling the contractile function, maintenance and growth of muscle. The energy of these reactions is derived from ATP. Quantitatively, the majority of the enzymes in muscle are dealing with the energy metabolism of the fibre. When the animal is stunned and bled, the fibres continue their metabolism as they would in an anaerobic situation in a living animal. The basic need to maintain the physiological ATP level is done by degradation of muscle polysaccharide glycogen and by subsequent anaerobic glycolysis. As a result, a drop of pH from the level of resting muscle of 7.2 to 5.5 will occur. Finally, the reduced pH and temperature will decrease the activity of glycolytic enzymes which in turn results in a gradual decrease of the ATP level. Subsequently changes in membranes, in cytoplasm and sarcolemma and denaturation of the fibre commence. Simultaneously, the main contractile protein complexes, which are normally kept separate by ATP, are irreversibly bound together, a phenomenon that is a called *rigor mortis*.

When ATP level falls to the subnormal level, fibre degradation is started by endogenous proteases, that is Ca^{2+}-activated calpains. The level of activity of calpains is dependent on the activators (Ca^{++}-content, pH and temperature) and inactivator calpastatin. They degrade the structural protein calpastatin and finally also calpains, which mean that they eliminate themselves in the long run.[7] The rate of activation and inactivation depends on time, pH and temperature in a very complex manner. The cooling regime causes considerable variation in tenderness depending on the stress history of animals before slaughtering and time–temperature–pH

combinations post mortem. These factors seem to be mainly responsible for the gradual loosening of the myofibrillar network soon after the formation of *rigor mortis*.

Cathepsins, another group of muscle proteases, are located in lyzosomes. Cathepsins degrade various myofibrillar components including myosin F-actin and troponin.[8]

In connective tissue, located within or between muscles, collagen molecules array to fibrils of 10–500 nm diameter and of unknown length. The collagen molecules of the fibrils are bound together with cross-bridges that are stabile in physiological conditions and make the fibrils inextensible. The degradation of collagen requires collagenase and another neutral protease activity. Once cleaved the collagen, fibril fractions can be degraded by a wide range of proteases even to the peptide and amino acid level. Denatured collagen is more easily degraded by proteases. However, degradation of native collagen is very slow.[5] Endogenic proteolytic system of meat itself is responsible for tenderization during natural aging. However, when tenderization is desired to be enhanced or tough pieces of meat need to be tenderized, plant or microbial enzymes can be added to boost tenderization. Tenderization with non-meat proteases is discussed in Section 12.4.

12.2.4 Factors in meat processing

12.2.4.1 *Heating*

Heating causes dramatic changes in meat protein systems. The myofibrillar proteins denature and thus open up and aggregate, thereby loosing a large proportion of their water binding. Additionally, there will be an increase of cross-bridges between the filaments, thereby reducing the space between the filaments. Upon heating, the collagen in connective tissue also denaturizes and shrinks, creating a contractive force within the meat. Collagen also is partly dissolved by heating, depending on the type of collagen, the age of the animal and temperature–time–pH of the heating process.[9] The endogenic proteolytic enzymes will also be denatured, but the muscle proteins become more susceptible to exogenous proteolytic enzymes. Heating and acid pH renders collagen, resistant to proteolysis in its native state, more susceptible to proteolysis.[5]

12.2.4.2 *Water binding*

Water binding is the most important technological property in meat and meat products. Good water binding is generally obtained when the myofibrillar proteins carry high (in most cases negative) net charge, when there are as little as possible cross-bridges in and between the structural elements and when the connective tissue membranes are thin and the collagen fibrils are non-mature.[10]

The great cause for water binding and variation therein are interactions between and in the myofilaments (actin and myosin). The amount of the water bound is determined by the net charge of the proteins, causing an increase in binding and by the number and strength of cross-bridges that limit the binding.[11]

The swelling depends on pH. Without salt there is a relative maximum of swelling at pH 3.0, a minimum at pH 5.0 (the average isoelectric point of meat proteins) and from there is a constant increase within the physiological pH range of 5.0–7.5. The water binding increases also with increasing salt content up to 5% NaCl.[10–14]

12.2.4.3 *Mechanical treatments*

Mechanical treatments such as mincing or chopping also affect the properties of meat. Mincing keeps the fibre bundles and fibres intact, but cut them across the longitudinal axis. In addition to that, chopping disintegrates the fibres also to some extent. By chopping, usually salt (and sometimes phosphates) and water are added, resulting in a swelling of myofilaments or a chemical disintegration and solubilization of myofibrillar proteins. Also connective tissue membranes are damaged to various extent, depending on the method of comminution. These phenomena may expose the structural proteins, for example added enzymes.

12.3 ENZYMES USED FOR MEAT PROCESSING

12.3.1 Proteases and peptidases

Since proteases have an important role in meat processing in tenderization, various exogenous proteases capable of digesting connective tissue and muscle proteins have been chosen for this purpose. Papain (EC 3.4.22.2), bromelain (EC 3.4.22.33) and ficin (EC 3.4.22.3) are being used at commercial scale meat tenderization.[15] Furthermore, proteases have been used for bone cleaning and flavour formation in the meat industry.

Proteases are ubiquitous enzymes, that are present in a wide variety of sources including plants, microbes and animals. They are essential for cell growth and differentiation. They catalyze the hydrolytic cleavage of the peptide bond present in proteins and peptides. Proteases constitute the most important group of industrial enzymes currently in use in terms of tonnages and sales value. They have a long history of applications in the food, detergent and leather industries.

Proteases are a very diverse group of enzymes in terms of action, structure and protein properties. They are commonly classified according to their origin (microbial, plant, animal), their mode of action (endopeptidases and exopeptidases) or the nature of the catalytic site. Endopeptidases cleave peptide bonds located internally in the protein. Exopeptidases cleave from the end of the polypeptide chain and are further classified to aminopeptidases or carboxylpeptidases cleaving either from an amino or a carboxyl terminus, respectively. Based on the catalytic group involved in the nucleophilic attack at the carbon of the scissile peptide bond in the substrate, proteases can be classified as serine proteases (EC 3.4.21), cysteine proteases (EC 3.4.22), aspartic proteases (EC 3.4.23) and metallo proteases (EC 3.4.24). The cleavage sites of some commercial endopeptidases are given in Table 12.3.

Papain from papaya latex (EC 3.4.22.2) is the most commonly used proteolytic enzyme in meat tenderization. In addition to papain, bromelain from pineapple stem (EC 3.4.22.32) and ficin from fig tree latex (EC 3.4.22.3) are known meat tenderizers.[16] Some examples of their experimental use are shown in Table 12.4. These enzymes have broad substrate specificity and they hydrolyze both myofibrillar proteins and connective tissue.[17,18] Papain preferably cleaves peptide bonds involving basic amino acids and it also has an esterase activity. Papain degrades myosin and actin at similar rates, whereas bromelain degrades myosin preferentially.[19] The common plant proteases do not hydrolase native collagen, but they act upon the gelatine, the heat-denatured form of collagen[16] generated during cooking.

The pH optimum of papain varies with the nature and concentration of the substrate. The pH optimum range is from 5.0 to 7.0. The optimum is pH 5.0 for gelatine and pH

Table 12.3 Cleavage site of some commercial endopeptidases.

Enzyme	EC number	Cleavage site (P1)
Serine proteases		
Chymotrypsin	3.4.21.1	tyr-, trp-, phe-, leu-
Trypsin	3.4.21.4	arg-, lys-
Subtilisin	3.4.21.12	Hydrophobic amino acids
Cysteine protease		
Cathepsin B	3.4.22.1	arg-, lys-, phe-X
Papain	3.4.22.2	arg-, lys-, phe-X
Ficin	3.4.22.3	phe-, tyr-
Bromelain	3.4.22.4	lys-, arg-, phe-, tyr-
Aspartic proteases		
Pepsin	3.4.23.1	Aromatic, leu-, asp-, glu-
Chymosin	3.4.23.4	phe105-met106 in casein
Metallo protease		
Thermolysin	3.4.24.27	ile-, leu-, val-, phe-

7.0 for casein and haemoglobin. Papain concentrate demonstrates optimum stability over the range of pH 5.0–9.0 at 60–70°C.[20] The enzyme is extremely temperature stable in comparison to other proteases. Effective activity is demonstrated over the temperature range of 10–90°C. Temperatures above 90°C rapidly inactivate the enzyme.[21] Either freezing or heat treatment can be used to inactivate the enzyme and prevent over-tenderization and sensory defects.

The latter is obviously unsuitable for raw products. Alternative methods for enzyme inactivation have also been investigated. Potato protein and ascorbic acid have been studied as possible inhibitors for papain, but the results have not been very promising.[22]

Plant proteases mainly used to tenderize meat have limited activity on collagen. Attempts to tenderize collagen-rich connective tissue with tenderizing proteases inevitably led to extensive hydrolysis of non-collagen proteins and resulted in too soft meat. Collagenases (EC 3.4.24.3) are proteases that degrade the native collagen, which consists of fibrils composed of laterally aggregated rod-like tropocollagen. Tropocollagen unit consists of three helically twisted polypeptide chains, having glycine residues at every third position and an abundance of proline and hydroxyproline.[23] Currently, food-grade collagenase preparations are not commercially available although the need is evident.

Most of the reported collagenases are from human and other mammalian sources. Bacterial collagenases also exist, usually from pathogenic strains. Microbial collagenases are more unspecific attacking various sites along the collagen helix.[24] The most thoroughly studied microbial collagenase is from *Clostridium histolyticum.*[25] In addition collagenases from *Achromobacter iophagus*,[26] *Pseudomonas aeruginosa*[27] and from a marine bacterium[28] have been reported.

Sugasawara and Harper[29] reported on purification of three collagenases of *Cl. histolyticum* with molecular weights of 96 000, 92 000 and 76 000 Da. *Cl. histolyticum* collagenases have their pH optima at 7–9 and they require Ca^{2+} for activity.[30] Crude preparations of *Cl. histolyticum* contain several other proteases, such as a sulfhydryl protease[31] and a trypsin-like protease.[32] Due to its origin *Cl. histolyticum* collagenase is not suitable for meat applications.

Actinidin (EC 3.4.22.14) from kiwifruit tenderizes meat and it has been found to hydrolyze also collagen to a certain degree[33,34] (Table 12.6). According to Morimoto *et al.*,[35] actinidin

Table 12.4 Examples of the use of plant proteases (and protease-containing fractions) in meat tenderization.

Protease	Raw material meat	Processing conditions	Effect on meat texture and other quality factors	References
Papain	Turkey drumsticks, baking hens and roasters	Injection with marinade containing papain	All products significantly more tender than the controls. The flavoured marinade probably masked some of the off-flavour caused by papain	90
Papain	Beef meat	Injection with papain and treating with high pressure (100–300 MPa for 10 min)	Papain and pressurization to 100 MPa significantly increased tenderness. Higher pressures did not lead to further tenderization	91
Bromelain	Beef cubes	Dipping in enzyme solution before freeze drying	Improved texture and marked effect on collagen solubilization	82
Bromelain	Beef plates	Injection into beef plates processed into beef bacons	Some effect on texture, negative effect on visual appearance	92
Bromelain	Beef round muscles	Injection with bromelain solution	Improved tenderness, although salt and phosphate injection was more effective in some cases	93
Ficin	Ground beef	Ficin-tenderized meat in sausage manufacturing	More protein solubilization in meat and improved water-holding capacity, emulsion stability and other quality factors of sausages	88
Actinidin or papain	Beef semitendinosus steaks	Incubation enzyme solution for 30 min before cooking	Actinidin did not hydrolyze as many myofibrillar proteins as papain but resulted in the same degree of meat tenderization without over-tenderized surface	33
Crude actinidin	Beef semitendinosus muscle and achilles tendon	Immersion in crude actinidin and treating with high pressure at 0–500 MPa	Protease decreased shear force values but pressure treatment had no significant effect on it. Protease solubilized some non-heated α-chain collagen, pressure increased effect slightly	34
Crude ginger extract	Beef steak and sliced beef	Marinating	Significantly increased tenderness	94
Ginger extract	Sheep meat chunks	Marinating (24 h at 4°C)	Decreased shear force and increased cooking yield, water-holding capacity and collagen solubility	95

Table 12.4 (*Continued*)

Protease	Raw material meat	Processing conditions	Effect on meat texture and other quality factors	References
Ginger extract	Buffalo meat chunks	Marinating (48 h at 4°C)	Improved tenderness of tough buffalo meat, no adverse effects on other meat quality parameters	96
Ginger extract	Goat meat chunks	Marinating 24 h at 4°C, then ground	Increased protein, especially collagen, solubility, tenderness and shelf life	79
Ginger powder	Spent-hen muscles	Immersion in water containing ginger powder	Lower shear values and higher sensory scores and proteolysis	97
Ginger extract, cucumis extract or papain	Pork chunks	Curing in solutions containing 7.5% cucumis or 9% ginger extract or 0.50% papain powder, then prepared to enrobed products	All treatments lowered shear force values and enhanced overall acceptability compared to control. Ginger additionally increased shelf life	98

had no collagenase activity under acidic conditions, although it was capable of hydrolyzing atelocollagen (pepsin-hydrolyzed collagen). Mostafaie *et al*.[36] have recently shown that actinidin can hydrolyze collagen types I and II at neutral and alkaline conditions, but the action is inhibited in an acidic pH range. Nevertheless, it has been shown that the pretreatment of beef connective tissue with kiwifruit juice before heating significantly decreased the shear force of connective tissue after heating in comparison with other pretreatments without protease activity.[37]

Along with collagen, elastin contributes to the toughness of connective tissue of meat. Elastase enzymes from *Bacillus* (EC 3.4.4.7)[38,39] have been shown to improve meat tenderness. Elastolytic activity of elastase from alcalophilic *Bacillus* sp. Ya-B was found to be clearly higher than that of papain or bromelain, and the enzyme preferred elastin and/or collagen to myofibrillar proteins.[38]

There is a current interest to discover novel meat tenderizing proteases both from plant and microbial origin. One example of a source for a novel potent enzyme is Kachri fruit, *Cucumis trigonus* Roxburghi, which traditionally has been used as a meat tenderizer in the Indian subcontinent and contains a cucumicin-like (cucumicin EC 3.4.21.25) serine protease activity.[40] Recently proteases from ginger rhizomes (*Zingiber officinale*) have also gained a lot of interest due to their collagenolytic activity. Choi and Laursen[41] reported about two ginger proteases (GP-I and GP-II) and showed that they belong to the papain family of cysteine proteases.

12.3.2 Lipases

Lipases (triacylglycerol lipase EC 3.1.1.3) can be used for flavour formation in sausage production. Endogenous enzymes, enzymes from starter cultures or isolated commercial enzymes can be used in the application. Lipases are ubiquitous in nature and are found in

various organisms including animals, plants, fungi and bacteria. Lipases are esterases which are key enzymes involved in fat digestion. They convert insoluble triacylglycerols to more soluble fatty acids and di-and monoacylglycerols, which can be assimilated by the organism. All known lipases belong to the family of α/β-hydrolases, which share a common fold composed of a central hydrophobic eight-stranded β-sheet packed between two layers of amphiphilic α-helices.[42,43] They also share a common catalytic mechanism. The active sites of lipases are composed of a Ser-Asp/Glu-His motif, which compose a catalytic triad similar in arrangement to serine proteases.[44,45]

Most microbial lipases exhibit maximum activity in the temperature range of 30–40°C.[46] Thermophilic lipases retaining activities in the temperature range of 50–65°C have been isolated from both filamentous fungi (*Aspergillus niger*, *Thermomyces lanuginosus*) and some bacteria (*Pseudomonas* and *Bacillus* sp.). Maximum activity of most microbial lipases is displayed in the pH range of 5.6–8.5 and maximum stability in the neutral pH range.[46] Some clearly alkaline lipases having pH optima around 9.5 have been discovered, for example from *Bacillus* and *Pseudomonas* species.[47,48] Lipases are commercially available from different suppliers also in food grade form.

12.3.3 Transglutaminase

TGase (EC 2.3.2.13) can be used for structure engineering for tailoring the structural properties of different processed and heated meat products. The enzyme is an acyltranferase and it generates formation of covalent linkages in the food matrix. The acyl-transfer reactions are formed between a γ-carboxyamine group of a peptide-bound glutamyl residue (acyl-donor) and a primary amino group (acyl-acceptor) of various substrates including the ε-amino group of lysyl residues in certain proteins.[49] The formed linkage is an ε-γ-glutamyl-lysine isopeptide bond. TGases have been reported to cross-link many food proteins as has been described in various reviews.[50–52] TGase-catalyzed reactions lead to inter- or intramolecular cross-linking in proteins, depending on whether the glutamyl and lysyl residues are located on the same or different proteins.

TGases are widely distributed enzymes discovered in many different animal tissues and body fluids, fish, birds, invertebrates, amphibians, plants and microbes. They are involved in several biological functions including blood clotting, wound healing, epidermal keratinization and in a number of human disease states.[49,53] Discovery of microbial TGases from *Streptomyces* and *Streptoverticillium* species in late 1980s[49] enabled fast development of TGase for various food applications including meat processing.[54] TGases are commercially available from Ajinomoto Inc. and Yiming Biological products Co. Ltd. TGases from different origins differ in their cross-linking ability towards myofibrillar proteins. Mammalian TGases[55–57] tend to modify in addition to myosin also actin, while microbial TGases originating from *Streptomyces* have only a limited activity on actin. In addition to myosin and actin, troponin T has also been found to be modified by microbial TGase.[58,59]

Depending on the source, properties of TGases vary considerably. Both guinea pig liver and *Streptomyces mobaraensis* (former *Streptoverticillum mobaraense*) TGases are monomeric proteins with a molecular mass of 75 and 38 kDa, respectively.[60] In contrast to mammalian TGases the activity of *Streptomyces* TGases is independent of Ca^{2+}. The enzyme shows pH optimum between 5 and 8, and has substantial activity at pH 4–9.[60] The enzyme retains full activity at 40°C for 10 min but is totally inactivated within a few minutes at 70°C.

12.3.4 Oxidative enzymes

Oxidative enzymes can be an alternative for TGases to generate cross-links in protein matrices. Oxidoreductases including tyrosinases and laccases are reported to cross-link meat proteins.[1,58] These enzymes have been tested in only few applications, mainly because of limited availability.

Tyrosinases (EC 1.14.18.1) are capable of generating cross-links in a protein matrix as such or together with some small molecular weight compounds.[61] The physiological role of tyrosinases is related to melanin and eumelanin synthesis. In fruits and vegetables, tyrosinase is responsible for enzymatic browning reactions and in mammals for pigmentation. In fungi the role of tyrosinase is correlated with cell differentiation, spore formation, virulence and pathogenesis. Tyrosinases are copper-containing proteins, containing two type-3 copper atoms in their active site to shuttle electrons from the substrate to molecular oxygen, which is a terminal electron acceptor. The most extensively investigated fungal tyrosinases both from a structural and functional point of view are from *Agaricus bisporus*[62,63] and *Neurospora crassa*.[64] An interesting tyrosinase was recently discovered from the filamentous fungus *Trichoderma reesei*.[65] Sigma and Fluka sell crude *Agaricus* tyrosinase for research purposes.

Laccases (EC 1.10.3.2) have also been shown to cross-link proteins and peptides.[66–68] Their best known role in nature is related polymerization and depolymerization processes of lignin.[69] Laccases are also copper-containing enzymes. They contain four copper atoms in their active site and use molecular oxygen as a terminal electron acceptor. Laccases show surprisingly broad substrate specificity, being capable of oxidizing various phenolic compounds, for example diphenols, polyphenols, different substituted phenols, diamines and aromatic amines.[69] Where tyrosinase-catalyzed cross-linking is based on quinone formation, laccase-catalyzed cross-linking is based on free radicals and their further reactions. Laccases oxidize their substrates with a one-electron removal mechanism.[69] The unstable radicals undergo further non-enzymatic reactions including polymerization.

12.3.5 Glutaminase

L-Glutamic acid is a well-known flavour enhancing amino acid. For instance, the unique flavour of fermented soya sauce is mainly due to glutamic acid. L-Glutaminase (L-glutamine aminohydrolase EC 3.5.1.2) produced by starter cultures has an important role in flavour formation, for example in sausage production. By supplementing glutaminase to fermented seasoning agents, such as miso, soya sauce and pickles, the glutamic acid content of these products can be bolstered, making it possible to prepare food products with increased 'umami' taste. Glutaminases catalyze the hydrolytic deamidation of L-glutamine to L-glutamic acid, a flavour enhancer and ammonia, an acidity neutralizer. They belong to a large subfamily of serine-dependant β-lactamases and penicillin-binding proteins (for a review see Nandakumar *et al.*[70]). L-Glutaminase is strictly specific to L-glutamine and thus differs from glutaminase–aspariginase (EC 3.5.1.1) which catalyzes hydrolysis of both glutamine and asparagine with similar efficiency.

Glutaminases are ubiquitous enzymes in bacteria and eukaryotes but seem to be lacking in archaea, thermophiles and plants.[70] The majority of the microbes having glutaminases have been isolated from soil and a few from marine environments. Glutaminase from Bacillus amyloliquefaciens is commercially available as industrial enzyme preparation from Amano Enzyme Inc. Glutaminases have been purified and characterized for instance from *Aspergillus oryzae*,[71,72] *Rhizobium etli*[73] and *Debaryomyces*.[74] Most of the reported glutaminases are

intracellular enzymes although some extracellular glutaminases have been reported. The main cellular function of glutaminases is proposed to be associated with the control of intracellular concentration of glutamine, which is an essential nitrogen metabolite.[75]

Many reported glutaminases work optimally at a temperature range of 40–50°C and at neutral pH.[70] High salt concentrations can markedly inhibit glutaminases. For example, *A. oryzae* glutaminase is inhibited at 3 M NaCl concentrations,[70] which can hamper the use of the enzyme in applications requiring high salt concentration. Therefore salt-tolerant glutaminases have been screened for. Some marine bacteria including *Micrococcus luteus* K-3 have been shown to tolerate up to 16% (w/v) concentrations of NaCl.[76]

12.4 MEAT TENDERIZATION WITH ADDED ENZYMES

Of all the attributes of eating quality, texture and tenderness are presently rated as most important by the average consumer. Methods to increase tenderness include, for example natural aging, electric stimulation, mechanical blade tenderization and use of added proteolytic enzymes (see Section 12.3). The most widely used exogenous enzymes in meat tenderization are the plant enzymes papain, bromelain and ficin. Plant proteases, especially papain and bromelain, have been studied for tenderization purposes for decades. Some examples of the use of plant proteases or protease-containing fractions in experimental meat tenderization are given in Table 12.4.

The way of applying tenderizing enzymes in meat industry depends on the actual target. If the aging time of high-grade meat parts has to be shortened, the main action of protein hydrolysis should be on myofibrillar proteins. If the tenderness improvement of lower grade meat cuts or meat from, for example aged animals is desired, the connective tissue proteins, mostly collagen, should be the target of proteolysis. The methods and challenges to tenderize meat sold raw to consumers differ from those needed for cooked meat. Plant proteases mainly used to tenderize meat act unfortunately more actively on other meat proteins than on collagen. Therefore attempts to tenderize collagen-rich connective tissue inevitably led to too extensive hydrolysis of non-collagen proteins, resulting in too soft (mushy) meat. To tenderize meat pieces with a high connective tissue content it is evident that an enzyme having pronounced activity against connective tissue but limited activity against myofibrillar proteins should be used. Potential collagenases come mainly from microbial origins, but unfortunately up till now there are no commercial food-grade collagenases available.

Although not suitable for food manufacturing purposes, some collagenases have been studied in meat tenderization. Foegeding and Larick[17] studied *Cl. histolyticum* collagenase in tenderization of beef steak but the results were not very promising. Clear positive effects were achieved in the study where microbial collagenases were evaluated for their ability to degrade collagen in a restructured beef product. Extreme thermophilic bacteria species have been searched in order to find proteases which would be active against collagen during a controlled cooking period of meat and show only limited activity during often uncontrollable cold storage periods.[77,78]

Interesting new proteases from plant origin are cucumis and ginger extracts. Powdered cucumis extract from the Kachri fruit as well as ginger proteases has successfully tenderized meats from different species (see examples in Table 12.4). Ginger extract has proven to be especially effective in increasing collagen solubilization.[79]

12.4.1 Methods for enzyme application in meat tenderization

Due to biochemical consistence and structure of meat it can be challenging to evenly distribute the tenderizing protease into meat pieces. Possible methods are, for example spraying, injection, dipping and marinating. During past decades many reports about pre slaughter injection of inactivated plant protease – mainly papain – solution into the living animals' vascular system have been published. This is done in order to achieve thorough distribution of enzyme throughout the carcass. This has been described for lamb[80] and beef[81] and this *antemortem* process has been used at least previously industrially in many countries.

Dipping of meat pieces in solution containing proteolytic enzymes or marinating in such solution has been widely used (e.g. Quaglia *et al.*[82] and Naveena and Mendiratta[83]). The problem with that method is the poor penetration of the enzyme into the meat pieces and the resulting possible over-tenderized surface and mushy texture whereas the interior remains unaffected.[16]

Injection of proteolytic enzyme solution directly into meat pieces has been shown to be a more effective way of tenderization than marinating in enzyme-containing solution. Much higher dosages of papain were needed in marinating compared to injection to achieve the same level of tenderization. Most likely this is due to restricted enzyme–substrate contact area in marinating.[84] Even the carrier solution used for enzyme injection may have significant effect on meat quality attributes.[85] Huerta-Montauti *et al.*[86] found that treating beef muscles with papain containing brine solution in a vacuum tumbler permitted the throughout distribution of the enzyme to the entire muscle and allowed the breakdown of structural proteins.

The cooking procedures used in meat manufacturing highly affect the activity of tenderizing enzymes. The more time the product is kept at or near the temperature optimum of used enzyme, the more protein hydrolysis and also tenderization are expected to happen.[87] For optimized quality the processing conditions should be adjusted to suit the activity, but also the inactivation of the enzyme(s) used. Meat industry can use enzymatically tenderized meat to produce high-quality ready-to-eat products, for example sausages. Tenderization of meat with enzymes increases solubility of meat proteins, which may have marked positive effect when they are used as raw material in processed meat products. Tenderization of beef meat with ficin before using it in sausage manufacturing, substantially improved water-holding capacity, emulsion stability and other quality factors (e.g. taste) of sausages.[88] When the enzyme-aided tenderization takes place in meat industry facilities, the process is highly controllable. This is in contrast to the situation where enzyme is applied to meat which is then sold raw to consumers.

Different enzymes or enzyme mixtures are suitable for different meat cuts. For fine-tuned applications, the substrate specificity and activity profiles of applied enzyme preparations should be known to adjust the enzyme levels and processing conditions to suit each other. The use of plant extracts, for example plant puree or juice, rich in tenderizing enzymes can contribute to good tenderizing effect with supposedly lowered costs compared to commercial enzymes.[37,89]

In the future it could be possible by means of modern microbial methods to produce different kind of fine-tuned proteases for different needs of meat tenderization: one for accelerating the tenderization of high-quality red meat (effect on myofibrils), one for degrading the connective tissue of lower grade meat from old animals sold as raw (effect on collagen at low temperatures) or used as raw material in industry (effect on denatured collagen).

12.5 ENZYMATIC GENERATION OF FLAVOUR IN MEAT PRODUCTS

Flavour is defined in the literature as multimodal phenomenon in which several independent sensory modalities such as taste, aroma, trigeminal sensation and texture are involved.[99] Flavour has a major role, along with tenderness, in acceptability of meat by consumers.[100] Because of the relationship of flavour and consumer acceptability, it is important to understand the factors influencing meat flavour in order to produce good-quality meat products. The flavour of raw meat is quite bland. However, it contains non-volatile constituents that are essential flavour precursors which during processing and storage affect the taste of the meat product.[16,101] In general, the flavour of processed meat is a result of either enzymatic action or chemical reactions such as pyrolysis of amino acids and peptides, sugar degradation, degradation of ribonucleotides, Maillard reactions, thiamine degradation and degradation of lipids. The main enzymatic reactions affecting meat flavour or formation of flavour precursors are proteolysis and lipolysis. Both groups of reactions are due to the contribution of either endogenous proteases and lipases, enzymes of microbial origin naturally present in the product or enzymes added during the manufacturing process.

Most of the literature about the use of enzymes or starters in flavour formation deals with fermented meat products. Literature covers mainly the flavour formation of Mediterranean type dry-cured products such as Iberian, Serrano, Parma and Bayonne hams or Italian salami, Spanish chorizo or French saucission sec and less of Northern European dry-cured fermented meat products. Typical to Mediterranean-type products is a slow curing process with no nitrite and no smoking. Whereas in the Northern European countries, nitrite is used and the smoking is an integral part of curing.[102,103] Due to the numerous studies devoted to optimization of flavour and ripening of dry-cured meat products by enzymes, attention is paid mostly to the meat product types discussed below.

12.5.1 Proteolysis and lipolysis in meat flavour development

Dry-cured meat products are appreciated for their unique flavour. The compounds implicated in flavour generation arise from many sources, such as spices, sugar metabolism, lipolysis and lipid oxidation, proteolysis and amino acid degradation.[104–106] During ripening proteolysis takes place, yielding for example polypeptides, peptides and free amino acids, which are involved in taste and flavour development of meat products. Meat protein hydrolysis is mainly catalyzed by endogenous enzymes, such as cathepsins (described in Section 12.3.2) and trypsin-like peptidases as well as proteases (described in Section 12.3.1) produced by microorganisms are involved in the ripening process. These enzymes are mainly originating from *Micrococcaceae* but also from moulds and yeasts in those dry sausages in which they are present.[107] Glutaminase addition to protease has an important role from the point of view of sausage production, especially regarding the deamidation of glutamine, since hydrolysis of the glutamine amide group produces ammonia, an acidity neutralizer and umami flavour. Umami can be described as savoury or broth-like taste with ability to enhance other flavours (glutaminase is described in Section 12.3.5).

Lipolysis constitutes another important group of enzymatic reactions which are related to aroma formation of fermented sausages. Phospholipases and lipases hydrolyze phospholipids and triacylglycerols forming free fatty acids (lipases are described Section 12.3.2). Unsaturated fatty acids are further oxidized to aroma volatile compounds. This oxidation may lead

to the formation of aliphatic hydrocarbons, alcohols, aldehydes and ketones. Further alcohols react with free fatty acids forming some esters.[101,108–110]

12.5.2 Effect of enzymes on ripening of dry-cured meat products

Dry-cured meat products need a long period of ripening in order to allow the transformation of free amino acids and fatty acids through microbial (oxidative deaminations, decarboxylations) and/or chemical (Maillard reactions) ways to yield aromatic compounds (aldehydes, ketones, lactones, alcohols and esters). Because long ripening time involves a high cost of storage until a suitable matured state is reached, many attempts have been made to shorten this period. Proteases and lipases have been used for this purpose. However, it has been found that addition of proteinases and lipases alone is not useful in shortening the ripening time. This is because the final flavour also depends on subsequent generation of volatile compounds through lipid oxidation and amino acid catabolism. Therefore, to shorten the ripening of sausages, it is necessary to create conditions, for example by adding an efficient starter or by adding other types of enzymes, which stimulate formation of volatiles in a shorter time than usual. Table 12.5 shows examples of the use of enzymes for flavour formation and for shortening the ripening time.

The most promising method to shorten ripening time of fermented sausages has been obtained by the incorporation of cell-free extracts from lactic acid bacteria and moulds. The addition of cell-free extract from *Lactobacillus paracasei* sbsp. *paracasei* has been found to accelerate the ripening and improve the sensory quality of sausages.[111] The addition of cell-free extracts from moulds such as *Mucor racemosus* and *Penicillium aurantiogriseum* also leads to improved sensory properties of fermented sausages by increasing the generation of ammonia and volatile compounds derived from amino acid catabolism.[112–114] Bolumar *et al*.[115–117] purified and characterized two proteinases (PrA and PrB) and two aminopeptidases (arginyl aminopeptidase and prolyl aminopeptidase) from cell-free extract of the yeast strain *Debaryomyces hanseii* CECT 12 487. The enzymes were used to catalyze the hydrolysis of sarcoplasmic proteins and, together with amino acid converting enzymes, to generate ammonium and thereby increase the pH. Bolumar *et al*.[118] used above-mentioned cell-free extract together with the extract from *Lactobacillus sakei* CECT 4808 containing high exoproteolytic activity to accelerate the proteolytic pathway and thereby improve the sensory quality of fermented meat products. By addition of these extracts the sensory quality of fermented meat products was improved by promoting the generation of volatile compounds derived from lipid oxidation and carbohydrate fermentation.

12.6 STRUCTURE ENGINEERING BY CROSS-LINKING ENZYMES

Apart from the hydrolytic enzymes affecting the tenderness of meat or generation of flavour, the functional properties of meat proteins can be modified by cross-linking enzymes. These enzymes are used to bind fresh meat pieces together and to tailor the structural properties of various processed meat products. The main target protein in meat for cross-linking enzymes is the myofibrillar protein myosin. Cross-linking enzymes are generally capable of positively affecting gelation and consequently the texture of meat gels. Potential cross-linking enzymes

Table 12.5 Examples of the use of enzymes in generation of meat flavour.

Enzyme	Type	Application	Effect on meat flavour	References
Papain	Thiol proteinase	Spanish dry-fermented sausages (*salchichón*)	No significant differences in sensory properties compared to control sausage	119
Pronase E	Mixture of proteinase, amino- and carboxypeptidases	Spanish dry-fermented sausages (*salchichón*)	No significant differences in sensory properties compared to control sausage, except in high amounts excessive softening occurs	120
Palatase M 200 L, Novozymes and Protease P 31 000 Solvay Enzymes	Mixture of acid, neutral and alkaline proteases	Spanish dry-fermented sausages	Slight softening of the product, no other sensory differences observed	121
Protease E and fungal extract *Penicillium aurantiogriseum*	Mixture of proteinase, amino- and carboxypeptidases	Spanish dry-fermented sausages (*salchichón*)	The combined action of protease and fugal extract improved both flavour and texture characteristics	112
Protease Flavourzyme and Lipase Novozyme 677BG, Novozymes	Mixture of proteases with both exo- and endopeptidase activities	Spanish dry-fermented sausages	Significant increase in the concentration of some esters and acids but no increase in amino acid derivates. Effect on sensory properties not mentioned	122
Lactococcus lactis Subs. *cremoris* NCDO 763, α-ketoglutarate and papain with proteolytic, peptidolytic and aminotransferase activities	–	Dry-fermented sausage	*Lactococcus lactis* Subs. *cremoris* NCDO 763 and α-ketoglutarate together increased content of the volatile compounds responsible for the ripened flavour and sensory quality	123
Fungal protease Epg222	Serine protease	Spanish dry-fermented sausages (*salchichón*)	Higher aroma intensity and lower values of hardness compared to control	124
Cell-free extract from *Lactobacillus sakei* and *Debaryomyces hansenii* with aminopeptidase and proteinase activities	Arginyl and prolyl aminopeptidase and endoprotease	Dry-fermented sausage	Addition of *D. hansenii* and *L. sakei* together promoted the generation of volatile compounds derived from lipid oxidation carbohydrate fermentation	118
Starter culture (*Penicillium chyrsogenum and Debaroymyces hansenii*) having proteolytic activity	–	Dry-cured ham	No remarkable differences in volatile profile compared to reference. Better overall acceptability due to the improved texture	125

Table 12.5 (*Continued*)

Enzyme	Type	Application	Effect on meat flavour	References
Starter culture (*Staphylococcus xylosus* CVS11 and FVS21 and *Lactobacillus curvatus*) having proteolytic and lipolytic activity	–	Italian fermented sausage (salami)	Experimental sausages had less ripened flavour and less greasy and decreased pH compared to control sausage	126

for structure engineering of meat systems are summarized in Table 12.6. Hitherto TGase has been the main cross-linking enzyme studied and applied industrially in meat protein modification. The ability of TGase to cross-link meat proteins has been generally known for over two decades. Although the most obvious source of TGase for the meat industry would be blood, microbial enzymes have superseded the mammalian ones due to their Ca^{2+}-independency, favourable pH and temperature profiles and commercial availability for industrial use.

12.6.1 Restructuring of unheated meat

High-quality meat products at moderate prices demanded by consumers have been the driving forces to develop methods to restructure low-value cuts of poorer quality to improve their market value by making them palatable steaks resembling intact muscle and to maximize the efficiency of carcass utilization. Traditionally salt and phosphates in conjunction with heat treatment have been used to bind meat pieces together. Unheated comminuted products are usually frozen to enhance binding. Nowadays, when consumers demand fresh, unfrozen meat as well as lower salt contents, technologies have been developed to eliminate the need for freezing and to enable the use of less salt. One of these technologies is the enzyme-aided restructuring,[52] which has been used on a commercial scale for some time and is still the main TGase application in the meat sector. TGase has been found to improve the strength of restructured meat protein gels with or without added salt and phosphates.[52, 136–139]

Kuraishi *et al.*[137] reported that restructured meat products that are traditionally prepared using salt and phosphates to promote extraction of proteins, can be prepared without added

Table 12.6 Cross-linking enzymes for structure engineering.

Enzyme	Reaction	Cross-link	Application	References
Transglutaminase (EC 2.3.2.13), acyltransferase	Formation of an isopeptide bond	Protein-bound glutamine–lysine	Production of restructured meat	52, 127
			Increased firmness of heated meat products	128–133, 156
Tyrosinase (EC 1.12.18.1), non-radical forming oxidase	Oxidation of tyrosine residues	Tyrosine–tyrosine	Increased gelation of meat proteins, increased firmness of meat gels	134, 135
		Tyrosine–lysine Tyrosine–cysteine		
Laccase (EC 1.10.3.2), radical forming oxidase	Oxidation of tyrosine	Tyrosine–tyrosine	Firmness improvement of meat gels	67

Table 12.7 TGase applications in processed meat systems.

Application	References
Gelation of meat proteins	
Beef	141, 142
Pork	143
Poultry	59, 88, 134, 144–147
Texture of meat model systems	
Beef	129, 148, 149
Pork	150–153
Poultry	135, 154, 155
Texture of sausages and hams	128, 130, 156

salts using TGase. Binding strength can be boosted at low temperatures with caseinate added to the system as an extender. Caseinate is an excellent substrate for TGase. No data, however, have been reported on how the treatment affected the product characteristics in a heated state. Lee and Park[138] showed that hardness, chewiness and springiness increased significantly by adding TGase to the restructured ground lean pork. Water-holding capacity (WHC) of unheated samples decreased due to TGase, but the enzyme had no effect on the cooking loss. Kolle and Savell[140] reported a study on consumers' attitudes towards restructured beef muscles. The beef muscles studied were free of 'kernel' fat and heavy connective tissue. TGase and caseinate were used as binding agents to restructure the meat. In the study, consumers rated cooked restructured products higher than non-restructured controls in several palatability traits, for example juiciness, flavour and overall like.

12.6.2 Processed meat systems

In addition to binding fresh meat pieces together, the effects of TGase have been studied in isolated meat protein systems and model meat products aiming at improved textural properties (Table 12.7).

Although meat protein cross-linking has hitherto been carried out almost solely by TGase, tyrosinases and laccases are also known to be capable of cross-linking meat proteins and increasing the firmness of meat systems.[6,67,135,157,158]

TGase-catalyzed formation of additional covalent bonds in structural meat proteins leads, by definition, to firmer gel structures as has been clearly shown in many studies (Table 12.7). However, excessive increase in gel firmness due to additional formation of transverse bonds in the myofibrillar protein network may cause constraint in protein mobility and flexibility of myofibrils, leading to an undesired decrease in water holding.[106] Decreased WHC is mostly an unwanted phenomenon and has been observed to take place particularly in heated comminuted meat systems particularly when salt concentrations are low.[10,133,159] Effects of TGase from *Streptomyces mobaraense* on textural properties and water holding in low-salt meat systems are summarized in Table 12.8.

TGase has not been shown unequivocally to be an adequate salt replacer from the water-holding viewpoint, but at high enough salt levels ensuring sufficient protein solubility, TGase is capable of improving both textural and water-holding properties. However, when salt levels are low ($\leq$2% NaCl), introduction of strong cross-links in proteins by TGase has not generally been found to improve water holding of heated meat systems, although textural properties have been improved.[128,132,133,155,159] The reason is most probably the increased protein–protein interaction and thus reduced protein–water interaction. Due to

Table 12.8 Effects of *Streptomyces mobaraense* TGase on texture and water-holding properties of heated or high-pressurized low-salt meat gels.

Raw materials	Processing conditions	Effect on texture	Effect on water holding	References
Beef and pork meat chopped with TGase, 26% fat, 18% ice, 1.65% nitrite curing salt, TPP	Cooking to a core temperature of 70°C	Improved breaking strength	No effect on cooking loss	128
Pork batter (10% protein), 0.4–2% NaCl, TPP	TGase-pretreatment, cooking to a core temperature of 70°C at various heating temperatures	Increased gel hardness and chewiness at all NaCl levels	Decreased cooking loss at all NaCl levels	160
Minced chicken breast meat, 2% NaCl, 0.05–0.3% TPP, 30% water, 10% pork fat, 3% soya or milk protein treated with TGase	TGase-treated non-meat proteins added to sausage batters after which the sausages heated at 75°C for 30 m	Improved breaking stress at both studied tripolyphosphate levels	Not studied	161
Homogenized chicken thighs, 1% NaCl, 30% water, 10% egg yolk, 10% dehydrated egg white, 0.3% TPP	High pressure (500 MPa, 30 min, 40°C) treatment after TGase addition	Improved gel hardness, chewiness, springiness	Decreased expressible moisture due to pressure and TGase	154
Homogenized chicken thighs, 1% NaCl, 30% water, 10% egg yolk, 10% dehydrated egg white, no TPP	High pressure (500, 700, 900 MPa, 30 min, 40°C) treatment after TGase addition	Improved gel hardness, chewiness, springiness	Increased expressible moisture due to TGase but not pressure	155
Pork meat pieces massaged with TGase, 13% water, 1–2% NaCl	Cooking for 65 min at 72°C and for 65 min at 78°C	Slightly improved firmness	No effect on cooking loss or juiciness	132
Pork batter, 15% water, no NaCl, 1% KCL, 20% fibre, 2% caseinate	Heating for 15 min at 40°C, then cooking to the final temperature of 70°C	Decreased gel hardness with TGase>TGase/fibre> TGase/KCL > TGase/caseinate	Increased cooking loss with TGase>TGase/KCL>TGase/fibre>TGase/caseinate	133
Pork, chicken or lamb batter, 13% water, 1.5% NaCl, TPP	Cooking for 30 min at 70°C	Improved gel hardness	Increased cooking loss	159
Ground chicken thighs or beef 50 g, water 30 g, NaCl 1.4 g, TPP 0.21 g	Heating at 40 or 80°C for 30 min	Improved breaking stress with both meat raw materials and at both studied heating temperatures	Not studied	156

(*continued*)

Table 12.8 (*Continued*)

Raw materials	Processing conditions	Effect on texture	Effect on water holding	References
Ground pork 36%, NaCl 2%, non-meat protein extenders 2%, water	Heating to internal temperature of 72°C and cooling to 20°C	Improvement of gel strength with all studied extenders	Reduced cooking loss and expressible moisture with blood plasma and caseinate as extenders	152

TPP: tripolyphosphate.

TGase-catalyzed covalent bond formation and subsequent limited protein solubility and swelling ability, the result is reduced WHC. Carballo *et al.*[159] have postulated that the combined effect of TGase and low amount of salt (1.5%) in a comminuted and heated meat system leads to increased cooking loss because of the reduced WHC of solubilized meat proteins cross-linked with TGase. This conclusion is very much in line with what has been known for years about water holding in unheated and heated meat systems[9,10,12,157,162] for reviews. Extra covalent cross-links between myofibrillar proteins may prohibit the swelling of the myofibrils, which is a prerequisite for good water holding.

However, improved water holding caused by TGase in low salt concentrations has also been reported at least by Tseng *et al.*[131,152,154,160] Tseng *et al.*[131] were able to increase cooking yield as a function of increasing TGase dosage in chicken meatballs containing 1% salt, 0.2% tripolyphosphate and 25% pork fat. The high fat amount, phosphate and no added water might have contributed to the result. Pietrasik and Li-Chan[160] studied the effects of TGase and various salt levels (0–2%) on pork batter gel properties. The authors found that low salt levels, as expected, caused decreased gel firmness and cooking yield, but TGase was able to improve both properties in the low-salt batters but not to the same level as with 2% salt. Trespalacios and Pla[154] reported significant reduction of the expressible moisture of low-salt chicken batters treated with TGase and high pressure. However, in their study, ground chicken meat was mixed with fresh egg yolk and dehydrated egg white, both at 10% of the meat mass. Although the reported effects of egg proteins on hydration properties of meat gels are said to be somewhat contradictory, they may have played a role in water holding in the studied chicken meat batters. Omitting tripolyphosphates (TPPs) from the recipe resulted, however, in markedly increased expressible moisture from the meat system. Pietrasik *et al.*[152] reported about a synergetic positive effect of TGase together with various non-meat protein extenders (2%) on cooking yield and expressible moisture of low-salt (2%) and low-fat pork gels.

WHC is a feature that is affected by many technological factors (salts, ionic strength and pH), but also by many intrinsic features of meat itself. Due to the complexity of WHC per se and the inconsistent findings about the effects of TGase in the development of WHC in various product applications reviewed in this section, generalizations concerning the role of TGase in the water holding of meat systems should not be made. To exploit TGase in meat product processing, the enzyme dosage and processing conditions must be optimized for every process and product. Often tools such as non-meat protein or hydrocolloid extenders [150–152,154,161,163,164] and sufficient amounts of salts, need to be added in order to obtain acceptable water binding and to induce sufficient protein–water interaction in the system. Nonetheless, the positive effect of TGase on the textural properties in various kinds of meat systems is beyond dispute.

12.7 OTHER APPLICATIONS

Proteolytic enzymes are also potential tools for valorization of different meat by-products. Protein hydrolyzates with strong meat flavour can be used in soups, sauces and in ready meals. Proteases can be applied for production of protein hydrolyzates from different meat by-products such as bones,[165] sheep visceral mass,[166] chicken by-products[167] or bovine by-products.[168] These hydrolyzates can be used as flavour enhancers, as seasoning additives, as nutritional additives to low-protein food products or as animal feed supplements when not suitable for food use. Optimization of the type proteolytic enzymes used in the treatments is needed in order to avoid formation of bitter hydrolysis products when food applications are targeted.[167,169,170] The flavour intensity depends on the free amino acid content and on the type of peptides present and their reactions during the process, and therefore the presence of endoproteases and exo-peptidases has to be optimal.[157] Enzymes can also be used for treatment of fresh bones to be suitable for gelatine production. This two-phase process produces meat extract and cleaned bones for subsequent gelatin manufacture.[157]

12.8 FUTURE PROSPECTS

Consumers rate tenderness as one of the most important sensory attributes of meat. Tenderness is a precondition for a fast and easy cooking, which appeals to consumers. Toughness of beef meat is perhaps not a global problem but, for example in Nordic countries, it seems as most of the beef on the market still comes from dairy cattle with less tender meat. Since connective tissue consisting of collagen, naturally resistant to degradation, is predominantly responsible for the toughness of meat, proteolytic enzymes degrading selectively collagen rather than red meat are needed. Such enzymes would enable tenderization of lower grade tough parts of meat. Powerful enzymes of this kind are not yet in the market for food use.

Besides tender meat, conscious consumers demand fresh meat products devoid of excessive amounts of fat and salt. In addition, manufactures want means to make the carcass utilization more effective to maximize the profits. Efficient exploitation of the carcass and its by-products is also required from an environmental viewpoint. Novel enzymatic techniques are surely worth considering when new possibilities for more effective use of slaughter animal carcasses are investigated. One solution to this need, that is enzymatic cold binding technology exploiting TGase, is already applied in the meat industry. Meat pieces, also low-value cuts and trimmings, from one or more animal species can be glued together with TGase to create meat products with tailor-made meat content, shape and size, which are stable both in cold and cooking conditions. Innovative potential for development of this type of meat products seems to be limitless.

Efficient carcass utilization necessitates environmentally sustainable applications from offal. Poultry feathers have been utilized on a limited basis for animal feed. However, the keratin protein is poorly digestible. Being heavily disulphide cross-linked and containing a high amount of hydrophobic amino acids, keratins may have great potential in the non-food sector, such as packaging, film and coating applications. Solubility is a prerequisite for application and is currently investigated using harsh chemicals. Environmentally benign manufacture of materials from feathers would benefit from specific proteases capable of efficient keratin hydrolysis.

REFERENCES

1. http://faostat.fao.org/
2. Walls, E.W. (1960) The microanatomy of muscle. In: *The Structure and Function of Muscle*, Vol. **1** (ed. G.H. Bourne). Academic Press, New York/London, pp. 21–61.
3. Macfarlane, J.J., Schmidt, G.R. and Turner, R.H. (1977) Binding of meat pieces: a comparison of myosin, actomyosin, and sarcoplasmic proteins as binding agents. *Journal of Food Science* **42**, 1603–1604.
4. Lawrie, R.A. and Ledward, D.E. (2006) Chemical and biochemical constitution of muscle. In: *Lawrie's Meat Science*, 7th edn (ed. R.A. Lawrie). Woodhead Publishing Limited, Cambridge, p. 76.
5. Bailey, A.J. and Light, N.D. (1989) *Connective Tissue in Meat and Meat Products*, 1st edn. Elsevier Science Publishers, London/New York, pp. 65–73.
6. Schweigert, B.S. and Payne, J.B. (1956) A summary of the nutrient content of meat. American Meat Institute Foundation, Bulletin No. 30.
7. Dransfield, E. (1994) Modelling post-mortem tenderisation – V: inactivation of calpains. *Meat Science* **37**, 391–409.
8. Penny, I.F. (1980) The enzymology of conditioning. In: *Developments in Meat Science*, Vol. **1** (ed. R.A. Lawrie). Elsevier Applied Science, London/New York, pp. 115–143.
9. Tornberg, E. (2005) Effects of heat on meat proteins – implications on structure and quality of meat products. *Meat Science* **70**, 493–508.
10. Ruusunen, M. and Puolanne, E. (2005) Reducing sodium intake from meat products. *Meat Science*, **70**, 531–542.
11. Hamm, R. (1972) *Kolloidchemie des Fleisches*. Paul Parey Gmbh, Berlin/Hamburg.
12. Offer, G. and Knight, P. (1988a) Structural basis of water-holding capacity in meat. Part 1. General principles and water uptake in meat processing. In: *Developments in Meat Science*, Vol. **4** (ed. R.A. Lawrie). Elsevier Applied Science, London/New York, pp. 63–171.
13. Whiting, R. (1988) Solute-protein interactions in a meat batter. *Proceedings of American Reciprocal Meat Conference* **41**, 53–56.
14. Cheng, Q. and Sun, D.-W. (2008) Factors affecting the water holding capacity of red meat products: a review of recent research advances. *Critical Reviews in Food Science and Nutrition* **48**, 137– 159.
15. Grzonka, Z., Kasprzykowski, F. and Wiczk, W. (2007) Cysteine proteases. In: *Industrial Enzymes Structure, Function and Applications* (eds J. Polaina and A.P. MacCabe). Springer, Dordrecht, Netherlands, pp. 181–195.
16. Lawrie, R.A. (1998) *Lawrie's Meat Science*, 6th edn. Woodhead Publishing Ltd., Cambridge.
17. Foegeding, E.A. and Larick, D.K. (1986) Tenderization of beef with bacterial collagenase. *Meat Science* **18**, 201–214.
18. Cronlund, A.L. and Woychik, J.H. (1987) Solubilization of collagen in restructured beef with collagenases and α-amylase. *Journal of Food Science* **52**, 857–860.
19. Kim, H.-J. and Taub, I.A. (1991) Specific degradation of myosin in meat by bromelain. *Food Chemistry* **40**, 337–343.
20. Glazer, A.N. and Smith E.L. (1971) Papain and other sulfhydryl proteolytic enzymes. In: *The Enzymes*, Vol. **3** (ed. P.D. Boyer). Academic Press, New York, pp. 501–546.
21. Kilara, A., Shahani, K.M. and Wagner, F.W. (1977) Preparation and properties of immobilized papain and lipase. *Biotechnology & Bioengineering* **14**, 1703–1714.
22. Ockerman, H.W., Harnsawas, S. and Yetim, H. (1993) Inhibition of papain in meat by potato protein or ascorbic acid. *Journal of Food Science* **58**, 1265–1268.
23. Harper, E. (1980) Collagenases. *Annual Reviews of Biochemistry* **49**, 1063–1078.
24. Seifter, S. and Harper, E. (1971) Collagenases. In: *The Enzymes*, Vol. **3** (ed. P. Boyer). Academic Press, New York, pp. 649–697.
25. Mandl, I., MacLennan, J., Howes, E., DeBellis, R. and Sohler, A. (1953) Isolation and characterization of proteinase and collagenase from Cl. histolyticum. *The Journal of Clinical Investigation* **32**, 1323–1329.
26. Welton, R.L. and Woods, D.R. (1975) Collagenase production by *Achromobacter iophagus*. *Biochimica et Biophysica Acta* **384**(1), 228–234.
27. Carrick, L. and Berk, R. (1975) Purification and partial characterization of a collagenolytic enzyme from Pseudomonas aeruginosa. *Biochimica et Biophysica Acta* **391**, 422.

28. Merkel, J., Dreisbach, J. and Ziegler, H. (1975) Collagenolytic activity of some marine bacteria. *Applied Microbiology* **29**, 145.
29. Sugasawara, R. and Harper, E. (1984) Purification and characterization of three forms of collagenase from *Clostridium histolyticum*. *Biochemistry* **23**, 5175.
30. Takahashi, S. and Seifter, S. (1970) Dye-sensitized photo-inactivation of collagenase A. *Biochimica et Biophysica Acta* **214**, 556.
31. Mitchell, W. (1968) Pseudocollagenase: a protease from Clostridium histolyticum. *Biochimica et Biophysica Acta* **159**, 554.
32. Peterkofsky, B. and Diegelmann, R. (1971) Use of a mixture of protease-free collagenases for the specific assay of radioactive collagen in the presence of other proteins. *Biochemistry* **10**, 988.
33. Lewis, D.A. and Luh, B.S. (1988) Application of actinidin from kiwifruit to meat tenderization and characterization of beef muscle protein hydrolysis. *Journal of Food Biochemistry* **12**, 147–158.
34. Wada, M., Suzuki, T., Yaguti, Y. and Hasegawa, T. (2002) The effects of pressure treatments with kiwi fruit protease on adult cattle semitendinosus muscle. *Food Chemistry* **78**, 167–171.
35. Morimoto, K., Kunii, S., Hamano, K. and Tonomura, B. (2004) Preparation and structural analysis of actinidain-processed atelocollagen of yellowfin tuna (*Thunnus albacares*). *Bioscience, Biotechnology and Biochemistry* **68**, 861–867.
36. Mostafaie, A., Bidmeshkipour, A., Shirvani, Z., Mansouri, K. and Chalabi, M. (2008) Kiwifruit actinidin: a proper new collagenase for isolation of cells from different tissues. *Applied Biochemistry and Biotechnology* **144** (2), 123–131.
37. Sugiyama, S., Hirota, A., Okada, C., Yorita, T., Sato, K. and Ohtsuki, K. (2005) Effect of kiwifruit juice on beef collagen. *Journal of Nutritional Science and Vitaminology* **51**, 27–33.
38. Takagi, H., Kondou, M., Hisatsuka, T., Nakamori, S., Tsai, Y.-C. and Yamasaki, M. (1992) Effects of an alkaline elastase from an alcalophilic *Bacillus* strain on tenderization of beef meat. *Journal of Agricultural and Food Chemistry* **40**, 2364–2368.
39. Qihe, C., Guoqing, H., Yingchun, J. and Hui, N. (2006) Effects of elastase from a *Bacillus* strain on the tenderization of beef meat. *Food Chemistry* **98**, 624–629.
40. Asif-Ullah, M., Kim, K.-S. and Yu, Y.G. (2006) Purification and characterization of a serine protease from *Cucumis trigonus* Roxburghi. *Phytochemistry* **67**, 870–875.
41. Choi, K.H. and Laursen, R.A. (2000) Amino-acid sequence and glycan structures of cysteine proteases with proline specificity from ginger rhizome *Zingiber officinale*. *European Journal of Biochemistry* **267**, 1516–1526.
42. Ollis, D.L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S.M., Harel, M., Remington, S.J., Silman, Y. and Schrag, J. (1992) The α/β hydrolase fold. *Protein Engineering* **5**, 197–211.
43. Cygler, M., Grochulski, P., Kazlauskas, R.J., Schrag, J.D., Bouthillier, F., Rubin, B., Serreqi, A.N. and Gupta, A.K. (1994) A structural basis for the chiral preferences of lipases. *Journal of the American Chemical Society* **116**, 3180–3186.
44. Brady, L., Brzozowski, A.M., Derewenda, Z.S., Dodson, E., Dodson, G., Tolley, S., Turkenburg, J.P., Christiansen, L., Huge-Jensen, B. and Norskov, L. (1990) A serine protease triad forms the catalytic centre of a triacylglycerol lipase. *Nature* **343**, 767–770.
45. Schmid, R.D. and Verger, R. (1998) Lipases: interfacial enzymes with attractive applications. *Angewandte Chemie* (International ed. in English) **37**, 1608–1633.
46. Malcata, F.X., Reyes, H.R., Garcia, H.S., Hill, C.G. and Amundson, C.H. (1992) Kinetics and mechanisms of reactions catalysed by immobilized lipases. *Enzyme and Microbial Technology* **14**, 426–446.
47. Watanabe, N., Ota, Y., Minoda, Y. and Yamada, K. (1977) Isolation and identification of alkaline lipase producing microorganisms, cultural conditions and some properties of crude enzymes. *Agricultural and Biological Chemistry* **41**, 1353–1358.
48. Rúa, M.L., Schmidt-Dannert, C., Wahl, S., Sprauer, A. and Schmid, R.D. (1997) Thermoalkalophilic lipase of *Bacillus thermocatenulatus*. Large-scale production, purification and properties: aggregation behaviour and its effect on activity. *Journal of Biotechnology* **56**, 89–102.
49. Yokoyama, K., Nio, N. and Kikuchi, Y. (2004) Properties and applications of microbial transglutaminase. *Applied Microbiology and Biotechnology* **64**, 447–454.
50. Zhu, Y., Rinzema, A., Tramper, J. and Bol, J. (1995) Microbial transglutaminase – a review of its production and application in food processing. *Applied Microbiology and Biotechnology* **44**, 277–282.
51. Motoki, M. and Seguro, K. (1998) Transglutaminase and its use for food processing. *Trends in Food Science and Technology* **9**, 204–210.

52. Kuraishi, C., Yamazaki, K. and Susa, Y. (2001) Transglutaminase: its utilization in the food industry. *Food Reviews International* **17**, 221–246.
53. Griffin, M., Casadio, R. and Bergamini, C.M. (2002) Transglutaminases: nature's biological glues. *Biochemical Journal* **368**, 377–396.
54. Nonaka, M., Tanaka, H. and Okiyama, A. (1989) Polymerization of several proteins by Ca^{2+}-independent transglutaminase derived from microorganisms. *Agricultural and Biological Chemistry* **53**, 2619–2623.
55. Kahn, D. and Cohen, I. (1981) Factor XIIIa-catalysed coupling of structural proteins. *Biochimica et Biophysica Acta* **668**, 490–494.
56. De Backer-Royer, C., Traoré, F. and Meunier, J.C. (1992) Polymerization of meat and soya bean proteins by human placental calcium-activated factor XIII. *Journal of Agricultural and Food Chemistry* **40**, 2052–2056.
57. Tseng, T.-F., Chen, M.-T. and Liu, D.-C. (2002) Purification of transglutaminase and its effects on myosin heavy chain and actin of spent hens. *Meat Science* **60**, 267–270.
58. Lantto, R., Puolanne, E., Kalkkinen, N., Buchert, J. and Autio, K. (2005) Enzyme-aided modification chicken breast myofibrillar proteins: effects of laccase and transglutaminase on gelation and thermal stability. *Journal of Agricultural and Food Chemistry* **53**, 9231–9237.
59. Ramirez-Suarez, J.C., Addo, K. and Xiong, Y.L. (2005) Gelation of mixed myofibrillar/wheat gluten proteins treated with microbial transglutaminase. *Food Research International* **38**, 1143–1149.
60. Ando, H., Adachi, M., Umeda, K., Matsuura, A., Nonaka, M., Uchio, R., Tanaka, H. and Motoki, M. (1989) Purification and characteristics of a novel transglutaminase derived from microorganisms. *Agricultural and Biological Chemistry* **53**, 2613–2617.
61. Selinheimo, E., Nieidhin, D., Steffensen, C., Nielsen, J., Lomascolo, A., Halaouli, S., Record, E., O'Beirne, D., Buchert, J. and Kruus, K. (2007) Comparison of the characteristics of fungal and plant tyrosinases. *Journal of Biotechnology* **130**, 471–480.
62. Wichers, H.J., Gerritse, Y.A. and Chapelon, C.G.J. (1996) Tyrosinase isoforms from the fruitbodies of *Agaricus bisporus*. *Phytochemistry* **43**, 333–337.
63. Seo, S.-Y., Sharma, V.K. and Sharma, N. (2003) Mushroom tyrosinase: recent prospects. *Journal of Agricultural and Food Chemistry* **51**, 2837–2853.
64. Lerch, K. (1983) Neurospora tyrosinase: structural, spectroscopic and catalytic properties. *Molecular and Cellular Biochemistry* **52**(2), 125–138.
65. Selinheimo, E., Saloheimo, M., Ahola, E., Westerholm-Parviainen, A., Kalkkinen, N., Buchert, J. and Kruus, K. (2006) Production and characterization of a secreted, C-terminally processed tyrosinase from the filamentous fungus *Trichoderma reesei*. *FEBS Journal* **273**, 4322–4335.
66. Færgemand, M., Otte, J. and Qvist, K.B. (1998) Cross-linking of whey proteins by enzymatic oxidation. *Journal of Agricultural and Food Chemistry* **46**, 1326–1333.
67. Yamaguchi, S. (2000) Method for cross-linking protein by using enzyme. US Patent 6121013.
68. Mattinen, M.-L., Kruus, K., Buchert, J., Nielsen, J.H., Andersen, H.J. and Steffensen, C.L. (2005) Laccase-catalysed polymerization of tyrosine-containing peptides. *FEBS Journal* **272**, 3640–3650.
69. Thurston, C. (1994) The structure and function of fungal laccases. *Microbiology* **140**, 19–26.
70. Nandakumar, R., Yoshimune, K., Wakayama, M. and Moriguchi, M. (2003) Microbial glutaminase: biochemistry, molecular approaches and applications in the food industry. *Journal of Molecular Catalysis B: Enzymatic* **23**, 87–100.
71. Thammarongtham, C., Turner, G., Moir, A.J., Tanticharoen, M. and Cheevadhanarak, S. (2001) A new class of glutaminase from *Aspergillus oryzae*. *Journal of Molecular Microbiology and Biotechnology* **3**(4), 611–617.
72. Yamamoto, S. and Hirooka, H. (1974) Production of glutaminase by Aspergillus sojae. *Journal of Fermentation Technology* **52**, 564–569.
73. Calderón, J., Huerta-Saquero, A., Du Pont, G. and Durán, S. (1999) Sequence and molecular analysis of the *Rhizobium etli* gls A gene, encoding a thermolabile glutaminase. *Biochimica et Biophysica Acta* **1444**, 451–456.
74. Dura, M.A., Flores, M. and Toldra, F. (2002) Purification and characterisation of glutaminase from *Debaryomyces spp*. *International Journal of Food Microbiology* **76**, 117–126.
75. Brown, G., Singer, A., Proudfoot, M., Skarina, T., Kim, Y., Chang, C., Dementieva, I., Kuznetsova, E., Gonzalez, C.F., Joachimiak, A., Savchenko, A. and Yakunin, A.F. (2008) Functional and structural characterization of four glutaminases from *Escherichia coli* and *Bacillus subtilis*. *Biochemistry* **47**, 5724–5735.

76. Moriguchi, M., Sakai, K., Tateyama, R., Furuta, Y. and Wakayama, M. (1994) Isolation and characterization of salt-tolerant glutaminases from marine *Micrococcus luteus* K-3. *Journal of Fermentation and Bioengineering* **77**, 621.
77. Wilson, S.-A., Young, O.A., Coolbear, T. and Daniel, R.M. (1992) The use of proteases from extreme thermophiles for meat tenderization. *Meat Science* **32**, 93–103.
78. Murai, A., Tsujimoto, Y., Matsui, H. and Watanabe, K. (2004) An *Aneurinibacillus* sp. strain AM-1 produces a proline-specific aminopeptidase useful for collagen degradation. *Journal of Applied Microbiology* **96**, 810–818.
79. Pawar, V.D., Mule, B.D. and Machewad, G.M. (2007) Effect of marination with ginger rhizome extract on properties of raw and cooked chevon. *Journal of Muscle Foods* **18**, 349–369.
80. Rhodes, D.N. and Dransfield, E. (1973) Effect of pre-slaughter injections of papain on toughness in lamb muscles induced by rapid chilling. *Journal of the Science of Food and Agriculture* **24**, 1583–1588.
81. Bradley, R., O'Toole, D.T., Wells, D.E., Anderson, P.H., Hartley, P., Berrett, S., Morris, J.E., Insch, C.G. and Hayward, E.A. (1987) Clinical biochemistry and pathology of mature beef cattle following antemortem intravenous administration of a commercial papain preparation. *Meat Science* **19**, 39–51.
82. Quaglia, G.B., Lombardi, M., Sinesio, F., Bertone, A. and Menesatti, P. (1992) Effect of enzymatic treatment on tenderness characteristics of freeze-dried meat. *LWT – Food Science and Technology* **25**, 143–145.
83. Naveena, B.M. and Mendiratta, S.K. (2004) The tenderization of buffalo meat using ginger extract. *Journal of Muscle Foods* **15**, 235–244.
84. Ashie, I.N.A., Sorensen, T.L. and Nielsen, P.M. (2002) Effect of papain and a microbial enzyme on proteins and beef tenderness. *Journal of Food Science* **67**, 2138–2142.
85. Janz, J.A.M., Pietrasik, Z., Aalhus, J.L. and Shand, P.J. (2005) The effects of enzyme and phosphate injections on the quality of beef semitendinosus. *Canadian Journal of Animal Science* **85**, 327–334.
86. Huerta-Montauti, D., Miller, R.K., Schuehle Pfeiffer, C.E., Pfeiffer, K.D., Nicholson, K.L., Osburn, W.N. and Savell, J.W. (2008) Identifying muscle and processing combinations suitable for use as beef for fajitas. *Meat Science* **80**, 259–271.
87. Fogle, D.R., Plimpton, R.F., Ockerman, H.W., Jarenback, L. and Persson, T. (1982) Tenderization of beef: effect of enzyme, enzyme level, and cooking method. *Journal of Food Science* **47**, 1113–1118.
88. Ramezani, R., Aminlari, M. and Fallahi, F. (2003) Effect of chemically modified soy proteins and ficin-tenderized meat on the quality attributes of sausage. *Journal of Food Science* **68**, 85–88.
89. Iizuka, K. and Aishima, T. (1999) Tenderization of beef with pineapple juice monitored by fourier transform infrared spectroscopy and chemometric analysis. *Journal of Food Science* **64**, 973–977.
90. Cunningham, F.E. and Tiede, L.M. (1981) Properties of selected poultry products treated with a tenderizing marinade. *Poultry Science* **60**, 2475–2479.
91. Schenková, N., Šikulová, M., Jeleníková, J., Pipek, P., Houška, M. and Marek, M. (2007) Influence of high isostatic pressure and papain treatment on the quality of beef meat. *High Pressure Research* **27**, 163–168.
92. Bruggen, K., McKeith, F.K. and Brewer, M.S. (1993) Effect of enzymatic tenderization, blade tenderization, or pre-cooking on sensory and processing characteristics of beef bacon. *Journal of Food Quality* **16**, 209–221.
93. Kolle, B.K., McKenna, D.R. and Savell, J.W. (2004) Methods to increase tenderness of individual muscles from beef rounds when cooked with dry or moist heat. *Meat Science* **68**, 145–154.
94. Lee, Y.B., Sehnert, D.J. and Ashmore, C.R. (1986) Tenderization of meat with ginger rhizome protease. *Journal of Food Science* **51**, 1558–1559.
95. Mendiratta, S.K., Anjaneyulu, A.S.R., Lakshmanan, V., Naveena, B.M. and Bisht, G.S. (2000) Tenderizing and antioxidant effect of ginger extract on sheep meat. *Journal of Food Science and Technology* **37**, 651–655.
96. Naveena, B.M., Mendiratta, S.K. and Anjaneyulu, A.S.R. (2004) Tenderization of buffalo meat using plant proteases from *Cucumis trigonus Roxb* (Kachri) and *Zingiber officinale roscoe* (Ginger rhizome). *Meat Science* **68**, 363–369.
97. Bhaskar, N., Sachindra, N.M., Modi, V.K., Sakhare, P.Z. and Mshendrakar, N.S. (2006) Preparation of proteolytic activity rich ginger powder and evaluation of its tenderizing effect on spent-hen muscles. *Journal of Muscle Foods* **17**, 174–184.
98. Garg, V. and Mendiratta, S.K. (2006) Studies on tenderization and preparation of enrobed pork chunks in microwave oven. *Meat Science* **74**, 718–726.

99. Taylor, A.J. and Hort, J. (2004) Measuring proximal stimuli involved in flavour perception. In: *Flavor Perception*, Vol. **1** (eds A.J. Taylor and D.D. Roberts). Blackwell Publishing, Oxford, pp. 1–34.
100. Aaslyng, M.D., Oksama, M., Olsen, E.V., Bejerholm, C., Baltzer, M., Andersen, G., Bredie, W.L.P., Byrne, D.V. and Gabrielsen, G. (2007) The impact of sensory quality of pork on consumer preference. *Meat Science* **76**, 61–73.
101. Calkins, C.R. and Hodgen, J.M. (2007) A fresh look at meat flavour. *Meat Science* **77**, 63–80.
102. Lücke, F.-K. (1994) Fermented meat products. *Food Research International* **27**, 299–307.
103. Flores, J. (1997) Mediterranean vs northern European meat products – processing technologies and main differences. *Food Chemistry* **59**, 505–510.
104. Toldrá, F., Aristoy, M.-C. and Flores, M. (2000) Contribution of muscle aminopeptidases to flavor development in dry-cured ham. *Food Research International* **33**, 181–185.
105. Gandemer, F. (2002) Lipids in muscles and adipose tissues, changes during processing and sensory properties of meat products. *Meat Science* **62**, 309–321.
106. Jurado, Á., Garćia, C., Timón, M.L. and Carrapiso, A.I. (2007) Effect of ripening time and rearing system on amino acid-related flavour compounds of Iberian ham. *Meat Science* **75**, 585–594.
107. Fernández, M., Ordóñez, J.A., Bruna, J.M., Herranz, B. and de la Hoz, L. (2000) Accelerated ripening of dry fermented sausages. *Trends in Food Science & Technology* **11**, 201–209.
108. Toldrá, F. (1998) Proteolysis and lipolysis in flavour development of dry-cured meat products. *Meat Science* **49** (Suppl 1), S101–S110.
109. Toldrá, F. (2006) The role of muscle enzymes in dry-cured meat products with different drying conditions. *Trends in Food Science & Technology* **17**, 164–168.
110. Molly, K., Demeyer, D., Civera, T. and Verplaetse, A. (1996) Lipolysis in a Belgian sausage: relative importance of endogenous and bacterial enzymes. *Meat Science* **43**, 235–244.
111. Hagen, B.F., Berdagué, J.L., Holck, A.L., Næs, H. and Blom, H. (1996) Bacterial proteinase reduces maturation time of dry fermented sausages. *Journal of Food Science* **61**, 1024–1029.
112. Bruna, J.M., Fernández, M., Hierro, E.M., Ordóñez, J.A. and de la Hoz, L. (2000a) Improvement of the sensory properties of dry fermented sausages by the superficial inoculation and/or the addition of intracellular extracts of *Mucor racemosus*. *Journal of Food Science* **65**, 731–738.
113. Bruna, J.M., Fernández, M., Hierro, E.M., Ordóñez, J.A. and de la Hoz, L. (2000b) Combined use of Pronase E and fungal extract (Penicillium aurantiogriseum) to potentiate the sensory characteristics of dry fermented sausages. *Meat Science* **54**, 135–145.
114. Bruna, J.M., Hierro, E.M., de la Hoz, L., Mottram, D.S., Fernández, M. and Ordóñez, J.A. (2001) The contribution of *Penicillium aurantiogriseum* to the volatile composition and sensory quality of dry fermented sausages. *Meat Science* **59**, 97–107.
115. Bolumar, T., Sanz, Y., Aristoy, M.C. and Toldrá, F. (2003a) Purification and characterization of prolyl aminopeptidase from *Debaryomyces hansenii*. *Applied and Environmental Microbiology* **69**, 227–232.
116. Bolumar, T., Sanz, Y., Aristoy, M.C. and Toldrá, F. (2003b) Purification and properties of an arginyl aminopeptidase from *Debaryomyces hansenii*. *International Journal of Food Microbiology* **86**, 141–151.
117. Bolumar, T., Sanz, Y., Aristoy, M.C. and Toldrá, F. (2004) Protease B from *Debaryomyces hansenii*: purification and biochemical properties. *International Journal of Food Microbiology* **98**, 167–177.
118. Bolumar, T., Sanz, Y., Aristoy, M.C., Toldrá, F. and Flores, J. (2006) Sensory improvement of dry-fermented sausages by the addition of cell-free extracts from *Debaryomyces hansenii* and *Lactobacillus sakei*. *Meat Science* **72**, 457–466.
119. Diaz, O., Fernández, M., García de Fernado, G., de la Hoz, L. and Ordóñez, J.A. (1996) Effect of the addition of papain on the dry fermented sausage proteolysis. *Journal of the Science of Food and Agriculture* **71**, 13–21.
120. Diaz, O., Fernández, M., García de Fernado, G., de la Hoz, L. and Ordóñez, J.A. (1997) Proteolysis in dry fermented sausages: the effect of selected exogenous proteases. *Meat Science* **46**, 115–128.
121. Ansorena, D., Zapelena, M.J., Astiasarán, I. and Bello, J. (1998) Simultaneous addition of palatase M and protease P to a dry fermented sausage (Chorizo de Pamplona) elaboration: effect over peptic and lipid fractions. *Meat Science* **50**, 37–44.
122. Ansorena, D., Astiasarán, I. and Bello, J. (2000) Influence of the simultaneous addition of protease Flavourzyme and the lipase Novozym 677BG on dry fermented sausage compounds extracted by SDE and analyzed by GC-MS. *Journal of Agricultural Food Chemistry* **48**, 2395–2400.
123. Herranz, B., Fernández, M., Hierro, E.M., Bruna, J.M., Ordóñez, J.A. and de la Hoz, L. (2003) Use of *Lactococcus lactis* Subs. *cremoris* NCDO 763 and α-ketoglutarate to improve the sensory quality of dry fermented sausages. *Meat Science* **66**, 151–163.

124. Benito, M.J., Rodríguez, M., Martín, A., Arand, E. and Córdoba, J. (2004) Effect of the fungal protease EPg222 on the sensory characteristics of dry fermented sausage 'Salchichón' ripened with commercial starter cultures. *Meat Science* **67**, 497–505.
125. Martín, A., Córdoba, J.J., Aranda, E., Córdoba, M.G. and Asensio, M.A. (2006) Contribution of a selected fungal population to the volatile compounds on dry-cured ham. *International Journal of Food Microbiology* **110**, 8–18.
126. Casaburi, A., Aristoy, M.C., Cavella, S., Monaco, R., Ercolini, D., Toldra, F. and Villani, F. (2007) Biochemical and sensory characteristics of traditional fermented sausages of Vallo di Diano (Southern Italy) as affected by the use of starter cultures. *Meat Science* **76**, 295–307.
127. Nielsen, G.S., Petersen, B.R. and Møller, A.J. (1995) Impact of salt, phosphate and temperature on the effect of a transglutaminase (F XIIIa) on the texture of restructured meat. *Meat Science* **41**, 293–299.
128. Hammer, G. (1998) Mikrobielle transglutaminase und diphosphat bei feinzerkleinerter brühwurst. *Fleischwirtschaft* **78**, 1155–1162, 1186.
129. Kerry, J.F., O'Donnell, A., Brown, H., Kerry, J.P. and Buckley, D.J. (1999) Optimisation of transglutaminase as a cold set binder in low salt beef and poultry comminuted meat products using response surface methodology. In: *Proceedings of the 45th International Congress on Meat Science and Technology*, Vol. **1**. Yokohama, Japan, pp. 140–141.
130. Mugumura, M., Tsuruoka, K., Fujino, H., Kawahara, S., Yamauchi, K., Matsumura, S. and Soeda, T. (1999) Gel strength enhancement of sausages by treating with microbial transglutaminase. In: *Proceedings of the 45th International Congress on Meat Science and Technology*, Vol. **1**. Yokohama, Japan, pp. 138–139.
131. Tseng, T.-F., Liu, D.-C. and Chen, M.-T. (2000) Evaluation of transglutaminase on the quality of low-salt chicken meat-balls. *Meat Science* **55**, 427–431.
132. Dimitrakopoulou, M.A., Ambrosiadis, J.A., Zetou, F.K. and Bloukas, J.G. (2005) Effect of salt and transglutaminase (TG) level and processing conditions on quality characteristics of phosphate-free, cooked, restructured pork shoulder. *Meat Science* **70**, 743–749.
133. Jiménez Colmenero, F., Ayo, M.J. and Carballo, J. (2005) Physicochemical properties of low sodium frankfurter with added walnut: effect of transglutaminase combined with caseinate, KCl and dietary fibre as salt replacers. *Meat Science* **69**, 781–788.
134. Lantto, R., Plathin, P., Niemistö, M., Buchert, J. and Autio, K. (2006) Effects of transglutaminase, tyrosinase and freeze-dried apple pomace powder on gel forming and structure of pork meat. *LWT – Food Science and Technology* **39**, 1117–1124.
135. Lantto, R., Puolanne, E., Kruus, K., Buchert, J. and Autio, K. (2007) Tyrosinase-aided protein cross-linking: effects on gel formation of chicken breast myofibrils and texture and water-holding of chicken breast meat homogenate gels. *Journal of Agricultural and Food Chemistry* **55**, 1248–1255.
136. Wijngaards, G. and Paardekoopper, E.J.C. (1988) Preparation of a composite meat product by means of enzymatically formed protein gels. In: *Trends in Modern Meat Technology*, Vol. **2** (eds B. Krols, P.S. van Room and J.H. Houben). Pudoc, Wageningen, pp. 125–129.
137. Kuraishi, C., Sakamoto, J., Yamazaki, K., Susa, Y., Kuhara, C. and Soeda, T. (1997) Production of restructured meat using microbial transglutaminase without salt or cooking. *Journal of Food Science* **62**, 488–490, 515.
138. Lee, E.Y. and Park, J. (2003) Microbial transglutaminase induced cross-linking of a selected comminuted muscle system – processing conditions for physical properties of restructured meat. *Food Science and Biotechnology* **12**, 365–370.
139. Serrano, A., Cofrades, S. and Jimenez Colmenero, F. (2003) Transglutaminase as binding agent in fresh restructured beef steak with added walnuts. *Food Chemistry* **85**, 423–429.
140. Kolle, D.S. and Savell, J.W. (2003) Using Activa^{TM} TG-RM to bind beef muscles after removal of excessive seam fat between *the m. longissimus thoracis* and *m. spinalis dorsi* and heavy connective tissue from within the *m. infraspinatus*. *Meat Science* **64**, 27–33.
141. Kim, S.-H., Carpenter, J.A., Lanier, T.C. and Wicker, L. (1993) Polymerization of beef actomyosin induced by transglutaminase. *Journal of Food Science* **58**, 473–474, 491.
142. Ionescu, A., Aprodu, I., Daraba, A. and Porneala, L. (2008) The effects of transglutaminase on the functional properties of the myofibrillar protein concentrate obtained from beef heart. *Meat Science* **79**, 278–284.
143. Xiong, Y.L., Agyare, K.K. and Addo, K. (2008) Hydrolyzed wheat gluten suppresses transglutaminase-mediated gelation but improves emulsification of pork myofibrillar protein. *Meat Science* **80**, 535–544.
144. Ramirez-Suarez, J.C. and Xiong, Y.L. (2002a) Transglutaminase cross-linking of whey/myofibrillar proteins and the effect on protein gelation. *Journal of Food Science* **67**, 2885–2891.

145. Ramirez-Suarez, J.C. and Xiong, Y.L. (2002b) Rheological properties of mixed muscle/nonmuscle protein emulsions treated with transglutaminase at two ionic strengths. *International Journal of Food Science and Technology* **38**, 777–785.
146. Stangierski, J., Zabielski, J. and Kijowski, J. (2007) Enzymatic modification of selected functional properties of myofibril preparation obtained from mechanically recovered poultry meat. *European Food Research and Technology* **226**, 233–237.
147. Stangierski, J., Baranowska, H.M., Rezler, R. and Kijowski, J. (2008) Enzymatic modification of protein preparation obtained from water washed mechanically recovered poultry meat. *Food Hydrocolloids* **22**, 1629–1636.
148. Pietrasik, Z. (2003) Binding and textural properties of beef gels processed with κ-carrageenan, egg albumin and microbial transglutaminase. *Meat Science* **63**, 317–324.
149. Dondero, M., Figueroa, V., Morales, X. and Curotto, E. (2006) Transglutaminase effects on gelation capacity of thermally induced beef protein gels. *Food Chemistry* **99**, 546–554.
150. Jarmoluk, A. and Pietrasik, Z. (2003) Response surface methodology study on the effects of blood plasma, microbial transglutaminase and κ-carrageenan on pork batter gel properties. *Journal of Food Engineering* **60**, 327–334.
151. Pietrasik, Z. and Jarmoluk, A. (2003) Effect of sodium caseinate and κ-carrageenan on the binding and textural properties of pork muscle gels enhanced by microbial transglutaminase addition. *Food Research International* **36**, 285–294.
152. Pietrasik, Z., Jarmoluk, A. and Shand, P.J. (2007) Effect of non-meat proteins on hydration and textural properties of pork meat gels enhanced with microbial transglutaminase. *LWT – Food Science and Technology* **40**, 915–920.
153. Herrero, A.M., Cambero, M.I., Ordonez, J.A., de la Hoz, L. and Carmona, P. (2008) Raman spectroscopy study of the structural effect of microbial transglutaminase on meat systems and its relationship with textural characteristics. *Food Chemistry* **109**, 25–32.
154. Trespalacios, P. and Pla, R. (2007a) Simultaneous application of transglutaminase and high pressure to improve functional properties of chicken meat gels. *Food Chemistry* **100**, 264–272.
155. Trespalacios, P. and Pla, R. (2007b) Synergistic action of transglutaminase and high pressure on chicken meat and egg gels in absence of phosphates. *Food Chemistry* **104**, 1718–1727.
156. Ahhmed, A.M., Kawahara, S., Ohta, K., Nakade, K., Soeda, T. and Mugumura, M. (2007) Differentiation in improvements of gel strength in chicken and beef sausages induced by transglutaminase. *Meat Science* **76**, 455–462.
157. Nielsen, P.M. and Olsen, H.S. (2002) Enzymic modification of food protein. In: *Enzymes in Food Technology* (eds R.J. Whitehurst and B.A. Law). CRC Press, Boca Raton, FL, pp. 109–143.
158. Lantto, R., Puolanne, E., Katina, K., Niemistö, M., Buchert, J. and Autio, K. (2007b) Effects of laccase and transglutaminase on the textural and water-binding properties of cooked chicken breast meat gels. *European Food Research and Technology* **225**, 75–83.
159. Carballo, J., Ayo, J. and Jiménez Colmenero, F. (2006) Microbial transglutaminase and caseinate as cold set binders: influence of meat species and chilling storage. *LWT – Food Science and Technology* **39**, 692–699.
160. Pietrasik, Z. and Li-Chan, E.C.Y. (2002) Binding and textural properties of beef gels as affected by protein, κ-carrageenan and microbial transglutaminase addition. *Food Research International* **35**, 91–98.
161. Mugumura, M., Tsuruoka, K., Katayama, K., Erwanto, Y., Kawahara, S. and Yamauchi, K. (2003) Soya bean and milk proteins modified by transglutaminase improves chicken sausage texture even at reduced levels of phosphate. *Meat Science* **63**, 191–197.
162. Offer, G. and Knight, P. (1988b) Structural basis of water-holding capacity in meat. Part 2. Drip losses. In: *Developments in Meat Science*, Vol. **4** (ed. R.A. Lawrie). Elsevier Applied Science, London/New York, pp. 173–243.
163. Kilic, B. (2003) Effect of microbial transglutaminase and sodium caseinate on quality of chicken döner kebab. *Meat Science* **63**, 417–421.
164. Ramirez-Suarez, J.C. and Xiong, Y.L. (2003) Effect of transglutaminase-induced cross-linking on gelation of myofibrillar/soy protein mixtures. *Meat Science* **65**, 899–907.
165. Vollmer, A.N and Rosenfield, R.G. (1983) Extraction of protein from pork bones. US patent 4402873.
166. Bhaskar, N., Modi, V.K., Govindaraju, K., Radha, C. and Lalitha, R.G. (2007) Utilization of meat industry by products: protein hydrolyzate from sheep visceral mass. *Bioresource Technology* **98**, 388–394.

167. Surówka, K. and Fik, M. (1994) Studies on the recovery of proteinaceous substances from chicken heads: II application of pepsin to the production of protein hydrolyzate. *Journal of the Science of Food and Agriculture* **65**, 289–296.
168. Webster, J.D., Ledward, D.A. and Lawrie, R.A. (1982) Protein hydrolyzates from meat industry by-products. *Meat Science* **7**, 147–157.
169. Fik, M. and Surówka, K. (1986) Preparation and properties of concentrate from broiler chicken heads. *Journal of the Science of Food and Agriculture* **37**, 445–454.
170. Kilara, A. (1985) Enzyme-modified protein food ingredients. *Process Biochemistry* **20**, 149–158.

13 Enzymes in protein modification

Per Munk Nielsen

13.1 INTRODUCTION

Proteins are widely used as ingredients in the food industry and can be isolated from many types of raw materials like milk (casein and whey), wheat (gluten), soya, meat (gelatine and meat extracts), etc. The opportunities to use proteins as ingredients in food products are often limited by the properties of the proteins. One of the methods to modify these properties is the hydrolysis of the proteins to smaller peptides. This technology has been used for many years with the production of low allergenic mother's milk substitutes as a good example of proteases used for reduction of the allergenicity of the proteins.

The market for hydrolyzed proteins ranges from relatively low-value products used for instance in pet food to high-value nutritional products, such as special peptides in enteral nutrition, baby food formulas or bioactive peptides. The properties of the hydrolyzed proteins are adjusted by the selection of the protease, pretreatment of raw material, the parameters of hydrolysis as well as the downstream processing of the hydrolyzate. Even though there are other enzymes than proteases for protein modification, they are not yet used commercially or have only very limited use for modifying protein ingredients. Examples of these alternative methods are mentioned below with references for the interested reader:

- Transglutaminase, which cross-links proteins by forming an isopeptide bond between glutamine and lysine and is used in modifying the proteins in a food product but hardly ever used to produce protein ingredients.[1,2]
- Tyrosinase, which by an oxidative reaction can form covalent tyrosine–tyrosine, tyrosine–cystein or tyrosine–lysine cross-links.[1]
- Laccase, which by an oxidative reaction can form tyrosine–tyrosine cross-links, forms disulphide bridges, oxidize thiol groups.[1]
- Peptidoglutaminase used in de-amidation of glutamine to glutamic acid, but so far only on a laboratory scale.[3]
- Sulfhydryl oxidase.[4]

This chapter is therefore limiting the discussion to the aspects of protein hydrolysis for production of hydrolyzed food protein products.

13.2 THE HYDROLYSIS REACTION

The protease catalyzed reaction cleaves a peptide bond in the protein as illustrated in Fig. 13.1.

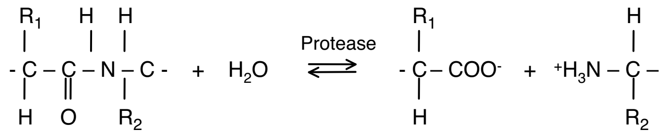


Fig. 13.1 Reaction scheme for protease catalyzed reaction (at pH near 6).

The reverse reaction of hydrolysis is normally not utilized for practical application. It is referred to as the plastein reaction.[5] The plastein reaction has been the subject of many research projects but so far it has not found its way into practical applications.

The extent or degree of hydrolysis is defined by the percentage of peptide bonds hydrolyzed:

$$\text{Degree of hydrolysis (DH)\%} = \frac{\text{peptide bonds cleaved}}{\text{total number of peptide bonds}} \times 100$$

There is one important detail about the hydrolysis reaction. When the peptide bond is cleaved one molecule of water is added. This will have a significant impact on the dry matter composition of the end product, since it is dependant upon the degree of hydrolysis. If for instance, a protein isolate with 90% protein in dry matter is hydrolyzed with DH = 25%, one molecule water for each four amino acids, corresponding to $18/(4*128) = 3.5\%$ water, will be added to the protein/peptides mixture. Anticipating that nothing is added or removed from the reaction mixture, it will contain 86.5% 'protein' on dry matter based on the normal method of measurement, that is nitrogen times the Kjeldahl factor. This can create a conflict with the definitions of isolates, which are normally defined as having at least 90% protein on dry matter.

13.3 CONTROLLING THE HYDROLYSIS REACTION

Several aspects of this reaction are utilized in the control of the DH. Some are related to the formation of the amino groups, others to the acidity of the peptides or to the change in other properties of the resulting peptides. Table 13.1 shows an overview of the methods that can be used for monitoring the DH.

The most accurate method for following the reaction is the pH-stat method.[7] The method has the advantage of being an online measurement but also has some severe drawbacks. The useable pH-range is limited to 7–9 where the carboxylic- and amino-groups are dissociated and where hydrolyzing one bond releases one H^+-ion. The ionization of the groups at a pH in the range of 4–7 is unclear and does not result in release of one mole H^+ or OH^-. Therefore, it is not possible to get a precise measurement of the DH by monitoring pH change or the amount of base equivalents required to keep pH constant in the pH-range of 4–7. Below pH 4 the pH-stat technique is, in principle, sufficiently precise to be used but this method is hardly used due to the lack of commercial enzymes active at this low pH. Another limitation is the addition of base during the titration which is quite often undesirable as this will accumulate as salt in the end product.

A good alternative for monitoring the reaction is the use of osmolality. It does not deliver an immediate answer but will within a few minutes of sampling. The measurement with

Table 13.1 Methods for measuring the degree of hydrolysis.

	Principle of the method	References
Based on amino group formation		
OPA	*O*-phtaldialdehyde reaction with primary amino groups to form a coloured detectable compound	6
TNBS	2,4,6-Trinitrobenzenesulfonic acid reaction with amino groups to form a coloured detectable compound	7, 8
Ninhydrin	Ninhydrin reaction with amino groups to form a coloured detectable compound	7
Formole titration	Titration of amino groups with formaldehyde	
based on acidity		
pH-stat	Keeping pH constant during hydrolysis; amount of titrant is equivalent to degree of hydrolysis	7
Titration to alkaline pH	Titrate acid formed during hydrolysis (at pH > 5.5) up to pH 8.0	
Change in pH	Follow pH during hydrolysis	9
Based on other properties		
Osmometry	Measurement of freezing point depression, which correlates to degree of hydrolysis	7
Brix	Refractive index correlating with soluble dry matter	7
Soluble nitrogen	Soluble nitrogen	10
TCA index	Amount of peptides soluble in trichloroacetic acid (above a certain molecular weight the peptides precipitate)	7
Peptide chain length	HPLC method based on gel permeation chromatography	
Viscosity	Follow change in viscosity during hydrolysis	7

an osmometer can be problematic if the viscosity of the sample and/or the concentration of soluble components like salts are too high. It should also be noted that at high reaction rates the DH can change during the time between sampling and analysis and this can cause an analytical error. In Fig. 13.2, pH and osmolality measurements for controlling a hydrolysis process are compared.

If the data from Fig. 13.1 are used for plotting pH versus osmolality it is clear that this is not a straight line, indicating that one of the methods is not correlating well with the degree of hydrolysis over the whole DH range; and it is the pH method that is incorrect.

Chemical methods used for detection of the amino groups are widely used. When sampling, the protease activity is stopped before the analysis by methods such as the addition of acid or by quick heat treatment.

Some methods of analysis use the correlation between degree of hydrolysis and properties of the protein itself. For example, at viscosity changes – normally viscosity is reduced upon protein hydrolysis – the protein becomes more soluble, which can be measured by the soluble nitrogen, TCA index and brix. These features are very important for many of the applications, but using changes in physical properties for process control is quite inaccurate

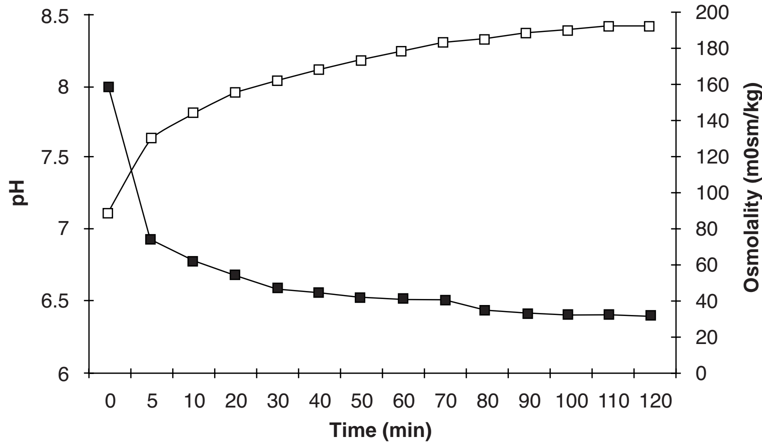


Fig. 13.2 Example of a hydrolysis curve monitored by osmolality and pH measurements. Alcalase hydrolyzing soya protein.[5]

and can only serve as indicative methods. Silvestre[11] reviewed the methods for analysis of protein hydrolyzates. In this review, more information about the potential and limitations of the different methods can be found.

13.4 PROTEASES

Proteases are classified according to the source of origin (microbial, animal, plant), the site they hydrolyze a protein (endo- or exo-peptidase) and the nature of the catalytic site (serine protease, metallo protease, aspartic protease). The proteases on the market differ widely with respect to purity. At one end of the scale, highly purified chymosin products for cheese coagulation are found which are sold as chromatographic purified products and at the other end crude pepsin is a good example. It is very common to use combinations of proteases to optimize the properties of the end product.

When cost effectiveness, food approvals, etc., have to be taken into account, the number of available proteases is rather limited. In Table 13.2, some of the typical available proteases are shown together with a description of their characteristics.

Due to differences in specificity of the enzymes the resulting peptides also widely differ. In Fig. 13.3, the molecular weight diagrams are shown from the hydrolysis of whey protein concentrate using four different proteases.

The figures (a)–(d) represent the molecular size distribution of peptides produced from whey protein concentrate at a DH of 4%. The enzymes used are trypsin, alkaline *Bacillus* protease, glutamic acid-specific *Bacillus* protease and *Mucor miehei* aspartic protease, respectively.[12] The aspartic protease on figure (c) is very different from the others by producing a relatively high number of very small peptides and a few medium-sized peptides. There are also significant differences between the patterns in the distributions obtained by the other proteases.

Figure 13.4 shows the peptide distribution when trypsin is used to produce a hydrolyzate with DH = 8%.[12] By comparing with Fig. 13.3(a), it is clear that the number of small peptides has increased.

Table 13.2 Proteases for production of food protein hydrolyzates.

Source	Type	Names/trade name	pH-range	Specificity
Pig pancreas	Serine	Trypsin, PTN	7–9	Lys, Arg
Pig pancreas	Serine	Chymotrypsin	7–9	Phe, Tyr, Trp
Pig pancreas	Aspartic	Pepsin	1–4	Aromatic, Leu, Asp, Glu
Calf stomach	Aspartic	Chymosin, rennet	3–6	Phe-Met in κ-casein
Papaya	Cystein	Papain	5–9	Broad
Pineapple	Cystein	Bromelain	5–8	Lys, Arg, Phe, Tyr
Figs	Cystein	Ficin	5–8	Phe, Tyr
Bacillus amyloliquefaciens	Metallo	Neutrase®	6–8	Broad
B. subtilis	Serine	Subtilisin	6–10	Broad
B. licheniformis	Serine	Alcalase®	6–10	Broad
B. stearothermophilus		Protease S	7–9	Broad
B. licheniformis	Serine	Glu-specific	7–9	Glu
Aspergillus oryzae	Mixture with aminopeptidase + carboxypeptidase	Flavourzyme®	5–8	Broad
Aspergillus niger	Aspartic	Acid protease A	2–3.5	
Mucor miehei	Aspartic	Rennilase®	3–6	A little broader than rennet
Rhizopus sp	Aspartic	Sumizyme RP	3–5	As pepsin
Fusarium	Asp, Lys	Specific	6–8	Asp, Lys

13.5 PROPERTIES OF HYDROLYZED PROTEIN

The properties of peptides differ from those of intact proteins. This is of course the basic reason for making protein hydrolyzates.

13.5.1 Taste

One of the most important properties for food protein hydrolyzates is taste. If this is not satisfactory it can become a very challenging task to formulate well-tasting products. With only a few exceptions consumers will not buy products they do not like. Exceptions are when they believe that the benefit they get is justifying 'suffering' when the product is consumed.

Intact proteins are tasteless in purified form.[13] When proteins are hydrolyzed there are two main contributions to the taste of the peptides. One is the release of flavour components 'hidden' in the protein structure, which are released when the protein is hydrolyzed. But most important is the formation of small peptides with a relative high content of hydrophobic amino acids which tend to create bitterness.[13] Adler-Nissen[7] made a detailed study of the bitterness problem and concluded that bitterness cannot be predicted from the theory of

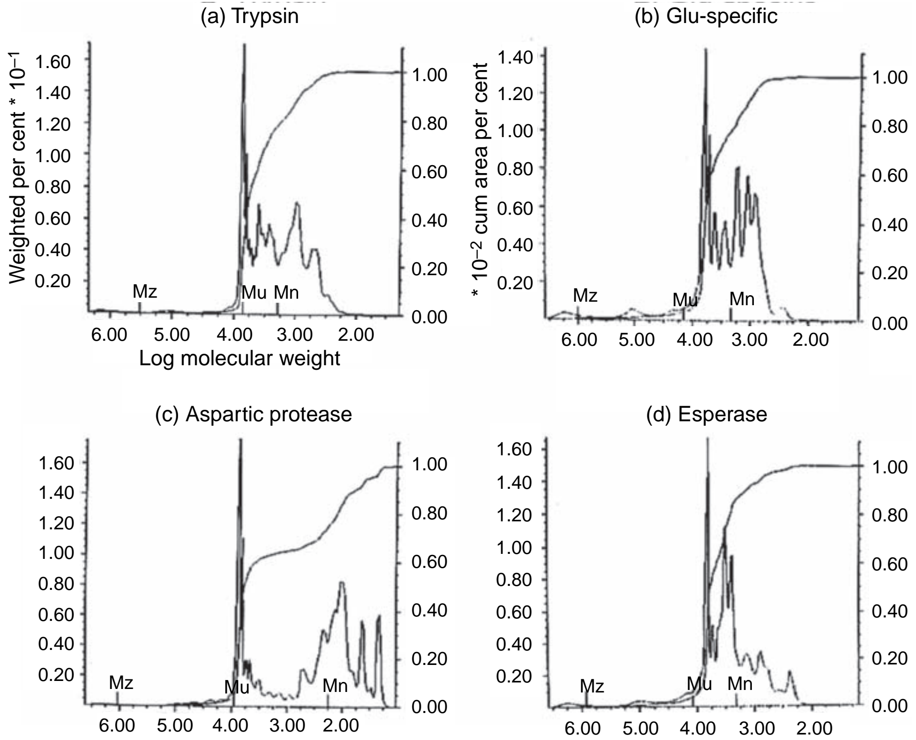


Fig. 13.3 Molecular weight distributions for whey protein hydrolyzates with DH 4% produced by different proteases. (a) Trypsin from pig pancreas, (b) glutamic acid-specific protease from *Bacillus*, (c) aspartic protease from *M. miehei* and (d) alkaline serien protease from *Bacillus* (Esperase®). (Nielsen unpublished data).

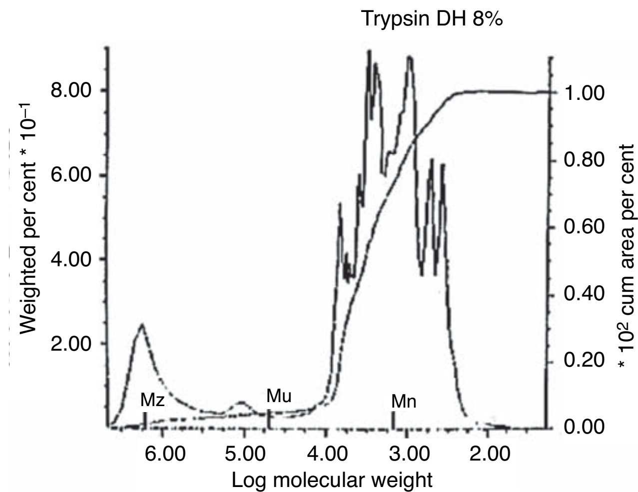


Fig. 13.4 Molecular weight distributions for whey protein hydrolyzates with DH 8% produced by trypsin. (Nielsen unpublished data).

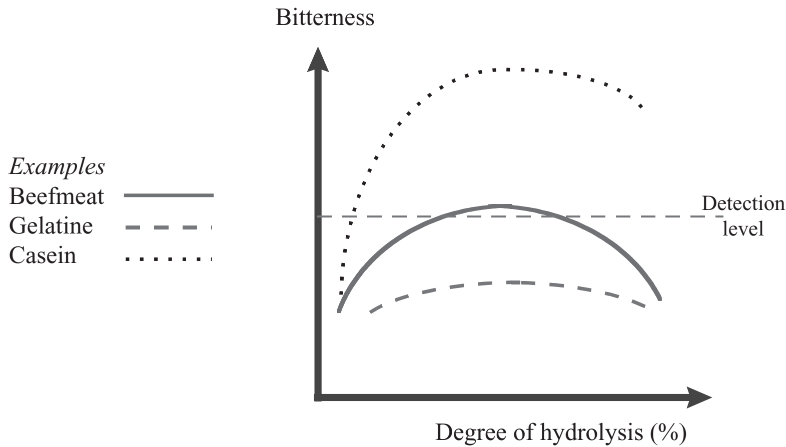


Fig. 13.5 A qualitative description of the bitterness development during hydrolysis of different protein raw materials.

hydrophobic peptides alone, but is a problem with many facets. He mentioned following main variables for bitterness control:

1. The average hydrophobicity of the hydrolyzed protein. Higher hydrophobicity is likely to result in high bitterness.
2. DH which influences both the concentration of soluble hydrophobic peptides and their chain length (see also Fig. 13.5).
3. The specificity of the enzyme which determines whether the terminal amino acids are hydrophobic or hydrophilic (see also Fig. 13.6).
4. Separation of the hydrolyzate, which may eliminate part of the bitterness if hydrophobic amino acids/peptides are eliminated by sedimentation at an appropriate pH.
5. Masking of bitterness as a way to decrease it.

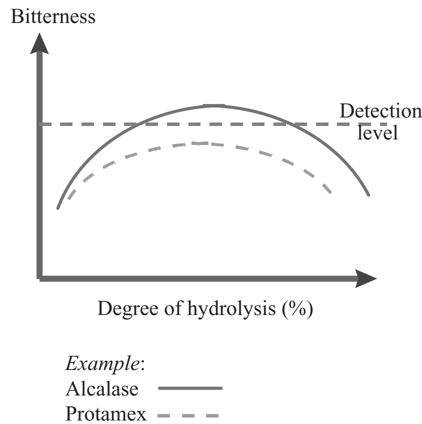


Fig. 13.6 Qualitative description of the development of bitterness during hydrolysis of protein raw material using different proteases.

The tendency of bitterness formation is different from one source of protein to another. Our experience is that the tendency of bitterness formation has the order: gelatin < meat < fish < pea < wheat < whey < soya < casein. This is a very general description, of course. In a recent study, Pedrosa *et al.*[14] investigated the palatability of low allergenic baby food formulas based on hydrolyzed proteins and found that the taste of formulas based on mixed protein hydrolyzates was better than formulas based on single-protein sources. Soya and rice formulas were rated higher than whey-based products with casein having lowest taste score. The study was based upon samples from the market and therefore differences in production methods and formulation were not taken into account.

Prevention of bitterness is achieved by selection of the type of protease(s) and operating conditions of the hydrolysis process. It appears that some specific proteases produce peptides with a superior taste compared to the proteases generally used in protein hydrolyzate production.[15] Recently, a protein hydrolyzate from casein was marketed by DSM, who claimed that the low bitterness of the product was achieved by using a proline-specific protease.

Two of the important parameters in the development of bitterness are illustrated in Figs 13.5 and 13.6. It is clear from Fig. 13.5 that there is a good chance of optimizing the bitterness by controlling the degree of hydrolysis. There is, however, a limitation to the trick of increasing the DH to eliminate the bitterness.[16] At high DH, for example >15%, there is a clear tendency that the bitterness is reduced at the expense of the development of a brothy taste caused by free amino acids and small peptides. This is not surprising when thinking about the very well-known savoury products soya sauce and hydrolyzed vegetable protein (HVP). Soya sauce is the result of enzyme activity and fermentation, and the proteins are extensively hydrolyzed resulting in a DH of approximately 70% with a lot of free amino acids present. HVP is produced by boiling proteinaceous raw materials in strong hydrochloric acid (HCl) and has an even higher DH. Especially HVP has a high content of glutamic acid that is utilized for flavour enhancement of the food products via the addition of HVP. This flavour enhancement property of glutamic acid is known as umami, which some di-peptides also have when glutamic acid is end-positioned at the C-terminus.[17]

Figure 13.6 is a qualitative illustration of the difference between various endoproteases. Alcalase is widely used in the food industry due to its effective hydrolysis of proteins, but does have some limitations with respect to bitterness development. This is partly solved when using another protease – Protamex®. Unfortunately, there is no scientific documentation of the difference between the mechanisms of the two proteases.

It is well known that bitterness can be prevented if endoproteases are combined with exo-peptidases. The exo-peptidases chop off amino acids from the amino-end or the carboxy-end of the peptides (aminopeptidases and carboxypeptidases, respectively). Often the end-positioned amino acids in the peptides are hydrophobic. When these are cleaved off, the peptides lose their bitterness and the cleaved off amino acids are themselves not very bitter.

From the review by Saha[18] it is clear that most work with enzymatic debittering has been focusing on the use of aminopeptidases and the positive effect of these enzymes has been proven. Besides the use of exo-peptidases two other approaches are suggested: (a) treatment with alkaline/neutral protease and (b) condensation reactions of bitter peptides using proteases (plastein formation). Saha[18] reviewed work that proved a positive effect of some enzymes on specific hydrophobic bitter peptides, but no commercial enzyme was mentioned for this concept. For the 'plastein-concept' the protease, under certain conditions, like high substrate dry solids and extreme pH, can gel some of the peptides. In the condensation reaction the bitter peptides gelate after, for example, papain treatment and can be eliminated by sedimentation or filtration.

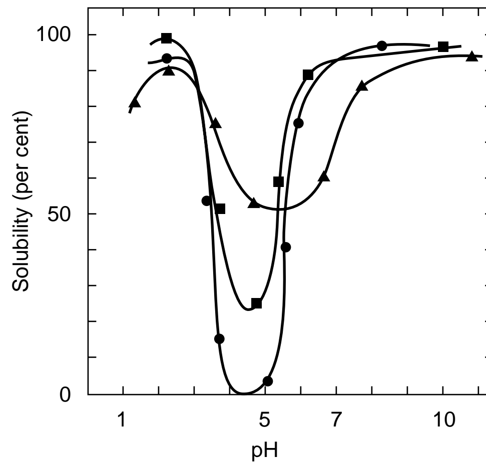


Fig. 13.7 pH-solubility profiles of native casein and of *Staphylococcus aureus* Glu-specific V8 protease modified casein. The solubility is expressed as per cent of total protein in solution. Circles – native casein; squares – DH = 2%; triangles – DH = 6.7% (with kind permission from Springer Science+Business Media (Ref. [21]) Jean-Marc Chobert, Mahmoud Sitohy and John R. Whitaker, Fig. 2.)

Some of the concepts for debittering have the drawback that yield losses can be significant if the debittering means removal of a fraction of the peptides. If bitterness cannot be eliminated in other ways it might be possible to mask it. Some of the ingredients in food products, in which peptides are used, actually help to mask the bitterness. Citric and malic acid and mixtures thereof are mentioned as examples.[7] Another possibility is the use of cyclodextrins. Cyclodextrins have such a shape and charge that bitter peptides tend to hide their hydrophobic parts in the interior ring structure of the cyclodextrin molecule, which will result in lower bitterness.[19] Tamura[19] also discussed the use of gelatinized starch and proteins like whey protein concentrates for the same purpose. Furthermore, polyphosphates have also been suggested.[20] Finally, the selection of flavour compounds can play an important role. The remaining bitterness fits better into the flavour of orange, lime and grapefruit flavoured formulas than into strawberry flavoured drinks.

13.5.2 Solubility

As most protein hydrolyzates are used as ingredients in beverages, their solubility is obviously very important. Native proteins have different solubilities, dependant, amongst other factors, on the pH of the solution. Figure 13.7 shows the effect of hydrolyzing casein to a DH of 2% and 6.7% on protein solubility and comparing this with native casein which is insoluble at its isoelectric pH around 4.5.[21]

Not only does the solubility of the casein increases dramatically, but it is also worth noticing that the pH at lowest solubility is shifting upwards, reflecting the change in charge of the proteins/peptides. The curve has some more information, that is the yield of casein soluble at the isoelectric pH is not more than approximately 50%. It is also evident that upon going from DH 2% to 6.75%, the solubility increases dramatically.

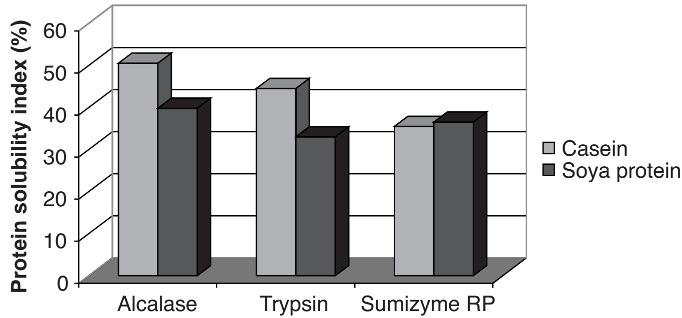


Fig. 13.8 Protein solubility index of protein hydrolyzate at isoelectric pH (adapted from Ref. [7]).

Figure 13.8 shows that the use of different endoproteases does not result in the same solubility at isoelectric pH of a protein/peptide at the same DH.[7] The three enzymes Alcalase, Trypsin and Sumizyme increase the solubility of casein and soya protein to approximately 50%, 45% and 35% and 40%, 33% and 35%, respectively.

This shows that there is no clear correlation between DH and solubility, as the latter is strongly dependent both on the enzyme and the protein substrate. This is also the conclusion in work from Mullally *et al.*[22] However, in general, for a specific enzyme and a specific substrate, the solubility will be higher at higher DH.

It should be mentioned that it is not possible to get a mixture of peptides, which is soluble at all pH values from hydrolyzing any protein, even when the DH is very high, for example 35%. There will always be a fraction of the protein that is insoluble at isoelectric pH and eventually will end as yield loss in the recovery of soluble peptides.

13.5.3 Viscosity

Normally the hydrolysis of proteins causes a decrease in the viscosity of the protein solution. This is for instance reflected in the method for using viscosity measurement to follow the degree of hydrolysis as was suggested by Richardson.[23] There are, however, many deviations from this 'rule'. The effect of hydrolysis of different proteins by Alcalase and Neutrase showed considerable variations both for the two enzymes tested and also among proteins.[24] Alcalase was more efficient in reducing viscosity than Neutrase. The viscosity of a gelatin solution gradually decreased with increasing DH up to 7%. Soya protein behaved very differently, as viscosity decreased dramatically even at DH = 3% and remained stable at higher DH. For corn gluten protein, the viscosity increased steeply during hydrolysis with Neutrase. The different behaviour of these three proteins was ascribed to the very different heterogeneity of the proteins. Gelatin is a homogeneous protein, and in hydrolyzed gelatin there is a good correlation between peptide chain length and viscosity. Soya protein is a heterogeneous mixture of several soluble proteins, whereas corn gluten is a highly heterogeneous mixture of slightly soluble protein particles.

There is a particular protease specificity that has been able to produce very interesting viscosity effects upon protein hydrolysis. This is the glutamic acid-specific endoprotease from *Bacillus licheniformis*[25] which has been studied extensively by researchers in Denmark and The Netherlands. During hydrolysis of whey proteins the viscosity increases as described in Fig. 13.9.[26] The viscosity remains low during hydrolysis until a DH of 4%, followed by a

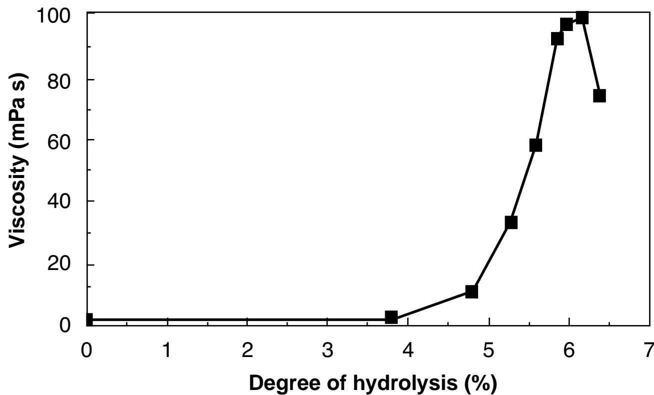


Fig. 13.9 Viscosity of whey protein hydrolyzate produced by a glutamic acid-specific endoprotease.[26]

steep increase until DH 6%, after which the viscosity decreases again. It was suggested that the high viscosity was caused by aggregation of the peptides causing a gelation.[27,28]

Detailed study of this surprising effect revealed that hydrolysis of the α-lactalbumin fraction by this enzyme in the presence of calcium leads to strong gel formation,[29] and further studies on this gel showed hollow linear filaments similar to microtubules.[25] A very detailed description of the self-assembly of hydrolyzed α-lactalbumin into nanotubes resulted from the work of Graveland-Bikker and is described in her PhD thesis.[30]

The concept of protein viscosity reduction is being applied industrially. One example is the use of protease in a fish meal stick water plant. Stick water is the soluble fraction of the processed fish protein which needs to be evaporated before drying. By adding protease the viscosity is reduced, which makes processing of the protein solutions much easier. This results in increased heat transfer and reduced formation of sediments on the hot surface in the evaporators. This even holds when protein solutions are dried to higher dry solids contents, thus leading to energy savings.[15] Generally, low viscosity is desirable in protein-fortified drinks and clinical nutrition products.

13.5.4 Emulsification

Native proteins like casein, soya and whey protein are widely used as emulsifiers in the food industry. Upon hydrolysis, their emulsifying properties are changed, since both the size of the molecules, their charge and the distribution of hydrophobic and hydrophilic regions are altered.

In a study by Adler-Nissen and Olsen,[24] the emulsifying capacity of soya protein hydrolyzate made with Alcalase and Neutrase was investigated. The study showed that the emulsifying capacity reached a maximum at a DH which was different for the two enzymes. Alcalase maximum was at DH = 5%, whereas Neutrase had high emulsifying capacity in the DH range of 2–6, but still lower than the Alcalase maximum (Fig. 13.10).

Other studies have found similar maximum values of emulsifying properties at a certain DH. Mietsch *et al.*[31] investigated Alcalase and Neutrase using soya protein and caseinate. They found that even when the emulsifying capacity increased as a result of the hydrolysis, there was no improvement in the stability of emulsions compared with un-hydrolyzed proteins. They also concluded that the change in emulsifying properties was different

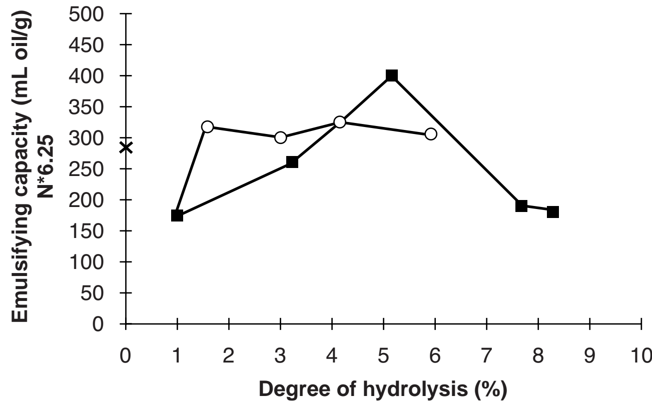


Fig. 13.10 Emulsifying capacity of soya protein at different DH produced by hydrolysis with Alcalase and Neutrase.[24]

between soya protein to caseinate; the change in soya protein emulsifying properties was much larger.

In a study of the effect of the glutamic acid-specific protease V8, Chobert *et al.*[32] found that the emulsifying properties of casein decreased at DH 2% and 6.7%. This indicates the importance of the specificity of the protease used for the hydrolysis. However, the result may have been different for other proteins. When trypsin was tested using casein and whey proteins Chobert *et al.*[33] concluded that the emulsifying activity was improved in the DH range up to 10%, whereas the stability of the emulsion was lower than control.

Hydrolyzing whey proteins up to a DH of 20% using Alcalase and Protamex, two *Bacillus* endoproteases, revealed differences mainly in the emulsion stability.[34] Data are presented in Fig. 13.11, showing a decline in both emulsifying capacity and stability at increasing DH above 5% and that the emulsion stability of Protamex-hydrolyzates is significantly better than when Alcalase was used.

Van der Ven *et al.*[35] made an extensive study of casein and whey protein hydrolyzates produced by 11 different commercially available proteases. The hydrolyzates represented a range in DH from 1% to 24%. They concluded that there is no direct relation between DH and emulsifying properties. Whey protein hydrolyzates had similar emulsifying properties in DH range of 5.5–24%. Casein hydrolyzates showed a different picture. These hydrolyzates were grouped into three groups which formed different emulsions. One group had a narrow emulsion droplet size distribution (DH $< 6\%$). Another set of emulsions made with similar

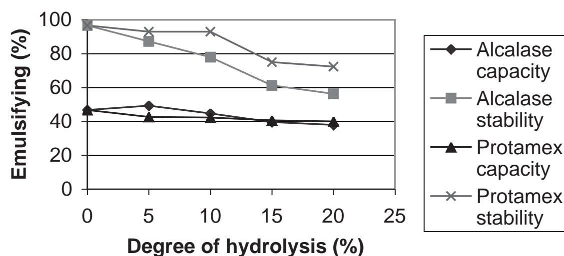


Fig. 13.11 Emulsifying capacity and stability of whey protein hydrolyzed with Alcalase and Protamex (adapted from Ref. [34]).

DH had a broad range of droplet size. The last group of hydrolyzates was characterized by a relatively large droplet size but within this group there was a relationship between DH and emulsion stability. Increasing DH resulted in decrease in emulsion stability.

The emulsifying properties of extensively hydrolyzed proteins are of importance in low allergenic baby food products. An indication of the decrease in emulsifying properties at very high DH in the range of 25–67% has been documented.[36] At these high DH values minimal emulsifying properties were found. The practical implications of the inferior emulsifying properties are that baby food formulations and other nutritional products made with extensively hydrolyzed proteins often require the addition of emulsifiers. The protein/peptide part is not effectively keeping the emulsion stable as is often seen with native proteins. A detailed discussion of the difference between whey protein and hydrolyzed whey protein has been made by Tirok *et al.*[37] who suggest use of lysolecithin to help stabilizing emulsions with hydrolyzed whey protein. A good emulsion in food products promotes the uptake of the nutrients besides giving a better appearance.

13.5.5 Foaming

The use of hydrolysis to improve the foaming properties has been suggested for making an egg white substitute from soya protein.[38] It was proposed that soya protein isolate at DH of 3% made by Alcalase could be used (see Fig. 13.12).

From this figure it is evident that the type of raw material used for making hydrolyzates is important. When using acid-precipitated soya protein, representing the typical production process for soya isolate, there is a narrow maximum for foaming expansion/capacity.

If instead ultrafiltration-derived soya isolate is used, the foaming properties are quite different and better.

In a process patented by Boyce *et al.*,[39] the use of a very specific protease is suggested for the hydrolysis of soya protein isolate to produce a high-quality egg white substitute.

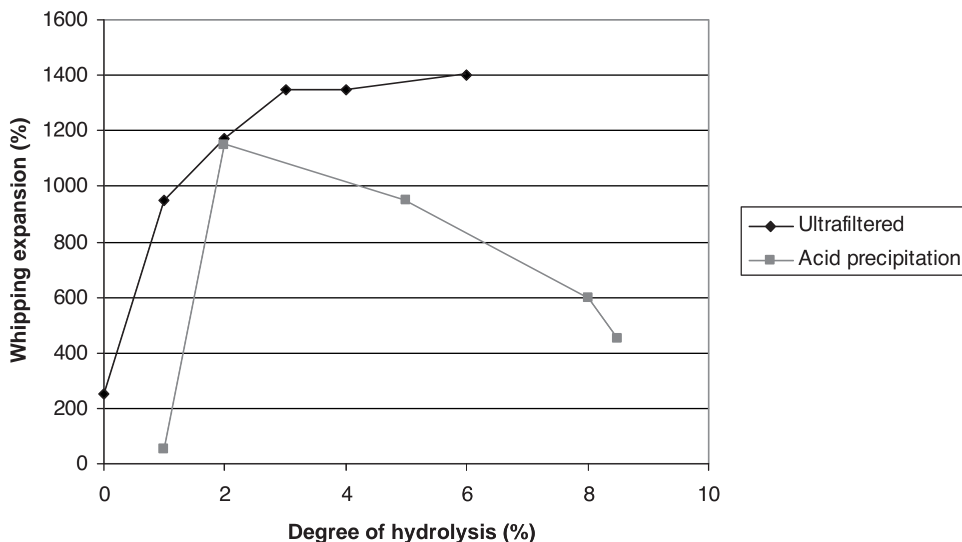


Fig. 13.12 Whipping expansion of soya protein hydrolyzates produced by Alcalase and Neutrase (adapted from Ref. [24]).

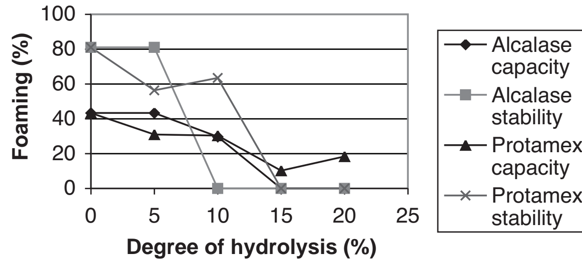


Fig. 13.13 Foaming properties of whey protein concentrate and the hydrolyzate produced by Alcalalse and Protamex (adapted from Ref. [34]).

The DH is very low, approximately 0.5% for optimum functionality. The enzyme used was *M. miehei* protease, which is also well known as microbial rennet for cheese production.

High DH negatively influences foaming properties. This has been documented by Severin and Xia,[34] who tested whey protein hydrolyzates (Fig. 13.13).

They also found that there are big variations in the foaming properties, dependent upon the type of enzyme used. They tested Alcalase and Protamex and found the properties of hydrolyzates made with Protamex to be best. However, hydrolysis by these enzymes resulted in a significant decrease in the stability of the foam starting at a DH of 5%. Unfortunately, they did not have any data in the DH range of 0–5% where optimal performance of hydrolyzates can be expected, according to the results shown in Fig. 13.12.

In another study showing the difference between using Alcalase and Neutrase, it was shown that a hydrolyzate made with Alcalase had a 12-fold improvement in foam expansion, whereas with Neutrase only four times better results were obtained compared with un-hydrolyzed protein. In both cases, the maximum foam expansion occurred at DH 3–4%.[40]

There is no consensus about the DH range for optimum foaming capacity. Don *et al.*[41] have found that soya protein hydrolyzed with a *Bacillus subtilis* protease had maximum foaming capacity at about 10% DH, whereas with *Aspergillus oryzae* protease an increased foaming expansion up to DH of 20% was obtained.

Some illustrative results were produced by Perea *et al.*,[42] showing the foaming capacity and foaming stability of an Alcalase-hydrolyzed whey protein concentrate in the DH range of 0–22% (Fig. 13.14). It appears that the foaming capacity is high when the foaming stability is relatively low and vice versa.

Van der Ven *et al.*[43] investigated the foaming properties of whey and casein hydrolyzates produced by 11 different commercial proteases. All casein hydrolyzates formed high initial foam volume but had lower stability than intact casein. Best stability was achieved at relatively low DH. For whey protein, the foaming properties differed with molecular weight distribution of the peptides. A large fraction of peptides with a molecular weight range of 3–5 kD had the best foam formation. The stability of foam produced with whey protein hydrolyzates was also lower than intact protein.

13.5.6 Gelling

The classic utilization of proteolytic gelling in foods is cheese production. By specific proteolysis, the κ-casein in milk is hydrolyzed at the peptide bond Phe_{105}-$Met_{106,}$ causing the charge of the casein micelle to change, thus enabling the gelation. One of the reaction products is casein macro peptide (CMP), which is released into the whey. Whey has been

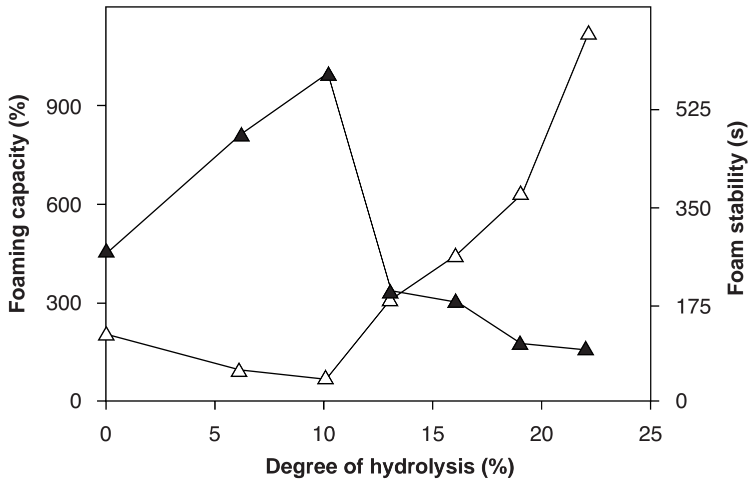


Fig. 13.14 The effect of the degree of hydrolysis on foaming properties of hydrolyzed whey protein concentrate made by Alcalase treatment. Foaming capacity △, foaming stability ▲. (Reprinted from Ref. [42], Copyright (1993), with permission from Elsevier.)

found to have properties that can be utilized by isolating the CMP and use it in functional food products. Instead of producing cheese from the curd it can be used for production of rennet casein. In this case, the curd is heated which contracts the gel and releases the whey, followed by drying of the casein.

The utilization of proteases has also been suggested for the coagulation of soya protein.[44–46]

In section 13.5.5, the use of a Glu-specific enzyme was mentioned which can cause viscosity increase and eventually gelling of whey protein solutions. This section describes gelling by a number of proteases in surprising conditions. Trypsin, papain, Pronase and a protease from *Streptomyces griseus* all caused gelling of whey protein. The DH for gelling was 27.1% for trypsin, 23.1% for papain, 28.2% for Pronase and 15.6% for the *S. griseus* protease.[47] To *et al.*[48] found that pepsin did actually improve the gel strength of whey protein concentrate but at lower DH.

A special term – plastein reaction – has been used for describing the gelling/aggregation taking place when a protein hydrolyzate/peptide mixture is incubated with a protease. Even though the plastein formation is hardly utilized in the production of protein ingredients, it is of interest since it can develop during the production of protein hydrolyzates. The most important parameters for plastein formation are type and size of peptides, concentration of peptides and pH of the reaction mixture. Generally, the DH of the protein hydrolyzate must be high to promote the plastein formation. Casein hydrolyzate with a molecular weight of 380–800 Dalton was the best substrate for plastein formation.[49]

Plastein formation generally takes place at a pH value different from that of the optimum for hydrolysis. Adler-Nissen[8] tested eight different proteases and showed that all but one had a pH optimum for plastein formation 2–3 pH units away from the optimum for hydrolysis. Only papain had the same pH optimum for hydrolysis and plastein formation.

It has been suggested by Fujimaki *et al.*[50] and Lalasdis and Sjoberg[51] that the plastein reaction can be used to reduce the bitterness of protein hydrolyzates. Several researchers have suggested improving the nutritive value of a protein hydrolyzate by incorporating essential

amino acids in the peptides by the plastein reaction.[52,53] Also the allergenicity of protein hydrolyzates can be reduced as result of plastein reaction.[54] Lorenzen and Schlimme[55] found that the plastein reaction also did occur at a peptide concentration lower than 20–30% and they suggested that it could influence the properties of protein hydrolyzates made under standard processing conditions.

Overall, it is difficult to control the plastein reaction and utilize it specifically for production of protein/peptide ingredients. The maximum yield that can be expected from a plastein reaction followed by separation is not more than around 30%, which economically limits the utilization. The interesting part of plastein reactions, taking place at normal low-concentration conditions when processing proteins, for practical applications seems to be the gelling effect and its influence on some of the other properties of hydrolyzates.

13.5.7 Allergenicity

The use of protein hydrolysis to lower the allergenicity of baby food formulations has been common practice for more than 60 years.[54] The technology enables production of mother's milk substitutes that are suitable both for the group of babies already suffering from cows' milk allergy and those that are at risk of developing it. These two segments require different products. The first needs a product where the protein is extensively hydrolyzed, that is DH $> 25\%$, whereas the other can benefit from a product with protein having a lower DH, since this can already reduce the risk of developing allergy.

β-Lactoglobulin is present in cows' milk at relatively high concentration (9.8% of the milk protein)[56] but is absent in human milk. Of the population allergic to milk proteins, 60–80% is allergic to β-lactoglobulin, 60% to casein, 50% to α-lactalbumin and 50% to serum albumin.[57] In literature, the attempts to reduce allergenicity normally focus on whey proteins or caseins. This is most likely due to the availability of these individual types of proteins as ingredients for baby food formulations. Most references study whey proteins which could be due to the slightly better amino acid composition but also to the inherent difficulties in producing a low-bitter product from casein.

Mahmoud *et al.*[36] investigated the reduction in antigenicity of casein using pancreatic protease. They measured the immunologically active casein at increasing DH up to approx. 70%. The results are presented in Fig. 13.15. The reduction in detectable casein (IAC value) was not as large as could be expected when compared with the results of Knights,[54] who got a 10^6 reduction in detectable casein in a high DH hydrolyzate.

Knights[54] also measured the molecular weight of the peptides in the hydrolyzate and found none above Mw 1200 D. Sixty-seven per cent were smaller than 500 D. A slightly different casein hydrolyzate with a maximum Mw of 5000 D, 0.3% in the range of 3500–5000 D and 0.5% in the range of 2500–5000 D induced anaphylaxis in guinea pigs. Other studies have shown that peptides from chymotrypsin hydrolyzed casein could raise antibodies in rabbits even though the maximum peptide size was 1000 D.[58]

13.5.8 Bioactive peptides

For many years there has been an interest in using peptides as bioactive components. From milk, for instance, a range of peptides with different physiological effects have been isolated and characterized. Saxelin *et al.*[59] summarized all bioactive peptides derived from cow milk protein and listed the following activities: Opioid antagonists, antihypertensive peptides, antithrombotic peptides, antimicrobial peptides, immunostimulants, mineral

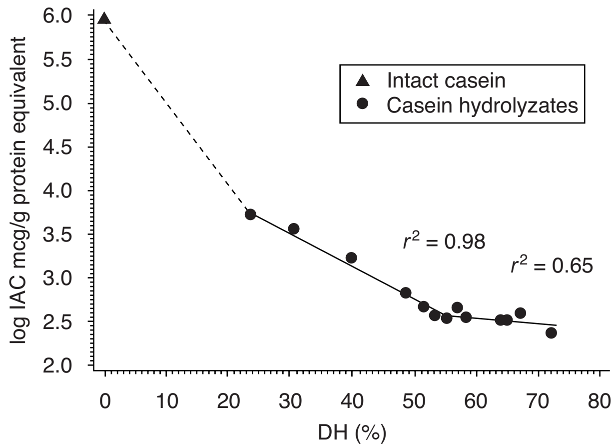


Fig. 13.15 Reduction in antigenicity of casein hydrolyzate at DH up to 70% using pancreatic protease. (Reprinted from Ref. [36].)

carriers and anti-stress peptides. The peptides originate from different parts of the caseins and whey proteins in the milk. In the body, these peptides can be formed by the enzyme activity in the digestion system and/or as result of the lactic acid fermentation in the intestine.

A recent review by Hartmann and Meisel[60] discussed the research on food-derived bioactive peptides. Besides the benefits mentioned above, such peptides can also have positive effects on the cardiovascular system (hypocholesterolemic and antioxidative). It has been hypothesized that upon intake of soya foods, the proteins are converted into bioactive peptides with the mentioned positive effects and that this explains the positive effect of consuming soya containing foods.

The technology for in vitro production of bioactive peptides takes both raw material selection and choice of protease into account. Table 13.3 shows examples of bioactive peptides made from food proteins.

Table 13.3 Bioactive peptides produced from food proteins.

Protein	Health claim	Peptide	Protease	References
Soya	Body weight control	Alcalase hydrolyzate	Alcalase	61
Casein	Anticariogenic	Casein phosphopeptide	Trypsin	62
Whey	Iron binding	Various	Different	63
β-lactoglobulin	Anti-microbial	Various	Alcalase, pepsin, trypsin	64, 65
α-lactalbumin	Angiotensin-converting enzyme inhibitor	Tyr-Gly-Leu-Phe	Pepsin	66
	Anti-microbial	Various	Trypsin	67
Collagen	Bone and joint disease	Gelatin	Alcalase	68

In a review covering the production and functionality of bioactive peptides from milk, Korhonen and Pihlanto[69] describe how the peptides can be produced in three different ways:

- Enzymatic hydrolysis by digestive enzymes.
- Enzymatic hydrolysis by enzymes from microorganisms or plants.
- Fermentation of milk by proteolytic starter cultures.

The first relates to the use of pepsin and trypsin, which are commercially available proteases in different formulations, and the second refers to publications utilizing Alcalase, chymotrypsin, pancreatin, pepsin and thermolysin. They also discuss the alternative technology to produce the peptides, which is expressing them in microbes and recover the peptides after a fermentation process.

13.6 PROCESSING ISSUES

The (enzymatic) processing of raw materials normally includes following process steps: preparing the raw material, heat treatment, separation, concentration and formulation. These will be discussed in details below. In Fig. 13.16 a general process lay out is shown for enzymatic processing of protein products.

13.6.1 Preparing the raw material

In the pretreatment of raw materials before hydrolysis it is generally of importance to get the raw material into conditions that can be handled further in the process. The correct pretreatment of raw materials can increase production yield. If, for example, the raw materials are bones from meat processing, it will be easier to fill and empty the hydrolysis tank if the bones are crushed to, for example 2–3 cm pieces. A bone crusher is standard equipment for several producers. Another aspect of the crushing is to get access to the protein in the marrow of the product, which will improve the yield and can improve the flavour intensity.

Meat like trimmings are easier to break down with proteases if they are homogenized and ground into a paste using wet milling. The milling can, however, have a negative influence on the separation of the fat after hydrolysis, but if mechanical separators (centrifuge or decanter) are available, this is not a problem. Generally, the processing of protein raw materials requires a relatively low protein concentration in order to keep viscosity of the substrate mixture low enough, that is approximately 10% protein.

Various heat treatments are always part of the process when carrying out enzymatic hydrolysis. The raw material suspension may need a pasteurization to keep microbial growth in control, the temperature of the raw material has to be adjusted to the temperature of hydrolysis and afterwards the enzymes are inactivated by heating. It is important to control the heat treatment so that excessive Maillard reactions are prevented.

13.6.2 Hydrolysis

Table 13.4 shows a list of the problems that can occur during the enzymatic hydrolysis process. The most likely reasons for the problems are described, as well as ways to eliminate these problems.

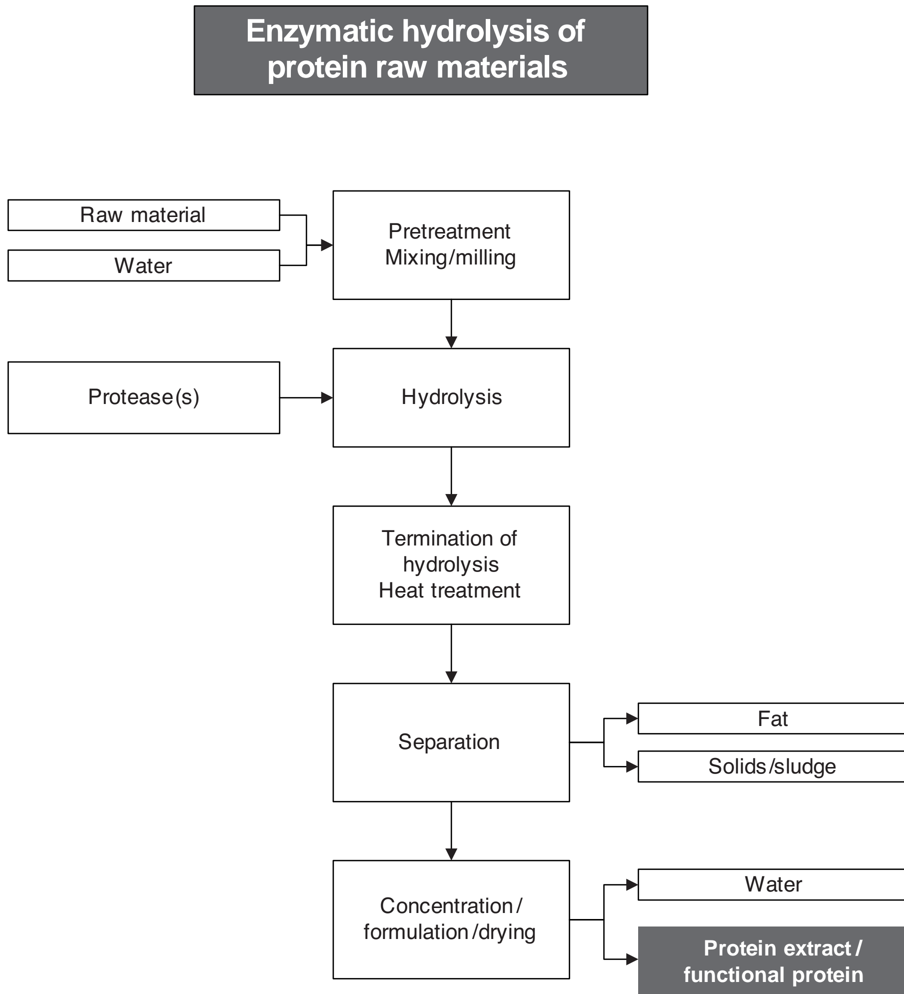


Fig. 13.16 General process layout for production of hydrolyzes protein/peptide products.

13.6.3 Inactivating the proteases

One key question in protein hydrolysis is related to the inactivation of the enzymes. In the end products, the amount of active enzyme must be zero or close to zero, which is normally secured by a heat treatment.

The basis for approval of proteases for food application is the No Observed Adverse Effect Level (NOAEL). For human consumption, this level is 0.44 Anson Units/kg/day, meaning that it has been demonstrated that humans can safely consume an amount of protease corresponding to at least 0.44 activity units per kg body mass per day. This level is based on feeding trials and a safety evaluation.

When producing a protein hydrolyzate for use in different applications, the safety margin can be estimated for each application. These are calculated from the maximum dosage of protease used per kilogram protein, the remaining level of intact enzyme protein after the

Table 13.4 Checklist for troubleshooting in enzymatic hydrolysis of protein raw materials.

Problem	Likely cause	Improvement
Product tastes bitter	Hydrolysis reaction is not optimized with respect to DH and/or choice of enzyme	Optimum enzyme choice is Protamex combined with Flavourzyme® DH to high or to low
	Too severe heat treatment	Reduce temperature during downstream processing
	Too long holding time at high temperature	Reduce holding time at high temperature especially when the product is concentrated
Product has an off flavour	Infection during processing	Check the raw material quality and/or reduce the holding time in process where microbes can grow. Alternatively pasteurize or add preservative, e.g. salt
Hydrolysis to slow	Temperature out of control	Check temperature using another thermometer
	Enzyme dosage too low	Increase dosage
	Enzymes have lost activity because of too warm and/or too long storage time	Make sure the enzyme is stored cool and not too long
	Enzyme diluted with water before usage	Only in original formulation the enzyme is stable. Do not keep diluted enzyme
	Protease inhibitor in raw material	Eliminate inhibitor, e.g. by heat denaturation or increase protease dosage
	Protein concentration different from expected concentration	Check the concentration and adjust if necessary with NaOH or HCl
	pH out of range	Check pH and adjust if necessary

inactivation and amount of daily intake of the hydrolyzed protein. For two typical applications the calculation looks as follows.

Production of the protein hydrolyzate with a high dosage, that is 50 AU kg^{-1} protein and a factor 1000 reduction in activity by heat treatment leaves 0.05 AU kg^{-1} protein.

In case of mother's milk substitute: A daily intake of 3.53 g protein/kg/day corresponds with approximately 0.18×10^{-3} AU/kg/day, which results in a safety margin of 2500.

Enteral nutrition: A daily intake of 2000 g of the product per day for a 60-kg person, which contain up to 3.5% protein means 1.17 g protein/kg/day, which corresponds with $\approx 0.058 \times 10^{-3}$ AU/kg/day, which results in a safety margin of 7500.

From this can be seen that the safety margin is well above the NOAEL level, even when a high enzyme dosage and an inactivation of a factor 1000 is anticipated. The NOAEL is a value estimated for each of the food proteases and is the basis for approval of the protease by the authorities for the different applications.

One of the key assumptions made in this evaluation is the inactivation of the protease. This can in practice be a difficult subject to handle. There is a well-documented example[7] of how the inactivation of the protease Alcalase is studied. The authors made a series of trials from which they could make a mathematical formula expressing the decimation time

(t_D – the time it takes to reduce activity by a factor 10) for the enzyme:

$$t_D = 1.19 \times 10^{(75-T)/8.31} \times (1 + S \times 10^{(75-T)/94}) \text{ min}$$

where T = temperature(°C), and S = substrate (protein) concentration (%).

The equation is valid in the pH-range of 6–9 and temperature range of 50–80°C.

If, for example, 80°C is used in the heating of a mixture containing 10% protein, the calculation is as follows:

$$t_D = 1.19 \times 10^{(75-80/8.31)} \times (1 + 10 \times 10^{(75-80)/94}),$$
$$t_D = 2.93 \text{ min}$$

A reduction in activity by a factor 1000 will then require approx. 9 min heating.

It should be noted that the enzyme is stabilized by the protein/peptide in the solution, therefore the t_D is directly proportional to S. Another issue is the question of how much the activity of the enzyme needs to be reduced to call the enzyme inactivated? In the example of the safety discussion above, a factor 1000 is suggested as a reasonable level. Others may want to secure even lower residual levels in the end product. This will call either for lower enzyme dosage and/or longer heating temperature/time. The pH effect on the inactivation is not included in the formula, unfortunately, as this is also an effective parameter, at least for some proteases, to utilize in the attempt to inactivate the enzyme. In a heating process there are some considerations to take into account, such as excessive production of Maillard reaction products or formation of lysino-alanine at severe heat treatment conditions, both of which can ruin the end product.

After hydrolysis, the temperature is typically increased to 95°C by indirect heating or by steam injection. Three types of batch heating can be used:

1. Heat the mixture in a steam jacket tank.
   This is a relatively slow method but allows full control of the temperature of the mixture, as well as the water content in the substrate. Again an efficient mixer in the tank is required to limit burnt material on the inner side of the tank.
2. Adding hot water to the substrate.
   This is a fast method to increase raw material temperature. An efficient mixer is needed in order not to cause spots with too high temperature. Dilution of the substrate limits this method of heating. This method can only bring the temperature up to what is needed for hydrolysis.
3. Directly heating by steam injection.
   An efficient mixer is needed and the steam must be of ‘food quality’. Problems with noise from the condensation of steam can be severe. However, it is an efficient and fast method for getting a temperature increase. Experience with flash heating equipment has shown to have a positive side effect as unwanted flavour components are flashed off during the process.

An alternative to batch heating is passing the hydrolyzate mixture through a continuous pasteurizer. If the hydrolyzate needs to be inactivated after a well-defined time, the time requirements of this process can present difficulties. If, however, the hydrolysis reaction has gone to completion, there may be no problem with the last part of the product having a longer holding time in the enzyme reactor.

Sometimes a heat treatment can be applied in order to intensify flavour. It is well known that heating causes Maillard reactions, which create flavoursome components. The most important parameters, aside from the composition of the product, are temperature, dry matter content and pH.

Temperature and dry matter composition influence the time it takes to attain a certain reaction whereas pH is more important in directing the reaction into different pathways.

To aid the development of specific flavour notes, a wide range of ingredients can be added to the protein hydrolyzate before the heating process. For example, cystein creates a distinct chicken flavour and addition of xylose can produce a fried type of flavour.

13.6.4 Recovery of the protein/peptide product

In many production processes, it is necessary to perform a separation step to recover the protein/peptide from the reaction mixture. The choice of separation process is dependent on the type of raw material in the process. The hydrolyzed mixture may need to be separated into three phases:

- Fat phase.
- Sediment: Larger particles of un-hydrolyzed protein or components in the substrate mixture are removed on a screen/sieve or separated by decantation or centrifugation.
- Water phase – the soluble product.

Removal of the fat fraction is most efficient at high temperature, that is 95°C. Equipment is available for performing the separation of all three fractions in one step.

1. Three-phase decanter.
   This equipment can handle large amounts of sludge and fat.
2. Three-phase centrifuge.
   Not so efficient when relative large amounts of sludge are present, as the centrifuge has to stop for de-sludging every time the sludge compartment is full.

A more versatile equipment set-up is that of a two-phase decanter or centrifuge followed by an oil separator. This is often preferred as it is easier to adjust to different products.

Omitting this relatively expensive equipment is possible under certain circumstances. When the raw material consists of bones and there is no requirement for a product with very low fat content, it is possible to do a relatively efficient separation in the tank by allowing the product to rest for 30–60 min at 95°C. The fat will settle at the top and the sludge on the bottom. When emptying the tank from the bottom and discarding the first couple of litres, it is possible to selectively isolate the protein solution without much fat and leaving the sediment in the tank, whereby the bone fraction acts as a filter.

In the recovery process, further steps can be taken to eliminate unwanted compounds, for example for improving the taste of the product. Some examples:

- Membrane filtration where ultrafiltration can produce a clear end product by keeping long chain peptides in the retentate and only allow the most soluble short chain peptides to pass to the end product.

- Isoelectric precipitation, which will eliminate a relatively large part of the hydrophobic peptides and result in a less bitter end product.[7]
- Activated carbon treatment which is another method to eliminate certain hydrophobic compounds.

13.6.5 Concentration, formulation and drying

Normally, the protein solution needs to be concentrated to become a stable product. Two types of unit operations can be used:

1. Reverse osmosis.
   By using membrane technology for concentration there is a limit to the maximum protein/dry matter that can be produced. Normally, 20% protein in solution is considered to be the maximum concentration that is economically feasible.
2. Evaporation.
   With this type of equipment it is possible to obtain a very high dry matter in the concentrate (approx. 50%). The evaporation normally takes place in vacuum which limits the effect heat can have on the quality of the end product. Heating the protein solution, especially at high dry matter content, causes Maillard reactions. Excessive heating at high dry matter content can result in off flavour formation and in the formation of bitterness in the product. In practice this means that a long holding time at high temperature must be avoided when concentrating and packing the product.

A stable formulation is needed when the product has to be kept for a longer period of time before being used. If, for instance, a soup stock protein extract from meat is the end product, it can be stabilized by concentrating to 50° Brix and the addition of 10% NaCl. The product must be filled hot into the container, that is at temperature above 80°C. By turning the container upside down immediately after filling, the lid is heated to kill spores of fungus and yeast. Immediate cooling is required to secure limited Maillard reactions.

It is very common to spray-dry the end products. Hydrolyzed proteins can be more difficult to dry, especially when they are extensively hydrolyzed. At high DH, dried products tend to become hydroscopic and lump formation can occur. Difficulties like deposits in the drier may need additions of other compounds to control the drying. A low DE maltodextrin can be used to mix with the concentrated hydrolyzate before drying.

13.7 PROTEIN HYDROLYZATES ON THE MARKET

Hydrolyzed protein/peptide products have been on the market for many years. A list of products which are or have been available is found in Table 13.5.

It is evident that there are many different applications where the hydrolysis technology has been utilized, where either the change in functional properties like solubility or taste are the major benefits, or where specific health effects are claimed.

Table 13.5 Examples from the market of properties and applications of hydrolyzed proteins/hydrolyzates from different raw materials.

Peptides from	**Product**	**Properties/application**	**Producer**
Whey protein	Whey protein hydrolyzate	Hypoallergenic baby food ingredient	Arla Foods, DMV
	Peptigen®	Fast absorbance peptides for dietic and sports nutrition	
	Whey protein hydrolyzate. BioZate	Reduction of blood pressure	Davisco
Casein	Low allergenic peptides	Hypoallergenic infant formula (Nutramigen®)	Mead Johnson
		Hypoallergenic baby food ingredient	Arla Foods, DMV, Fonterra
	PeptoPro®	Di-and tri-peptide peptides for sports nutrition (PeptoPro®) for insulin stimulation	DSM
		Casein-phosphopeptides for dental care product	Recaldent
	CE90GMM	Casein-phosphopeptides for better mineral absorption	DMV
	C12 Peption	ACE-inhibitor peptides for healthy blood pressure	DMV
	InsuVital™	Manage blood glucose level – help type 2 diabetics	DSM
	TensGuard™	Milk protein tri-peptide for blood pressure control	DSM
Soya	Soya protein hydrolyzate	Immune enhancing enteral nutrition (Advera®)	Ross/Abbott
	Soya protein hydrolyzate	Fully soluble peptides for protein fortification of acid drinks (Pro Up®	Novo Nordisk
Pea	Pea protein hydrolyzate	Protein fortification of drinks	Arla Foods
Meat	Hydrolyzed meat protein extract	Soluble meat protein/meat stocks for flavour and nutrition	Meatzyme
Gelatin	Gelatin-hydrolyzate	Peptides for treating arthritis and osteoporosis	Gelita Group
Fish	Nutripeptin®	Lowers postprandial blood glucose	Copalis, France
	Salmon protein hydrolyzates	Nutritional food supplement	Marine Bioproducts
Wheat		High-glutamine peptide for recovery nutrition	DMV
Egg white	Benefit®	Protein fortification of drinks	Sanovo Foods

13.8 CONCLUSION

The use of proteases for modification of protein properties to produce protein ingredients with improved properties is well established in the market. The major concepts are low-allergenic mother's milk substitutes and highly soluble proteins/peptides for protein fortification. The

expansion of the market with new innovative hydrolyzed protein products is to a large extent dependent upon the availability of enzymes. It is evident that controlling the properties of the peptides is a very complex task and may require new proteases. Proteases with more well-defined specificity can provide the tools for production of, for example bioactive peptides or improving the properties of protein products in the market. Experimental proteases with new well-defined specificities have been tested to some extent but commercialization of these interesting proteases must be driven by sufficiently high value of the end product to pay for the development of the new enzymes. This will hopefully be possible in the near future and result in a larger palette of enzymes covering a range of different specificities. Furthermore, there may be a market for other enzymes that act on proteins as well. Cross-linking enzymes like transglutaminase, tyrosinase and laccase are on the market and are being used in many food products. However, they have not yet proven to provide functionality for protein ingredient products.

REFERENCES

1. Lantto, R. (2007) Protein cross-linking with oxidative enzymes and transglutaminase. Effects in meat protein systems. Academic dissertation, VTT Technical Research Centre, Finland.
2. Nielsen, P.M. (1995) Reactions and potential industrial applications of transglutaminase. Review of literature and patents. *Food Biotechnology* **9**, 119–156.
3. Yamaguchi, S. and Yokoe, M. (2000) A novel protein-deamidating enzyme from chryseobacterium proteolyticum sp. nov., a newly isolated bacterium from soil. *Applied and Environmental Microbiology* **66**(8), 3337–3343.
4. Swaisgood, H. (1980) Sulphydryl oxidase: properties and applications. *Enzyme and Microbial Technology* **2**, 265–272.
5. Nielsen, P.M. (1997) Functionality of food proteins. In: *Food Proteins and Their Applications* (eds S. Damodaran and A. Paraf). Marcel Dekker, New York, pp. 443–472.
6. Nielsen, P.M., Pedersen, D. and Dambmann, C. (2001) Improved method for determining food protein degree of hydrolysis. *Journal of Food Science* **66**(5), 642–646.
7. Adler-Nissen, J. (1986) *Enzymic hydrolysis of food proteins*. Elsevier Applied Science Publishers, New York.
8. Adler-Nissen, J. (1979) Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. *Journal of Agricultural and Food Chemistry* **27**, 1257.
9. Mozersky, S.M. and Panettieri, R.A. (1983) Is pH drop a valid measure of extent of protein hydrolysis? *Journal of Agricultural and Food Chemistry* **31**, 1313.
10. Margot, A., Flaschel, E. and Renken, A. (1994) Continuous monitoring of enzymatic whey protein hydrolysis. Correlation of base consumption with soluble nitrogen content. *Process Biochemistry* **29**, 257.
11. Silvestre, M.P.C. (1997) Review of methods for the analysis of protein hydrolysates. *Food Chemistry* **60**(2), 263–271.
12. Nielsen, P.M. (2008) Unpublished data.
13. Ney, K.H. (1971) Voraussage der Bitterkeit von Peptiden aus deren Amminosaurenzusammensetzung. *Z. Lebensm.-Untersuc. Forsch.*, **147**, 64.
14. Pedrosa, M., Pascual, C.Y., Larco, J.L. and Esteban, M.M. (2006) Palatability of hydrolysates and other substitution formulas for cow's milk-allergic children: a comparative study of taste, smell, and texture evaluated by healthy volunteers. *Journal of Investigational Allergology and Clinical Immunology* **16**(6), 351–356.
15. Christensen, F.M. (1989) Review. Enzyme technology versus engineering technology in the food industry. *Biotechnology and Applied Biochemistry* **11**, 249.
16. Lynglev, G.B. (2008) Personal communications – unpublished results.
17. Tada, M., Shinoda, I. and Okai, H. (1984) L-ornithyltaurine, a new salty peptide. *Journal of Agricultural and Food Chemistry* **32**, 992.

18. Saha, B.C. and Hayashi, K. (2001) Research review paper. Debittering of protein hydrolyzates. *Biotechnology Advances* **19**, 355–370.
19. Tamura, M., Mori, N., Miyoshi, T., Koyama, S., Kohri, H. and Okai, H. (1990) Practical debittering using model peptides and related peptides. *Agricultural and Biological Chemistry* **54**, 41.
20. Tokita, F. (1969) Enzymatische und nicht tnzymatische Ausschaltung des Bittergesmacks bei enzymatischen Eiweisshydrolysaten. *Zeitschrift für Lebensmittel-Untersuchung und -Forschung* **138**, 351.
21. Chobert, J.-M., Sitohy, M. and Whitaker, J.M. (1987) Specific limited hydrolysis and phosphorylation of food proteins for improvement of functional and nutritional properties. *Journal of the American Oil Chemists' Society* **64**(12), 1704.
22. Mullally, M.M., O'Callaghan, D.M., Fitzgerald, R.J., Donnelly, W.J. and Dalton, J.P. (1994) Proteolytic and peptidolytic activities in commercial pancreatic protease preparations and their relationship to some whey protein hydrolysate characteristics. *Journal of Agricultural and Food Chemistry* **42**, 2973.
23. Richardson, T. (1977) Functionality changes of proteins following action of enzymes. *Advances in Chemistry Series* **160**, 185.
24. Adler-Nissen, J. and Olsen, H.S. (1979) The influence of peptide chain length of taste and functional properties of enzymatically modified soy protein. In: *Functionality and Protein Structure* (ed A. Pour-El). American Chemical Society, Washington, DC, p. 125.
25. Breddam, K. and Meldal, M. (1992) Substrate preferences of glutamic-acid-specific endopeptidase assessed by synthetic peptide substrates based on intramolecular fluorescence quenching. *European Journal of Biochemistry* **206**, 103–107.
26. Budtz, P. and Nielsen, P.M. (1992) Protein preparations. International Patent Application WO92/13964.
27. Creusot, N., Gruppen, H., Van Koningsveld, G.A., de Kruif, C.G. and Voragen, A.G.J. (2006) Peptide-peptide and protein-peptide interactions in mixtures of whey protein isolate and whey protein isolate hydrolysates. *International Dairy Journal* **16**(8), 840–849.
28. Otte, J., Lomholt, S.B., Ipsen, R., Stapelfeldt, H., Bukrinsky, J.T. and Qvist, K.B. (1997) Aggregate formation during hydrolysis of beta-lactoglobulin with a Glu and Asp specific protease from Bacillus licheniformis. *Journal of Agricultural and Food Chemistry* **45**, 4889–4896.
29. Ipsen, R., Otte, J. and Qvist, K.B. (2001) Molecular self-assembly of partially hydrolysed alpha-lactalbumin resulting in strong gels with novel microstructure. *Journal of Dairy Research* **68**, 277–286.
30. Graveland-Bikker, J. (2005) Self-assembly of hydrolysed α-lactalbumin into nanotubes. PhD Thesis, University of Utrecht.
31. Mietsch, F., Feher, J. and Halasz, A. (1989) Investigation of functional properties of partially hydrolyzed proteins. *Die Nahrung* **33**(1), 9–15.
32. Chobert, J.-M., Bertrand-Harp, C. and Nicolas, M.-G. (1988) Solubility and emulsifying properties of casein and whey proteins modified enzymatically by trypsin. *Journal of Agricultural and Food Chemistry* **36**, 883.
33. Chobert, J.-M., Sitohy, M. and Whitaker, J.M. (1988) Solubility and emulsifying properties of caseins modified enzymatically by Staphylococcus aureus V8 protease. *Journal of Agricultural and Food Chemistry* **36**, 220.
34. Severin, S. and Xia, W.S. (2006) Enzymatic hydrolysis of whey proteins by two different proteases and their effect on the functional properties of resulting protein hydrolysates. *Journal of Food Biochemistry* **30**, 77–97.
35. Van Der Ven, C., Gruppen, H., de Bont, D.B.A. and Voragen, A.G.J. (2001) Emulsion properties of casein and whey protein hydrolysates and the relation with other hydrolysate characteristics. *Journal of Agricultural and Food Chemistry* **49**, 5005–5012.
36. Mahmoud, M.I., Malone, W.T. and Cordle, C. (1992) Enzymatic hydrolysis of casein: effect of degree of hydrolysis on antigenicity and physical properties. *Journal of Food Science* **57**(5), 1223.
37. Tirok, S., Scherze, I. and Muschiolik, G. (2001) Behaviour of formula emulsions containing hydrolysed whey protein and various lecithin. *Colloids & Surfaces B: Biointerfaces* **21**, 149–162.
38. Adler-Nissen, J. and Olsen, H.S. (1982) Taste and taste evaluation of soy protein hydrolysates. In: *Chemistry of Food and Beverages – Recent Developments* (eds G. Charalambous and G.E. Inglett). Academic Press, New York, p. 149.
39. Boyce, C.O.L., Lanzilotta, R.P. and Wong, T.M. (1986) Enzyme modified soy protein for use as an egg white substitute. US Patent 4,632,903.
40. Olsen, H.S. (1995) Enzymes in food processing. In: *Enzymes, Biomass, Food, and Feed* (eds G. Reed and T.W. Nagodawithana). VCH, Weinheim, Germany, p. 663.

41. Don, L.S.B., Pilosof, A.M.R. and Bartholomai, G.B. (1991) Enzymatic modification of soy protein concentrates by fungal and bacterial proteases. *Journal of the American Oil Chemists' Society* **68**(2), 102.
42. Perea, A., Ugaide, U., Rodriguez, I. and Serra, J.S. (1993) Preparation and characterization of whey protein hydrolysates: applications in industrial whey bioconversion processes. *Enzyme and Microbial Technology* **15**, 418.
43. Van Der Ven, C., Gruppen, H., de Bont, D.B.A. and Voragen, A.G.J. (2002) Correlations between biochemical characteristics and foam-forming and – stabilizing ability of whey and casein hydrolysates. *Journal of Agricultural and Food Chemistry* **50**, 2938–2946.
44. Murata, K., Kusakabe, I., Kobayashi, H., Akaike, M., Park, Y.W. and Murakami, K. (1987) Studies on the coagulation of soymilk protein by commercial proteases. *Agricultural and Biological Chemistry* **51**(2), 385.
45. Murata, K., Kusakabe, I., Kobayashi, H., Kiuchi, H. and Murakami, K. (1987) Selection of commercial enzymes suitable for making soymilk curd. *Agricultural and Biological Chemistry* **51**(11), 2929.
46. Sato, K., Nakamura, M., Nishiya, T., Kawanari, M. and Nakajima, I. (1995) Preparation of a gel of partially heat-denatured whey protein by proteolytic digestion. *Milchwissenschaft: Milk Science International* **50**(7), 389.
47. Murata, K., Kusakabe, I., Kobayashi, H., Kiuchi, H. and Murakami, K. (1988) Functional properties of three soymilk curds prepared with an enzyme, calcium salts and acid. *Agricultural and Biological Chemistry* **52**(5), 1135.
48. To, B., Heibig, N.B., Nahai, S. and Ma, C.Y. (1985) Modification of whey protein concentrate to stimulate whippability and gelation of egg white. *Canadian Institute of Food Science and Technology Journal* **18**(2), 50.
49. Sukan, G. and Andrews, A.T. (1982) Application of the plastein reaction to caseins and to skim milk powder. 1. Protein hydrolysis and plastein formation. *Journal of Dairy Research* **49**, 265.
50. Fujimaki, M., Yamashita, M., Arai, S. and Kato, H. (1970) Plastein reaction – its application to debittering of protein hydrolysates. *Agricultural and Biological Chemistry* **34**, 483.
51. Lalasdis, G. and Sjoberg, L.-B. (1978) Two new methods of debittering protein hydrolysates and a fraction of hydrolysates with exceptionally high content of essential amino acids. *Journal of Agricultural and Food Chemistry* **26**, 742.
52. Hajós, G.Y., Szarvas, T. and Vámos-Vigázó, L. (1990) Radioactive methionine incorporation into peptide chains by enzymatic modification. *Journal of Food Biochemistry* **14**, 381.
53. Yamashita, M., Arai, S., Tsai, S.-J. and Fujimaki, M. (1971) Plastein reaction as a method for enhancing the sulfur-containing amino acid level of soybean proteins. *Journal of Agricultural and Food Chemistry* **19**, 1151–1154.
54. Knights, R.J. (1985) Processing and evaluation of the antigenicity of protein hydrolysates. In: *Nutrition for Special Need in Infancy.* Protein Hydrolysates (ed. F. Lifshitz). Marcel Dekker, New York, p. 105.
55. Lorenzen, P.C. and Schlimme, E. (1992) The plastein reaction: properties in comparison with simple hydrolysis. *Milchwissenschaft: Milk Science International* **47**, 499.
56. Walstra, P. and Jenness, R. (1984) *Dairy Chemistry and Physics*. John Wiley and Sons, Inc., New York.
57. Aas, K. (1988) The biochemistry of food allergens: what is essential for future research? In: *Food Allergy* (eds E. Schmidt and D. Reinhardt). Raven Press, Ltd., New York, p. 1.
58. Otani, H., Dong, X.Y. and Hosono, A. (1990) Antigen specificity of antibodies raised in rabbits injected with a chymotryptic casein-digest with molecular weight less than 1,000. *Japanese Journal of Dairy and Food Science* **39**, A31.
59. Saxelin, M., Korpela, R. and Mäyrä-Mäkinen, A. (2003) Introduction: classifying functional dairy products. In: *Functional Dairy Products* (eds T. Mattila-Sandholm and M. Saarela). CRC Press Woodhead Publishing Ltd, Cambridge, pp. 1–15.
60. Hartmann, R. and Meisel, H. (2007) Food derived peptides with biological activity: from research to food applications. *Current Opinion in Biotechnology* **18**, 63–69.
61. Vaughn, N., Rizzo, A. and Doane, D. (2008) Intracerebroventricular administration of soy protein hydrolysate reduces body weight without affecting food intake in rats. *Plant Foods for Human Nutrition* **63**, 41–46.
62. Adamson, N.J. and Reynolds, E.C. (1995) Characterization of tryptic casein phosphopeptides prepared under industrially relevant conditions. *Biotechnology and Bioengineering* **45**, 196–204.

63. Kim, S.B., Seo, I.S., Khan, M.A., Ki, K.S., Lee, W.S., Lee, H.J., Shin, H.S. and Kim, H.S. (2007) Enzymatic hydrolysis of heated whey: iron-binding ability of peptides and antigenic protein fractions. *Journal of Dairy Science* **90**, 4033–4042.
64. El-Zahar, K., Sitohy, M., Choiset, Y., Metro, F., Haertlé, T. and Chobert, J.M. (2004) Antimicrobial activity of ovine whey protein and their peptic hydrolysates. *Milchwissenschaft: Milk Science International* **59**(11–12), 653–656.
65. Philanto-Leppälä, A., Marnila, P., Hubert, L., Rokka, T., Korhonen, H.J. and Karp, M. (1999) The effect of α-lactalbumin and β-lactalbumin hydrolysates on the metabolic activity of *Escherichia coli* JM103. *Journal of Applied Microbiology* **87**(4), 540–545.
66. Mullally, M.M., Meisel, H. and FitzGerald, R.J. (1996) Synthetic peptides corresponding to α-lactalbumin and β-lactalbumin sequences with angiotensin-I-converting enzyme inhibitory activity. *Biological Chemistry Hoppe-Seyler* **377**(4), 259–260.
67. Pellegrini, A., Thomas, U., Bramaz, N., Hunziker, P. and von Fellenberg, R. (1999) Isolation and identification of three bactericidal domains in the bovine α-lactalbumin molecule. *Biochimica et Biophysica Acta* **1426**(3), 439–448.
68. Moskowitz, R. (2000) Role of collagen hydrolysate in bone and joint disease. *Seminars in Arthritis and Rheumatism* **30**(2), 87–89.
69. Korhonen, H. and Pihlanto, A. (2006) Bioactive peptides: production and functionality. *International Dairy Journal* **16**, 945–960.

14 Starch-processing enzymes

Marc J.E.C. van der Maarel

14.1 INTRODUCTION

Starch is a widely used renewable resource. It is present as a storage compound in the leaves, tubers, seeds and roots of many plants. Several of these starch-containing plants have been domesticated and are important agricultural crops. The most well known are corn, wheat, rice, potato and tapioca. Besides the use for direct human consumption, the roots, tubers and seeds are processed to harvest the starch. The starch is usually modified chemically or enzymatically to a wide variety of derivatives. In this chapter, the enzymatic conversion of starch into food ingredients is described. In addition to the use of amylases and related enzymes to convert starch into all kinds of syrups, recent developments in the use of glucanotransferases will be discussed.

14.2 STARCH AND STARCH-ACTIVE ENZYMES

Starch is composed of amylose, a virtually linear glucose polymer in which the glucose residues are linked via α-1,4 glycosidic linkages, and amylopectin, in which the majority of the glucose residues are linked via α-1,4 glycosidic linkages with up to 5% α-1,6 linked side chains (see Fig. 14.1 for the numbering of the C-atoms in the glucose moiety). These glycosidic linkages are stable at higher and neutral pH but hydrolyze chemically at lower pH. At the end of the polymeric chain a latent aldehyde group, known as the reducing end, is present. Amylose is a relatively small molecule ranging in size from several hundred to a few thousand glucose residues and containing one reducing and one non-reducing end. Amylopectin is a much larger molecule containing up to 100 000 glucose residues. It has one reducing and many non-reducing ends. Amylose and amylopectin are packed together in starch granules, the size and shape of which vary greatly among the different botanical sources. In principle, granules can be considered as relatively inert substrates that are only slowly degraded by enzymes. The amount of amylose in starch varies from almost nothing (waxy variants having almost 100% amylopectin) to 70% (high amylose corn variants). Wild-type starches on average have 20–25% amylose and 75–80% amylopectin.

A large variety of enzymes active towards amylose or amylopectin have evolved in nature.[1,2] Basically, starch-active enzymes are divided into two major groups depending on their mode of action: (1) exo- and endo-acting hydrolases that hydrolyze the α-1,4 and/or α-1,6 glycosidic linkage using water and (2) glucanotransferases that break an α,1-4

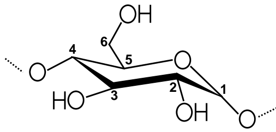


Fig. 14.1 Glucose, the building block of amylose and amylopectin. The numbers represent the different C-atoms present in glucose.

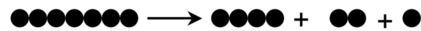
glycosidic linkage and form a new α-1,4 or α-1,6 glycosidic linkage (Fig. 14.2). Most of these enzymes have a retaining mechanism, that is they maintain the anomeric configuration of the hydroxyl group at the C4 atom. Some starch-active enzymes, such as β-amylase, are inverting enzymes, that is they change the anomeric configuration of the hydroxyl group at the C4 position from α to β. Amylases and related enzymes are categorized as glycoside hydrolases and are divided into different families according to the classification of Henrissat and co-workers.[3,4] A complete overview of all glycoside hydrolases known to date can be found on http://www.cazy.org/. The most well-known family of starch-active enzymes is the glycoside hydrolase family 13 containing more than 28 different reaction specificities.[2,5] Other glycoside hydrolase families containing starch-active enzymes are GH-15 (glucoamylase) and GH-57 (amylases, pullulanases, etc.).

14.3 STARCH HYDROLYSIS

For more than four decades, enzymes have been used to convert starch into a range of products. The first enzyme to be used on an industrial scale was the hydrolytic glucoamylase

Hydrolases acting on α,1-4 bonds

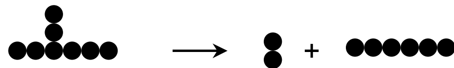
α-Amylase (E.C. 3.2.1.1)



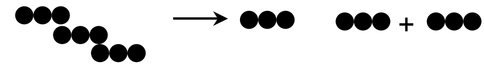
Maltogenic amylase (E.C. 3.2.1.133)

Hydrolases acting on α,1-6 bonds

Iso-amylase (E.C. 3.2.1.68)

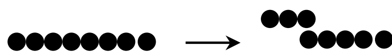


Amylopullulanase (E.C. 3.2.1.1/41)

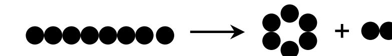


Transferases acting on α,1-4 or α,1-6 bonds

Glucan branching enzyme (E.C. 2.4.1.)



Cyclodextrin glycosyltransferase (E.C. 2.4.1.19)



Amylomaltase (E.C. 2.4.1.25)

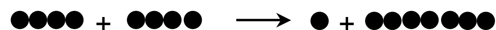


Fig. 14.2 Action patterns of some of the hydrolases and transferases of glycoside hydrolase family 13 enzymes.

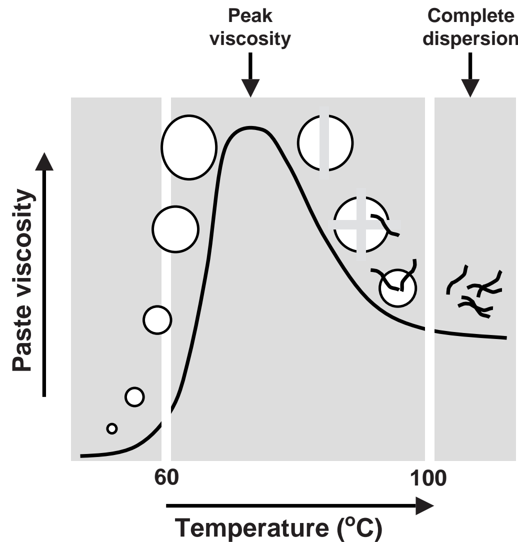


Fig. 14.3 Change in paste viscosity as a result of heating a starch slurry. Heating above the temperature at which the viscosity is maximal leads to breaking of the granules and complete dispersion of the amylose and amylopectin.

which showed benefits compared to the commonly applied acid hydrolysis. In the 1970s, glucose isomerase was introduced for the production of high fructose syrups. Glucose isomerase converts glucose into fructose, thus creating a product with a higher sweetness. Later, thermostable α-amylase and pullulanase were introduced for a faster and better hydrolysis of starch. Most enzymatic starch conversion processes start with heating a water-starch slurry to disrupt the granular structure and bring the two glucose polymers amylose and amylopectin into solution. The starch granule itself is more or less insoluble in water. However, upon heating the granules take up water, resulting in a swelling with a concomitant increase in the viscosity (Fig. 14.3). Continuation of the heating results in the disruption of the granules and a release of the amylose and amylopectin. The temperature that is needed for complete dissolution of starch granules depends on the source of the starch. For starches used in industrial processing the gelatinization temperature is as follows: corn, 72–76°C; wheat, 60–64°C; potato, 65–70°C; and tapioca 70–75°C. During cooling the free amylose and amylopectin side chains start to interact, forming a strong network. This starts at temperatures of 70–80°C, resulting in increasing viscosity. Finally, a white, opaque gel is formed. The interactions in this gel are so strong that the gel has become thermoirreversible, that is it cannot be brought into solution again by heating.

Starch processing is divided into three steps: gelatinization, liquefaction and saccharification. In the gelatinization step, a 30–40% starch slurry is heated quickly to break open the granules. This can be done in stirred tank reactors but the most preferred method is jet-cooking, in which pressurized steam is injected into the starch slurry. Prior to the steam injection the pH is adjusted, calcium (20–80 ppm) is added and a thermostable α-amylase such as that of the bacterium *Bacillus licheniformis* (Termamyl® of Novozymes) or *Bacillus amyloliquefaciens* is added. The dosage of the thermostable amylase is in the order of 0.5–0.6 kg ton^{-1}. The pH adjustment is necessary to create a pH at which the amylase functions best. In addition, the amylase requires calcium for its stability; without calcium the enzyme

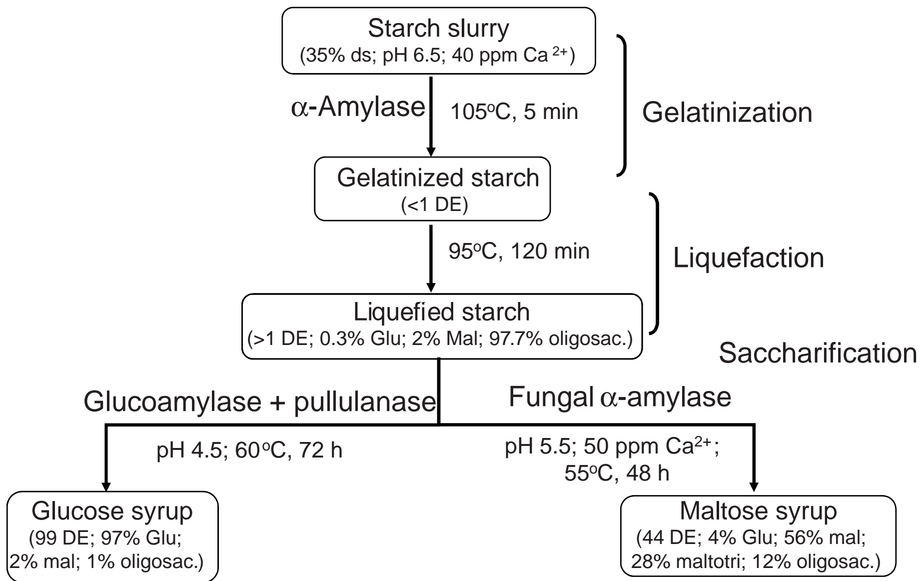


Fig. 14.4 Schematic overview of the various steps in industrial starch processing.

unfolds quickly and becomes inactive. Through a series of holding tubes the heated slurry is kept for 5 min at 105°C to ensure a proper gelatinization.

In the next step, the liquefaction (Fig. 14.4), the slurry is flash-cooled to a temperature of 95–100°C at which it is held for 1–2 h. The *B. licheniformis* amylase has a somewhat higher temperature stability compared with the *B. amyloliquefaciens* enzyme, thus determining the maximum temperature that can be applied during the liquefaction stage. During the liquefaction, the thermostable amylase hydrolyzes the α,1-4 linkages in both the amylose and the amylopectin to produce dextrins. Amylases like Termamyl work by an endo-action, that is they hydrolyze their substrate internally into smaller fragments. This reduces the amount of interactions (i.e. hydrogen bridges) with an accompanying reduction of the viscosity. The liquefaction process is allowed to proceed until the required dextrose equivalent (DE) value is obtained. The DE value is defined as the number of reducing ends relative to a pure glucose of the same concentration. Glucose has a DE of 100, while starch has a DE of near zero. The higher the DE value, the shorter the dextrins present. The maximum DE that can be obtained using these *Bacillus* amylases is around 40. Usually, the liquefaction step is stopped when a DE of 8–12 is reached. Prolonged incubation leads to the undesirable formation of maltulose (4-*O*-D-glucopyranosyl-D-fructose) that is resistant to degradation by glucoamylase and α-amylases.

Depending on the type of product to be produced, the reaction is either stopped or followed by a saccharification step. In this step, pullulanase, glucoamylase, β-amylase or an α-amylase are added to further degrade the liquefied starch into maltodextrins, maltose or glucose syrups. Saccharification is mostly performed as a batch process. The liquefied starch with a DE of 8–12 is pumped into a large stirred vessel, and after adjusting the pH to 4–5 and the temperature to about 60°C glucoamylase is added. The temperature must be lowered quickly to avoid retrogradation of the liquefied starch. Due to the pH adjustment the bacterial amylase used during the liquefaction phase will be inactivated. When the desired DE is

obtained the solution is heated to 85°C for a few minutes to stop the reaction. A prolonged incubation will lead to a fall in the DE because of the formation of isomaltose. The product obtained is filtered to remove fat and denatured protein and if needed further purified by passing through activated charcoal and/or ion exchange resins. Due to the hydrolysis process that consumes water, as for every glycosidic linkage broken one water molecule is needed, the dry matter content increases.

Glucoamylases mainly hydrolyze α-1,4 linkages but show some activity towards α-1,6 linkages. This makes them very suitable to use in achieving a more complete hydrolysis of the starch. With the commercialization of pullulanases such as the one of *Bacillus acidopullulyticus*, it became possible to specifically hydrolyze the α,1-6 glycosidic linkages and thus debranche the amylopectin efficiently. Addition of a pullulanase in combination with a glucoamylase gives an increase of the glucose yield of about 2%. When added together with a β-amylase the maltose yield is significantly increased. Other advantages of the addition of pullulanase are a reduction of the saccharification time, the possibility to increase the dry matter content and to reduce the amount of glucoamylase needed. At the end of the saccharification step, maltose, glucose or mixed syrups are obtained depending on the enzyme or enzyme mixtures used. Before enzymes were used to convert starch into various syrups, acid hydrolysis was used. Acid hydrolysis has some major drawbacks and limitations, such as the DE of the final product (28–55), corrosion of reactors and piping, colour formation, and increased salt content due to neutralization. With the use of enzymes, these drawbacks were overcome and a wider DE range became possible.

When glucoamylase and pullulanase are used for saccharification, a high glucose syrup is produced. To make a maltose syrup, traditionally barley β-amylase is added. However, because this enzyme is expensive, not thermostable and inhibited by copper and other metal ions, barley amylase has been replaced by a fungal acid α-amylase. This enzyme is added to liquefied starch solution with a DE of about 11 and the incubation is allowed to proceed for 48 h. By this time the fungal amylase has lost its activity. When in addition to the fungal amylase a pullulanase is also added even higher concentrated maltose syrups are obtained. Another enzyme suggested to be used for the production of maltose syrups is the thermostable cyclodextrin glycosyltransferase of the bacterium *Thermoanaerobacterium thermosulfurigenes*,[6] produced commercially by Danish company Novozymes and sold under the brand name Toruzyme™.[41]

Pullulanases (E.C. 3.2.1.41, pullulan α-1,6-glucanohydrolase) are enzymes that hydrolyze pullulan, a product of the fungus *Aerobasidium pullulans* composed of a repeating unit of three α,1-4 linked glucose residues joined together by an α-1,6 glycosidic linkage. Pullulanases are divided into type I that exclusively hydrolyze α-1,6 linkages and produce branched dextrins, and type II that hydrolyze both α-1,4 and α-1,6 linkages and produce mainly maltose and maltotriose.[7] Type II pullulanases have been commercialized and are used in the saccharification process. A group of enzymes similar to type I pullulanases are isoamylases (E.C. 3.2.1.68; glycogen α-1,6-glucanohydrolase). These enzymes specifically hydrolyze the α-1,6 glycosidic linkage in amylopectin or glycogen but they do not show any activity towards pullulan.[8,9] Currently, no thermostable isoamylases have been described. The most commonly known isoamylase is the one of the bacterium *Pseudomonas amyloderamosa*.[9] This enzyme is used for analytical purposes to determine the side chain composition of amylopectin.

Glucoamylases, also known as amyloglucosidase or saccharifying amylase (E.C. 3.2.1.3; 1,4-α-D-glucan glucohydrolase), resemble type II pullulanases in the fact that they also hydrolyze the α-1,4 and to a lesser extend α-1,6 glycosidic linkages of amylose and

amylopectin. They are inverting, exo-acting enzymes releasing β-glucose from the non-reducing ends of the substrate.[10] With glucoamylase alone an almost complete conversion of starch can be achieved. β-Amylases (E.C. 3.2.1.2; 1,4-α-D-glucan maltohydrolase) are also exo-acting enzymes releasing maltose units from the non-reducing end of the substrate. When acting on amylopectin, β-amylases stop at two to three glucose units before the branch point, thus leaving a short branched product. Commercially available β-amylases are produced from soya beans or barley. Because of the plant origin, these β-amylases are not very thermostable and cannot be used over a longer period of time at higher temperatures.

14.4 FRUCTOSE PRODUCTION WITH GLUCOSE ISOMERASE

Glucose syrups can further be converted into a glucose–fructose mixture using glucose isomerases (E.C. 5.3.1.18).[11] This enzyme was discovered in 1957 and developed in the second half of the 1960s. Glucose isomerase actually is a xylose isomerase converting xylose into xylulose but it also has activity towards glucose. Because of the side activity towards glucose, it was exploited commercially to produce a sweet alternative to sucrose derived from sugar beet. The first industrial process for the production of fructose syrup from glucose was described by Japanese researchers. In this process the enzyme was used in solution. Large-scale production was taken up in the US by the Clinton Corn Processing Company at the end of the 1960s. A drawback was and is that the enzyme is expensive, thus making the production of fructose syrup challenging. This was solved when it turned out that glucose isomerase can relatively easily be immobilized making re-use of the enzyme possible. Using immobilized enzyme, the Clinton Corn Processing Company was able to produce a 42% fructose syrup by 1968. Especially in the US, corn starch is converted en masse into high fructose corn syrups (HFCS) and used, for example in soft drinks. The enzyme can only establish a partial conversion of the glucose into fructose because it cannot discriminate between glucose and fructose. Thus an equilibrium of 50% glucose and 50% fructose is achieved. Furthermore care has to be taken to achieve optimal reaction conditions to obtain reasonable amounts of fructose. More than 8 million tons of HFCS is produced annually, representing the largest commercial application of immobilized enzymes in industry.

14.5 ISOMALTOOLIGOSACCHARIDES

Maltose can be converted into isomaltooligosaccharides (IMO) using specific α-glucosidases (E.C. 3.2.1.3; 1,4-α-D-glucan glucohydrolase). These are exo-acting enzymes that hydrolyze amylose, amylopectin and oligosaccharides including maltose from the non-reducing end producing glucose. However, when the concentration of maltose is sufficiently high, α-glucosidases also perform a transglycosylation reaction forming isomaltose, that is they couple two glucose residues together via an α-1,6 linkage. In a second step, the isomaltose is glycosylated and isomaltotriose is formed. Branched oligosaccharides such as panose and isopanose are also glycosylated.

IMO are commercially available in China and Japan and are sold as prebiotic fibers. The α-glucosidase used for the production of IMO is that of the fungus Aspergillus niger (Transglucosidase L-500 of Danisco-Genencor and Transglucosidase L of Amano). A

common production process for IMO is to take 30% liquefied corn starch (DE 5–15), add an α-amylase and a pullulanase to hydrolyze the starch and in addition the α-glucosidase to form the IMO (48 h, 58°C, pH 5.5). The dosage of the α-glucosidase is 0.5–1.0 kg tonnes^{-1} (0.05–0.1%). This results in a mixture containing 25% glucose, 5% maltose, 15% isomaltose, 2% maltotriose, 5% panose, 8% isomaltotriose and 40% oligosaccharides of DP 4 and higher. To obtain IMO with a higher DP, the use of a glucansucrase enzyme (E.C. 2.1.4.5; sucrose:1,6-α-D-glucan 6-α-D-glucosyltransferase) and sucrose[42] has been described. The glucansucrase hydrolyzes the sucrose and transfers the glucose molecule to the isomaltooligosaccharide acceptor with the formation of a new α-1,6 glycosidic linkage. Another production method is the use of a *Bacillus stearothermophilus* maltogenic amylase in combination with a *Thermotoga maritima* α-glucanotransferase.[12] With these two enzymes combined, a mixture containing up to 68% IMO could be made.

14.6 AMYLASES IN BAKING (SEE ALSO CHAPTER 6)

Amylases are also used on a large scale in the production of baked goods such as bread and cake. During dough preparation, starch granules are damaged as a result of the mixing and the starch becomes accessible for the endogenous wheat amylases. These start to degrade the starch into small oligosaccharides that serve as a substrate for the yeast. Fermentation of these sugars leads to the production of carbon dioxide and an increase in the loaf volume. Exogenous amylases can be added to speed up this process.

Amylose and amylopectin side chains present in freshly baked products are partially released during the baking stage and start to retrograde slowly during the cooling phase. This results in loss of product quality.[13] This process is described by the phenomenon of staling and is characterized by an increased firmness of the crumb, loss of crust crispness, decreased crust moisture content and loss of flavour. The retrogradation of especially amylopectin is correlated with the bread firming rate.[14] A consequence of the staling process is the loss of shelf life. Delaying or even preventing staling from occurring has been the focus of many research efforts. One of the anti-staling solutions is the addition of enzymes that shorten the side chains of the amylopectin and thereby partially prevent the retrogradation process. α-Amylases, debranching enzymes, branching enzymes, β-amylases and amyloglucosidases have been suggested to prevent staling.[2] The addition of α-amylases can give an anti-staling effect and improve the softness of the bread but already a slight overdose will result in sticky bread. This is attributed to the production of relatively long branched oligosaccharides.

Using exo-acting amylases instead of the endo-acting amylases prevents the retrogradation of baked products sufficiently to overcome the problems associated with it. Thermostable, maltogenic amylases that remove short oligosaccharides of DP 2–6 from the non-reducing ends of the amylose and amylopectin side chains turned out to be very good anti-staling enzymes. Currently, two commercial exo-acting amylases are available as anti-staling enzymes: Novamyl of Novozymes and Grindamyl of Danisco. Novamyl contains the maltogenic amylase of a specific *Bacillus stearothermophilus* strain (designated C599) isolated from an Icelandic hot water pool. This enzyme is a very efficient producer of maltose and was first launched on the market as Maltogenase.[15,16] It was the first commercial enzyme to be produced using a genetically modified microorganism. It turned out that this thermostable amylase is active during the baking process and prevents staling to a major extent. It was shown that the Novamyl maltogenic amylase has sequence and structural homology similar

to cyclodextrin glycosyltransferases (CGTase). A major difference is that in the Novamyl enzyme, a pentapeptide insertion (residue 191–195) is present that is absent in CGTase.[17] Deletion of this loop resulted in the production of cyclodextrin by the mutant Novamyl.

Recently, a second maltogenic-like anti-staling amylase was launched by the Danish ingredient company Danisco under the trade name Grindamyl.[43] This amylase, derived from a *Bacillus clausii* BT21 strain, is optimally active at 55°C and pH 9.5. It is active on soluble starch, amylopectin and amylose and produces mainly maltohexaose and maltotetraose via an exo-acting mode of action.[18] This maltogenic amylase is being marketed under the trade names GRINDAMYL™ PowerFresh for bread baking and PowerFlex™ for tortillas. Besides reducing the length of the amylopectin side chains, the Grindamyl amylase leads to a balanced fragmentation of the amylose resulting in an accelerated amylose recrystallization prior to amylopectin retrogradation. The final result is less network formation of recrystallized amylose and thus a further reduction in the crumb firming.[19]

14.7 GLUCANOTRANSFERASES

The most commonly used starch-active enzymes are those that hydrolyze soluble starch, amylopectin or amylose and produce smaller dextrins, maltooligosaccharides or glucose/maltose. Several enzymes, designated as glucanotransferases, related to these hydrolytic enzymes perform a transglycosylation reaction in which part of the donor molecule is transferred to an acceptor molecule (Fig. 14.2). The enzyme cyclodextrin glycosyltransferase is a good example of such a transferase type of enzyme. In the initial part of its reaction, it breaks an *O*-glycosidic linkage. In the second part of its reaction, this enzyme transfers the non-reducing end to the reducing end to form a cyclic molecule. This type of transfer reaction is designated as an intermolecular transfer, that is within the same molecule. The other type of transferase reaction is of the intramolecular type, that is another molecule is used as acceptor. Two types of glucanotransferase enzymes exist: the 4-α-glucanotransferases also known as amylomaltases that form an α-1,4 linkage, and the glycogen/starch branching enzymes that form an α-1,6 linkage (or branch point). Both types of enzymes have found commercial application in the processing of starch to food ingredients in the last decades.

14.8 CYCLODEXTRINS

Cyclodextrins are cyclic α-1,4 linked glucooligosaccharides containing 6, 7 or 8 glucose residues (named α-, β- and γ-cyclodextrins) and in early days named Schardinger sugars. Due to the arrangement of the glucose residues, the inner part of the cyclodextrins is hydrophobic while the outside is hydrophilic. This means that cyclodextrin can include hydrophobic guest molecules, modifying the physical and chemical properties of these molecules. This has attracted considerable attention and led to the development of various applications for cyclodextrins. An example is the use of cyclodextrins as a deodorizing agent in Febreze of Proctor and Gamble. In food products, cyclodextrins can be used to remove cholesterol, to stabilize volatile and labile compounds or for the reduction of unwanted taste or odour.[20–22] The larger γ-cyclodextrins are especially attractive to use.[23]

α-Cyclodextrins are marketed as a non-digestible, fully fermentable dietary fibre by Wacker Chemie. Cyclodextrins are produced by liquefying starch and then adding the enzyme

cyclodextrin glycosyltransferase (E.C. 2.4.1.19; 1,4-α-D-glucan 4-α-D-(1,4-α-D-glucano)-transferase (cyclizing)).[24] Wacker uses the enzyme from *Klebsiella oxytoca*, producing it by heterologous expression in *Escherichia coli* K12. Subsequently, α-cyclodextrin is obtained from the reaction mixture by precipitation with 1-decanol and subsequent decantation and steam distillation. α-Cyclodextrin is sold under the brand name CAVAMAX W6 by Wacker Chemie. It can be used as a dietary fibre in carbonated and non-carbonated transparent soft drinks, dairy products, baked goods and cereals.

Another group of cyclic glucose-containing molecules is cycloamyloses. They contain 16 or more glucose residues and form long hydrophobic cavities that can form inclusion complexes with larger hydrophobic molecules. Cyclic amyloses are formed by 4-α-gluconotransferases when acting on starch.[25] Low concentrations of high molecular weight amylose is incubated with relatively high concentrations of the enzyme. Besides the potential use of cycloamyloses as a chaperone to prevent proteins from misfolding, no commercial application of cycloamylose is known.

14.9 THERMOREVERSIBLE GELLING STARCH

Amylomaltases or 4-α-glucanotransferases (E.C. 2.4.1.25; 1,4-α-D-glucan:1,4-α-D-glucan 4-α-D-glycosyltransferase) of thermophilic bacteria such as *Thermus thermophilus*[26] and *Pyrobaculum aerophilum*[27] can be used to produce thermoreversible gelling starch. The enzyme transfers parts of the amylose to the non-reducing ends of the side chains of amylopectin. The resulting product consists solely of modified amylopectin molecules that due to their long side chains are able to form a white opaque network or gel.[26,28] The gelling of the amylomaltase-treated starch is thermoreversible, that is the enzymatically treated starch goes into solution upon heating. A similar thermoreversible gelling behaviour is also found with gelatin, a commonly used gelling ingredient. The Dutch potato starch company AVEBE sells amylomaltase-treated potato starch under the brand name Etenia™.[29] In addition to its thermoreversible gelling properties, Etenia is also a good fat replacer in dairy products.[30] The enzyme is produced via heterologous overexpression in *B. amyloliquefaciens* by the Dutch company DSM.[31] In contrast to most enzymes used in starch conversion, this amylomaltase is not excreted by the production host but remains inside the cells. This makes the downstream processing more complicated.

14.10 BRANCHED DEXTRINS

The second type of glucanotransferase enzymes that are exploited commercially are glycogen branching enzymes (E.C.2.4.1.18; 1,4-α-D-glucan:1,4-α-D-glucan 6-α-D-(1,4-α-D-glucano)-transferase). These enzymes initially break an α-1,4 glycosidic linkage and synthesize a new α-1,6 linkage. Branching enzymes are involved in the biosynthesis of amylopectin in plants (also known as Q enzymes) or glycogen, a storage compound of many microorganisms and animals. Glycogen branching enzymes differ from starch branching enzymes in the number of α-1,6 linkage they synthesize. Starch branching enzymes make about 3.5–5% α-1,6 linkages while glycogen branching enzyme can make up to 10% of these linkages.

The Japanese company Ezaki Glico uses the glycogen branching enzyme of the thermophilic bacterium *Aquifex aeolicus*[32,33] to convert maize starch into a product called cluster

dextrin (CCD). The enzyme is produced by Nagase Company (Japan). The CCD product is a branched cyclic dextrin that is added to sport drinks. There are indications that fluids containing the CCD ingredient influence the gastric emptying time positively.[34,35] In addition, studies with swimming mice gave indications that the CCD ingredients had a positive effect on their endurance.[36]

Another application of glycogen branching enzymes is the production of dextrins that at high concentrations have a low viscosity and do not retrograde. Treatment of liquefied starch with a proper, thermostable glycogen branching enzyme leads to the transfer of parts of the amylose and long side chains of the amylopectin to other amylopectin molecules with the formation of new, short side chains. The branched dextrin has such a side chain composition that only weak interactions between amylopectin molecules occur, leading to solutions with a relatively low viscosity. The Danish enzyme company Novozymes has patented the use of the glycogen branching enzyme of the thermophilic bacterium *Rhodothermus obamensis* to produce branched dextrin.[44] The enzyme has a temperature optimum of 65°C and is stable up to 80°C,[36] making it suitable for the processing of liquefied starch. Until now, no branched dextrins as food ingredients are being marketed, mainly due to the lack of a commercially available enzyme.

One aspect of glycogen branching enzymes that remains to be explored is their use in the production of slowly digestible dextrins. It is to be expected that with increasing branching points, the pancreatic human amylase will experience more and more difficulties in degrading the branched dextrin. This will lead to a lower amount of glucose produced and thus a lower blood glucose level. Extreme fluctuations in blood glucose levels are undesirable since this can lead in the long run to type-2 diabetes and heart and coronary problems. Recently, a patent application was published[37] on the use of the glycogen branching enzyme of the extremophilic bacterium *Deinoccocus radiodurans* to produce a slow digestible dextrin from starch.[37] Another approach to create a slowly digestible starch product is to treat a branched dextrin having a moderately increased amount of branching with a β-amylase.[40] However, such an application of glycogen branching enzymes is still in its infancy and it will take considerable time before commercial products become available.

14.11 CONCLUSIONS

Starch is a widely used ingredient for the production of syrups and related products. Enzymatic starch processing has been used for about four decades and has grown into an example of the industrial use of enzymes for food ingredient production. The production of fermentable sugars from starch using the same processing steps as outlined in Fig. 14.2 has become popular due to the increasing demand for cost-competitive bio-alcohol production. Over the last decade, two main growth areas for starch-processing enzymes have emerged: exo-acting amylases as anti-staling enzymes and the use of glucanotransferases for the production of thermoreversible gelling agents, fat replacers and branched dextrins. Besides large-scale use in food or fuel production, starch-acting enzymes are also used as analytical tools. Isoamylase is used as a debranching enzyme in analyzing amylopectin side chain composition. One of the enzymes awaiting commercialization is the α-1,4-glucan lyase of red seaweeds producing α,1-5 anhydrofructose from α-glucans.[37,38] With the enormous amount of information becoming available from whole genome sequence projects, new starch-active enzymes might be discovered. The finding of a new family of glycogen branching enzymes belonging to family GH57 is a good example of this.[39]

REFERENCES

1. Bertoldo, C. and Antranikian, G. (2002) Starch-hydrolysing enzymes from thermophilic archaea and bacteria. *Current Opinions in Chemical Biology* **6**, 1515–1160.
2. Van Der Maarel, M.J.E.C., Van der Veen, B.A., Uitdehaag, J.C.M., Leemhuis, H. and Dijkhuizen, L. (2002) Properties and applications of starch-converting enzymes of the alpha-amylase family. *Journal of Biotechnology* **94**, 137–155.
3. Henrissat, B. and Romue, B.C. (1995) Families, superfamilies and subfamilies of glycosylhydrolases. *Biochemical Journal* **311**, 350–351.
4. Henrissat, B. and Davies, G. (1997) Structural and sequence based classification of glycoside hydrolases. *Current Opinions in Structural biology* **7**, 637–644.
5. Stam, M.R., Danchin, E.G., Rancurel, C., Coutinho, P.M. and Henrissat, B. (2006) Dividing the large glycoside hydrolase family 13 into subfamilies: towards improved functional annotations of alpha-amylase-relates proteins. *Protein Engineering Design and Selection* **19**, 555–562.
6. Wind, R.D., Uitdehaag, J.C., Buitelaar, R.M., Dijkstra, B.W. and Dijkhuizen, L. (1998) Engineering of cyclodextrin product specificity and pH optima of the thermostable cyclodextrin glycosyltransferase from *Thermoanaerobacterium thermosulfurigenes* EM1. *Journal of Biological Chemistry* **273**, 5771–5779.
7. Domán-Pytka, M. and Bardowski, J. (2004) Pullulan degrading enzymes of bacterial origin. *Critical Reviews in Microbiology* **30**, 107–121.
8. Yokobayashi, K., Misaki, A. and Harada, T. (1970) Purification and properties of Pseudomonas isoamylase. *Biochimica et Biophysica Acta* **212**, 458–469.
9. Amemura, A., Chakrabotry, R., Fujita, M., Noumi, T. and Futai, M. (1988) Cloning and nucleotide sequencing of the isoamylase gene from *Pseudomonas amyloderamosa* SB-15. *Journal of Biological Chemistry* **263**, 9271–9275.
10. Sauer, J., Sigurskjold, B.W., Christensen, U., Frandsen, T.P., Mirgorodskaya, E., Harrison, M., Roepstorff, P. and Svensson, B. (2000) Glucoamylase: structure/function relationships, and protein engineering. *Biochmica et Biophysica Acta* **1543**, 275–293.
11. Bhosale, S.H., Rao, M.B. and Deshpande, V.V. (1996) Molecular and industrial aspects of glucose isomerase. *Microbiology Reviews* **60**, 280–300.
12. Lee, H.S., Auh, J.H., Yoon, H.G., Kim, M.J., Park, J.H., Hong, S.S., Kang, M.H., Kim, T.J., Moon, T.W., Kim, J.W. and Park, K.M. (2002) Cooperative action of alpha-glucanotransferase and maltogenic amylase for an improved process of isomaltooligosaccharide (IMO) production. *Journal of Agricultural and Food Chemistry* **50**, 2812–1817.
13. Kulp, K. and Ponte, J.G. (1981) Staling white pan bread: fundamental causes. *Critical Reviews in Food Science and Nutrition* **15**, 1–48.
14. Champenois, Y., Della, V.G., Planchot, V., Buleon, A. and Colonna, P. (1999) Influence of alpha-amylases on the bread staling and on retrogradation of wheat starch models. *Sciences des Aliments* **19**, 471–486.
15. Outtrup, H. and Norman, B.E. (1984) Properties and applications of a thermostable maltogenic amylase produced by a strain of *Bacillus* modified by recombinant DNA techniques. *Starch/Stärke* **12**, 405–411.
16. Diderichsen, B. and Christiansen, L. (1988) Cloning of a maltogenic alpha-amylase from *Bacillus stearothermophilus*. *FEMS Microbiology Letters* **56**, 53–60.
17. Beier, L., Svendsen, A., Andersen, C., Frandsen, T.P., Borchert, T. V. and Cherry, J. R. (2000) Conversion of the maltogenic alpha-amylase Novamyl into a CGTase. *Protein Engineering* **13**, 509–513.
18. Duedahl-Oleson, L., Kragh, K.M. and Zimmerman, W. (2000) Purification and characterisation of a maltooligosaccharide-forming amylase active at high pH from *Bacillus clausii* BT21. *Carbohydrate Research* **329**, 97–107.
19. http://www.thebaker.co.za/vol11no3snippets.html
20. Szejtli, J. (1994) Medical applications of cyclodextrins. *Medicinal Research Reviews* **14**, 353–386.
21. Szejtli, J. and Szente, L. (2005) Elimination of bitter, disgusting tastes of drugs and foods by cyclodextrins. *European Journal of Pharmaceutics and Biopharmaceutics* **61**, 115–125.
22. Somogyi, G., Posta, J., Buris, L. and Varga, M. (2006) Cyclodextrin (CD) complexes of cholesterol-their potential use in reducing dietary cholesterol intake. *Die Pharmazie* **61**, 154–156.
23. Li, Z., Wang, M., Wang, F., Gu, Z., Du, G., Wu, J. and Chen, J. (2007) gamma-Cyclodextrin: a review on enzymatic production and applications. *Applied Microbiology and Biotechnology* **77**, 245–255.

24. Biwer, A., Antranikian, G. and Heinzle, E. (2002) Enzymatic production of cyclodextrins. *Applied Microbiology and Biotechnology* **59**, 609–617.
25. Terada, Y., Fujii, K., Takaha, T. and Okada, S. (1999) *Thermus aquaticus* ATCC 33923 amylomaltase gene cloning and expression and enzyme characterization: production of cycloamylose. *Applied and Environmental Microbiology* **65**, 910–915.
26. Van Der Maarel, M.J.E.C., Capron, I., Euverink, G.J.W., Bos, H.T., Kaper, T., Binnema, D.J. and Steeneken, P.A.M. (2005) A novel thermoreversible gelling product made by enzymatic modification of starch. *Starch/Stärke* **57**, 465–472.
27. Kaper, T., Talik, B., Ettema, T.J., Bos, H.T., Van Der Maarel, M.J.E.C. and Dijkhuizen, L. (2005) Amylomaltase of *Pyrobaculum aerophilum* IM2 produces thermoreversible starch gels. *Applied and Environmental Microbiology* **71**, 5098–5106.
28. Riis Hansen, M., Blennow, A., Pedersen, S., Nørgaard, L. and Engelsen, S.B. (2008) Gel texture and chain structure of amylomaltase-modified starches compared to gelatin. *Food Hydrocolloids* **22**, 1551–1566.
29. www.etenia.nl
30. Alting, A.C., Van Der Velde, F., Kanning, M.W., Burgering, M., Mulleners, L., Sein, A. and Buwalda, P. (2009) Improved creaminess of low-fat yoghurt: the impact of amylomaltase-treated starch domains. *Food Hydrocolloids* **23**(3), 980–987.
31. http://www.dsm.com/en_US/downloads/about/Micro-organisms_table_en_1.pdf
32. Takata, H, Ohdan, K., Takaha, T., Kuriki, T. and Okada, S. (2003) Properties of branching enzyme from hyperthermophilic bacterium *Aquifex aeolicus*, and its potential for production of highly-branched cyclic dextrin. *Journal of Applied Glycoscience* **50**, 15–20.
33. Van Der Maarel, M.J.E.C., Vos, A., Sanders, P. and Dijkhuizen, L. (2003) Properties of the glucan branching enzyme of the hyperthermophilic bacterium *Aquifex aeolicus*. *Biocatalysis and Biotransformation* **21**, 199–207.
34. Takii, H., Takii Nagao, Y., Kometani, T., Nishimura, T., Nakae, T., Kuriki, T. and Fushiki T. (2005) Fluids containing a highly branched cyclic dextrin influence the gastric emptying rate. *International Journal of Sports Medicine* **26**, 314–319.
35. Takii, H., Ishihara, K., Kometani, T., Okada, S. and Fushiki, T. (1999) Enhancement of swimming endurance in mice by highly branched cyclic dextrin. *Bioscience Biotechnology Biochemistry* **63**, 2045–2052.
36. Shinohara, M.L., Ihara, M., Abo, M., Hashida, M., Takagi, S. and Beck, T.C. (2001) A novel thermostable branching enzyme from an extremely thermophilic bacterial species, *Rhodothermus obamensis*. *Applied Microbiology and Biotechnology* **57**, 653–659.
37. Van der Maarel, M.J.E.C., Binnema, D.J., Semeijn, C., Buwalda, P.L. and Sanders, P. (2008) Novel slowly digestible storage carbohydrate. WO/2008/082298.
38. Bojsen, K., Yu, S., Kragh, K.M. and Marcussen, J. (1999) A group of alpha-1,4-glucan lyases and their genes from the red alga Gracilariopsis lemaneiformis: purification, cloning, and heterologous expression. *Biochimica et Biophysica Acta* **1430**, 396–402.
39. Murakami, T., Kanai, T., Takata, H., Kuriki, T. and Imanaka, T. (2006) A novel branching enzyme of the GH-57 family in the hyperthermophilic archaeon Thermococcus kodakaraensis KOD1. *Journal of Bacteriology* **188**, 5915–5924.
40. Fuertes, P., Roturier, J.-M. and Petitjean, C. (2005) Highly branched glucose polymers. EP1548033.
41. Norman, B.E. and Hendriksen, H.V. (2002) Method for producing maltose syrup by using a hexosyltransferase. WO/2002/010427.
42. Vercauteren, R., Leontina, M. and Nguyen, V.S. (2004) Process for preparing isomaltooligosaccharides with elongated chain and low glycemic index. WO/2004/068966.
43. Kragh, K., Larsen, B., Duedahl-Olesen, L., Zimmermann, W.E.K. (2000) Non maltogenic exoamylase from B. clausii and its use in retarding retrogradation of a starch product. WO/2000/058447.
44. Takeda, Y., Hanashiro, I., Ihara, M. and Takagi, S. (2003) Method for producing dextrins using enzymes. WO/2003/106502.

15 Lipases for the production of food components

David Cowan

15.1 INTRODUCTION

One of the first attempts to summarize the then current knowledge of the industrial application of enzymes was made by Godfrey and Reichelt in 1983.[1] This book contained contributions from industrial, academic and government writers and was widely recognized as a major contribution to disseminating enzyme knowledge. Comprehensive though this volume was, the amount of space allocated to lipases was one of the smallest and only one lipase from calf stomach was listed in the appendix of enzyme types. At that time, the main recognized application was in the dairy industry for the accelerated ripening of cheese and production of high-intensity cheese flavours.

However, within a very short time frame the number of actual and potential lipase applications had increased vastly and within 10 years it had grown to include both food-related and technical applications. The reason for this explosion in applications can be seen to be a result of a number of different discoveries in enzyme biochemistry, linked with new production technologies and significant research resources being applied by the lipase producing and using companies.

The first enzyme applications were in the field of flavour generation and used the hydrolytic action of the lipase to generate free fatty acids that increased the 'cheesy' flavour of milk products. Short chain fatty acids from milk liberated by starter culture lipases were known to be involved in the natural ripening of some cheese types and the cheese makers tried to mimic this process. However, this application was limited in potential due to the relatively small amount produced of this cheese type. In 1984, however, Zaks and Klibanov[2] demonstrated that enzymes could function in micro aqueous environments, indicating the possibility that lipases could function in non-aqueous systems. Synthesis reactions, in which hydrolysis is reversed, became possible and opened up a new research area into lipase function.

Within this sector, one of the first products were specific fats (cocoa butter equivalents) as described by Coleman and Macrae[3] and Matsuo *et al.*[4] They utilized an immobilized lipase to exchange fatty acids on a triglyceride with the desired ones, to produce a cocoa butter-like fat. Macrae[5] describes the use of a lipase product absorbed onto a kieselguhr matrix to convert a mixture of palm mid fraction and stearic acid into a cocoa butter equivalent (CBE)-like product containing increased levels of the desired triglycerides, 1(3)-palmityl-3(1)-stearyl-2-monooleine (POSt) and 1,3-distearyl-2-monooleine (StOSt). The immobilized lipase was prepared by making a slurry of the enzyme with kieselguhr and then adding a solvent such as acetone or an alcohol (ethanol or methanol) to precipitate the enzyme onto the inorganic

particulate material. The immobilized enzyme was then separated by filtration, dried and then stored until required for use.

However, this type of process was difficult to control and did not become widely applied in the food industry. The cost of the immobilized enzyme was too high to permit general application and the wider use of lipases would occur only when this was reduced.

Two further developments were required before lipases could be more generally applied within industry. The first of these was the ability to produce large amounts of lipase under conditions of industrial fermentation. Eriksen[6] described the introduction of the first commercial lipase produced by molecular cloning of a lipase gene from *Humicola lanuginosa* in the filamentous ascomycete *Aspergillus oryzae*. This host microorganism could be cultivated and produced in industrial scale fermentation vessels and the technology could be applied to a range of different lipases. The broader application of lipases in the food and other industries is a result of the introduction of this new production technology, since it has resulted in a substantial improvement in the economy of lipase production and the possibility to produce lipases that hitherto had only been available in laboratory scale amounts.

The second development required for the expansion of the basic CBE process was the development of a range of different immobilization systems for the lipase to have both hydrophobic and hydrophilic carriers of the enzyme. The development of these was recently reviewed by Holm and Cowan.[7]

Today the four main areas in which lipolytic enzymes (lipases and phospholipases) are applied are as follows:

- Interesterification, to modify melting properties for fats for margarines and shortenings, without production of trans fats or by-products.
- Degumming, to render water soluble the phospholipid gums in a range of oils to allow them to be removed without yield loss.
- Ester synthesis within the cosmetic/oleochemical industry to produce esters and waxes with lowered energy requirements and by-product formation.
- Speciality fats, the synthesis of speciality fats of nutritional importance such as high omega-3 fish oils.

15.2 ENZYME BIOCHEMISTRY

Lipases are like all enzymes, protein molecules and soluble in water. Their normal function is to hydrolyze triglycerides into diglycerides, monoglycerides, fatty acids and glycerol. A second group of enzymes, esterases were characterized by their ability to hydrolyze carboxylic ester bonds. Originally, it was believed that all lipases had a three-dimensional structure where part of the amino acid chain of the protein formed a lid that covered the active site of the enzyme. This lid moved away from the active site when the enzyme was in contact with the oil–water interface. However, lipases without lids have been found and also constructed, so the current definition is based on the chain length of the fatty acids in the molecule that can be hydrolyzed. Enzymes which attack substrates with an acyl chain length greater than 10 carbon atoms are now considered as lipases and less than 10 are esterases.[8] However, this is just one of the various definitions of lipases in comparison with esterases. None of them is particularly satisfactory and a better way of describing this enzyme class and its components is required.

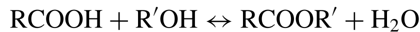
The individual reactions of lipases can be summarized as follows:

(i) Hydrolysis:

$$RCOOR' + H_2O \leftrightarrow RCOOH + R'OH$$

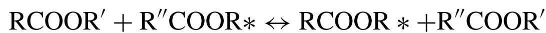
(ii) Synthesis:

(a) Esterification



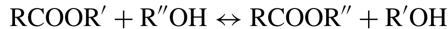
$$RCOOH + R'OH \leftrightarrow RCOOR' + H_2O$$

(b) Interesterification



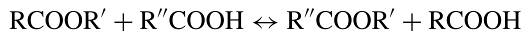
$$RCOOR' + R''COOR* \leftrightarrow RCOOR * + R''COOR'$$

(c) Alcoholysis



$$RCOOR' + R''OH \leftrightarrow RCOOR'' + R'OH$$

(d) Acidolysis



$$RCOOR' + R''COOH \leftrightarrow R''COOR' + RCOOH$$

All of these reaction mechanisms are employed in the industrial production and modification of lipids.

In addition to chain length preferences, lipases also will show regioselectivity. Most regioselective lipases act preferentially on ester bonds at the *sn*-1 and *sn*-3 position of the triglyceride structure, whereas few lipases are active at the *sn*-2 position. Lipases without regioselectivity also exist and are particularly useful where complete hydrolysis of a fat is required. Finally, lipases may also differentiate between saturated and non-saturated fats, showing preferential hydrolysis or modification of one type or another.

15.3 INTERESTERIFICATION

Control of the melting properties of fats is the key to their use within foods. The traditional fat sources such as animal fats or butter do not have the characteristics required and have largely been replaced by fats of vegetable origin. The solid fat content (SFC), which is defined as the percentage of solid fat in a sample at a defined temperature, is a key parameter in defining fat properties. A margarine suitable for making pastry fats should have an SFC of 38–45% at 15°C. This will allow the fat to be mixed with the flour and other ingredients and worked to give the correct structure. A vegetable oil such as rapeseed or soya bean oil will be totally liquid at this temperature and will not be suitable for pastry production. Initially, simple blending was used to obtain the required melting profile but often even this was not able to provide the desired characteristics of solid: liquid ratio and crystallization. For this reason, techniques were developed to modify the melting properties of fats so as to provide the correct SFC profile (Table 15.1).

Table 15.1 Techniques of fat modification.

Technique	Principle
Fractionation	Uses the different melting points of the fat components to separate fractions with significantly altered SFC by a process of fractional crystallization
Hydrogenation	Uses a catalytic hydrogenation reaction to increase saturation of the fat
Chemical interesterification (CIE)	Uses a chemical catalyst operating at high temperature to promote random exchange of fatty acids between two triglycerides
Enzymatic interesterification	Uses an enzyme catalyst to promote either a random or a 1.3 specific (depending on lipase type) exchange of fatty acids between two triglycerides

The techniques of fractionation and hydrogenation have been used for many years[8] whereas chemical interesterification (CIE) is a more recent technology.[9]

15.4 HYDROGENATION AND CHEMICAL INTERESTERIFICATION

By the 1960s, there had been a large swing away from dairy fats due to the increased convenience and lower cost of margarines and the suggestion that they were healthier. Unfortunately, in many cases the fats used were partially hydrogenated and consequently contained high trans fat levels. The trans fat level in a soft (unsaturated) oil such as soya bean and rapeseed increases with the decrease in saturation, reaches a peak and then declines again, reaching zero at full saturation. The degree of hydrogenation required to give the desired SFC also was very close to that giving maximum trans fat content. Subsequent blending of this hydrogenated fat with an unsaturated oil reduced the overall trans fat content but this was mitigated by an overall increase in fat consumption through bakery and similar products.

Studies in the 1960s concluded that the cholestrol-raising effect of hydrogenated fats was slightly lower than that of saturated fats, supporting to some extent the health claims made earlier. However, in 1990 it was observed that although trans fatty acids increase LDL cholesterol to a similar degree to saturated fat, they decrease HDL cholesterol relative to both *cis*-unsaturated and saturated fats. These initial studies have been followed up by a wide range of investigations where the overall conclusion remains that trans fatty acid consumption substantially increases the risk of coronary heart disease.[10]

Legislation has been introduced in a number of countries to control the trans fat level in foods and food components, and this has largely had the effect of removing hydrogenated fats from the market. In Europe and the US, fat modification by hydrogenation has effectively stopped and the facilities to produce this type of product have been closed.

CIE of fats that is catalyzed by a sodium methoxide catalyst was then widely employed as an alternative process for changing the melting properties of fats. This process operates at moderately high temperatures but uses an explosive catalyst which is non-specific, requires removal from the fats after the reaction and requires significant post treatments to remove colour and other by-products of the reaction. The nature and level of these by-products and the associated yield loss have now made the alternative process of enzymatic interesterification more attractive.

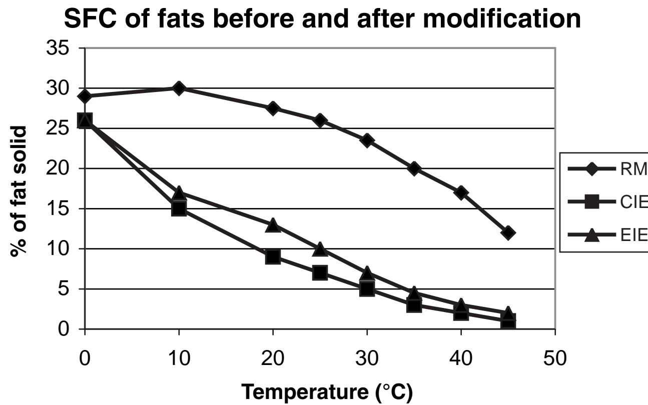


Fig. 15.1 Solid fat content of a 75% soya bean: 25% fully hardened soya bean oil before (RM) and after chemical or enzymatic interesterification with Lipozyme TL IM.

15.5 ENZYMATIC INTERESTERIFICATION

The development and implementation of an enzymatic interesterification (EIE) process for margarine and other bulk fats was dependent on two of the technology developments previously described, a low cost source of lipase and a robust and inexpensive immobilization system.

The immobilized lipases used initially for CBE production were neither robust nor inexpensive and this limited their application to relatively highly priced fat products. The new EIE process uses a lipase immobilized into a silica particle that both carries the enzyme and provides a non-compressible support system. In use, the enzyme remains within the carrier particle and does not leach out into the fat blend. A comparison of the SFC curve for CIE and EIE is shown in Fig. 15.1.

The change in melting properties that can be obtained is dependent on the fats used in the interesterification reaction and the proportions of the two fats employed. This is illustrated for blends of palm stearin and palm kernel oil in Fig. 15.2.

The trend diagram shows the change in SFC between the initial blend and the blend after interesterification. In this example EIE raises the portion of fat solid at lower temperature and decreases the portion solid at higher temperatures. This type of modification is typical of what might be required for a component of a margarine that would also be blended with a soft oil.

The enzyme used is a 1.3 specific lipase derived originally from *Thermomyces lanuginosus* and would normally not be expected to produce a randomization of the fat. However, a combination of the lipase and the immobilization system where the oil blend is in contact with both the enzyme and the silica carrier results in a preferential exchange of fatty acids (Fig. 15.3). The small difference in SFC curve between CIE and EIE shown in Fig. 15.1 is partly due to this preferential exchange but is mainly a result of the EIE process producing lower levels of diglycerides (yield loss) than the chemical process.

Typically batch enzyme reactions are conducted in the laboratory to establish the correct blend and interesterification conditions. The enzyme is dosed at 4% w/w and the reaction is conducted at 70°C, with samples being withdrawn at intervals and the SFC profile determined. From these results the correct blend for continuous interesterification can be determined.

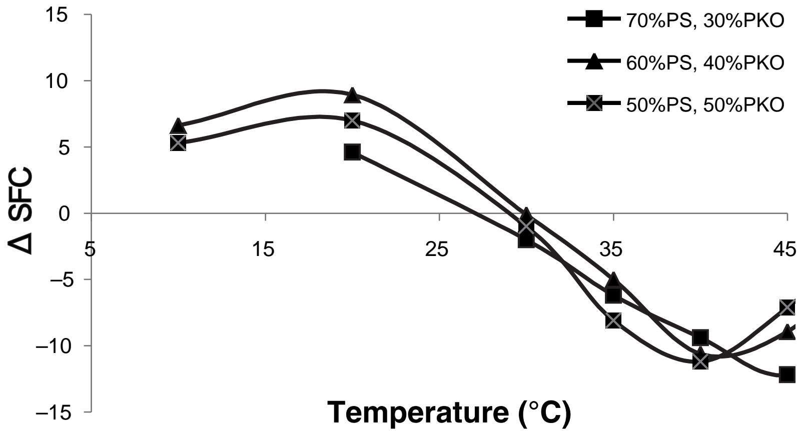


Fig. 15.2 Trend diagram for change in SFC for different blends of palm stearin and palm kernel oil before and after EIE.

The enzyme is filled into columns arranged in a series configuration and oil is pumped through the enzyme bed, with four to six columns in one line being the optimum arrangement (Fig. 15.4). In normal operation, the oil blend enters through the top of reactor no. 1 and passes down through the enzyme bed before exiting the reactor and transferring to reactor no. 2. Each reactor has an insulted water jacket to keep the internal temperature at 70°C and the enzyme itself sits on a sieve plate at the base of the reactor. This acts as a support for the enzyme particles and as a filter to prevent that the enzymes are washed through the reactors in this down flow configuration. The non-compressibility of the particles results in low pressure drop across the reactor (<0.5 bar/reactor) and an average flow of 1.5–2.0 kg oil/kg enzyme/h.

In steady state operation, each reactor contributes to the conversion of the fat, so that it leaves the reactor chain fully interesterified. Enzyme activity is gradually lost from reactor no. 1 due to the presence in the oils of low levels of oxidation compounds and acids. Eventually, the enzyme activity is depleted and the spent product is removed from reactor no. 1 and it is recharged with fresh enzyme. At this point the valves are altered so that the oil now flows first to reactor no. 2 and then finally exits via reactor no. 1 containing the fresh enzyme

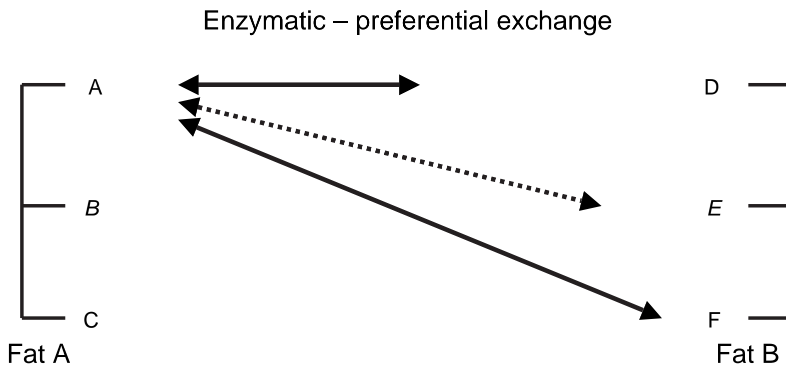


Fig. 15.3 Preferential exchange of fatty acids during EIE with Lipozyme TL IM.

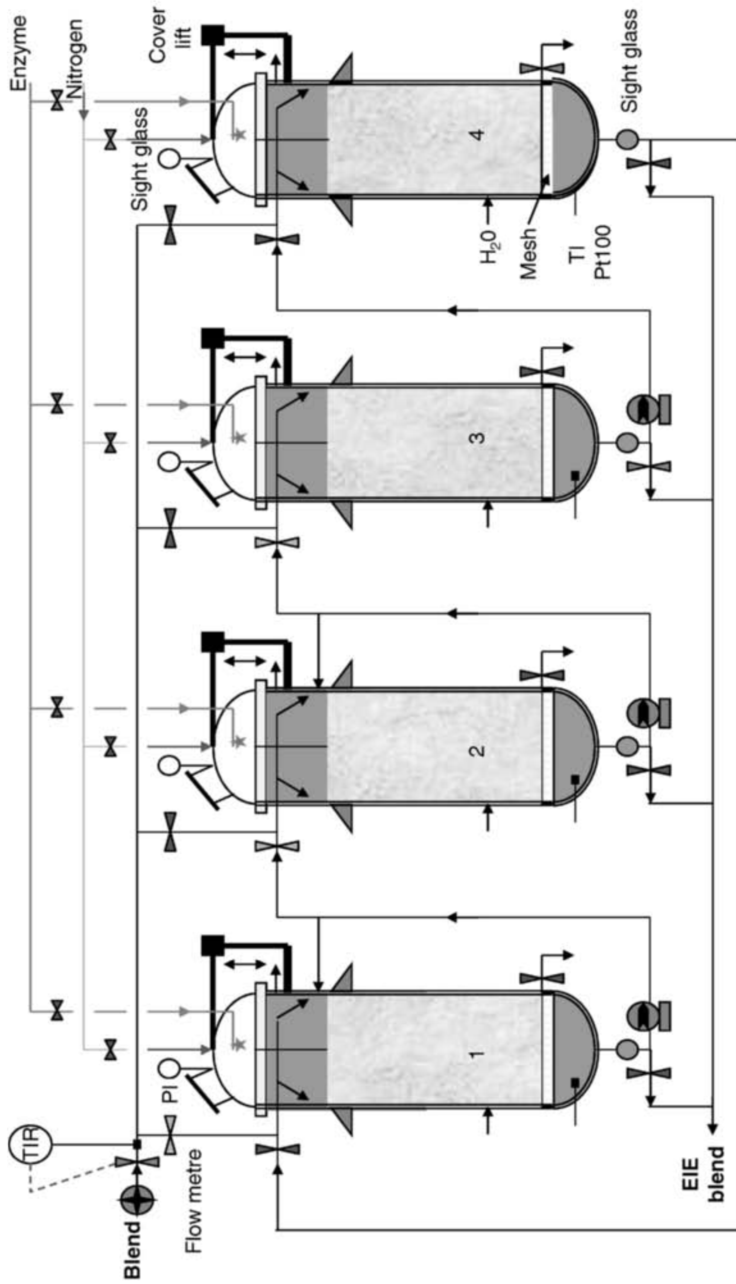


Fig. 15.4 Layout of reactors for EIE. (Also see Colour Plate 4.)

product. This process is repeated with one reactor being exchanged at approximately every 14 days of operation. In this mode of operation, the unconverted oil meets first of all the oldest, lowest activity enzyme and hence if there are high levels of undesired oxidation or other compounds in the oil, the older enzyme will act as a sacrificial column, protecting the subsequent columns.

15.5.1 Oil quality specifications

In any immobilized enzyme process, the productivity of the system (kg converted/kg enzyme product) determines the overall economy. In EIE, three factors have been identified as causing enzyme inactivation and/or a loss of conversion efficiency. These are:

1. The presence of particulates, for example soaps or phosphatides, which can cover the enzyme particle and prevent the oil from diffusing into the granule.
2. Excessive levels of oxidation compounds measured as peroxide values or anisidine content.
3. Residual acids coming from phosphoric acid degumming and/or acid-activated bleaching earths used in the refining of the oil before EIE.

The values recommended for these components are shown in Table 15.2 and are similar to those required for CIE and so quite normal within the industry with the exception of the acid extract value. When these oil quality parameters are met then EIE will operate at maximum efficiency and the most economical use of the enzyme will be obtained.

The presence of oxidation compounds and components coming from their breakdown are routinely monitored as indicators of oil stability. Peroxide value (PV) measures the level of hydroperoxides formed from the reaction between oxygen and unsaturated oils. The p-anisidine value (AV) is a measure primarily of aldehydes, 2-alkenals and 2,4 dienals, through a spectrophotometric assay at 350 nm. The peroxides are themselves unstable and will tend to decompose and form aldehydes and ketones. These two groups of compounds are normally associated with oxidative and hence flavour stability of oils and the chemical determination of the values are used as a quality indicator being simpler to determine than organoleptic measurements.

The oxidation compounds are also a factor in enzyme stability as both the peroxides and aldehydes have the potential to interact with protein molecules causing a loss of activity. Osório *et al.*[11] observed that oil blends with a high oxidation risk gave lower enzyme productivity than blends where oxidation was absent. This has been further studied by Cowan *et al.*,[12] who described an alternative analysis method for the determination of the influence of oil quality on enzyme productivity. In this one aliquot of the enzyme is sequentially brought

Table 15.2 Oil quality specifications for EIE.

Type	Identity	Level required
Particle occluding	Soaps	<1 ppm
	Phosphorus	<3 ppm
	Nickel	<0.2 ppm
Oxidation compounds	Peroxide value	<2 meqO_2/kg
	Anisidine	<5
Mineral acids	Acid extract value	pH 6.0–9.0

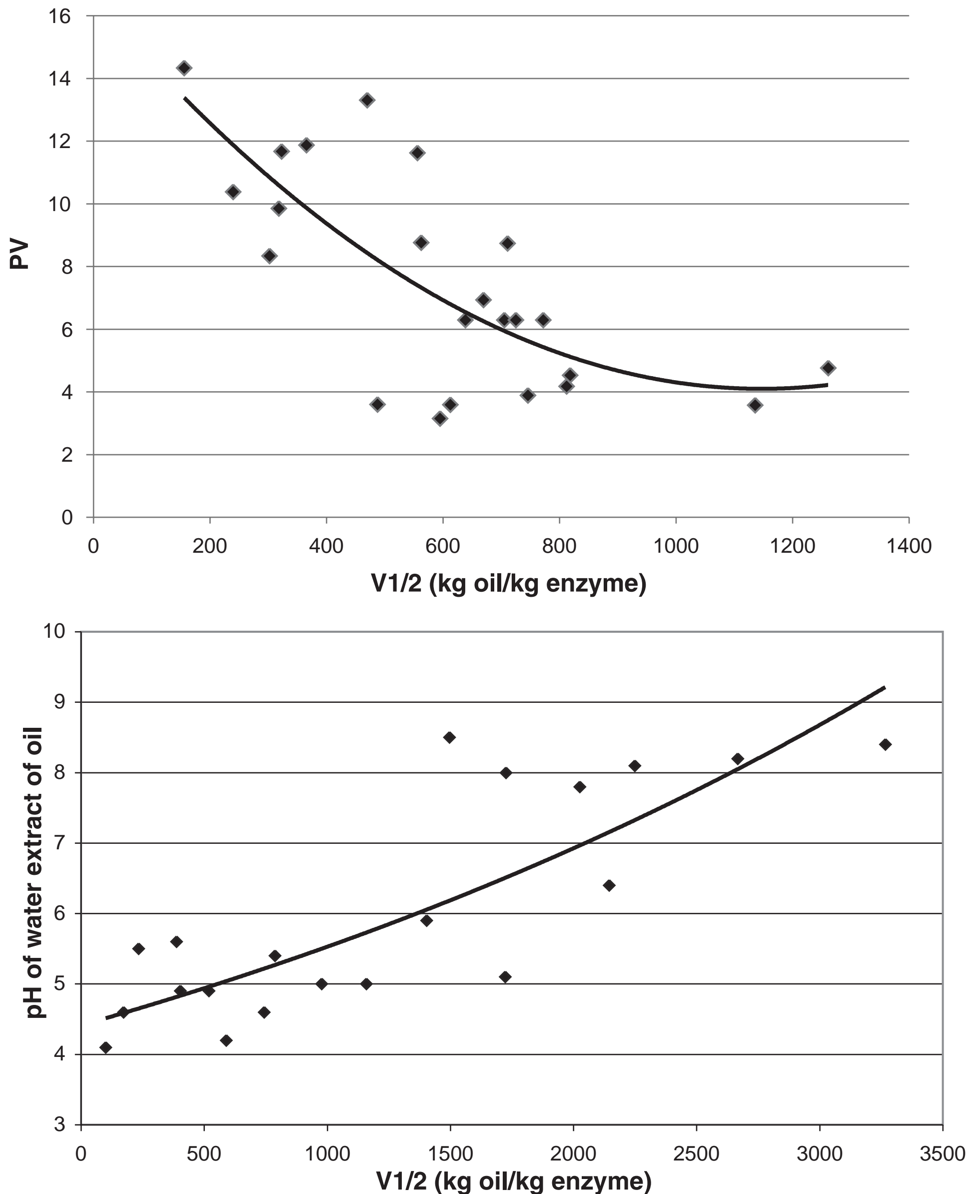


Fig. 15.5 Influence of PV on enzyme productivity (V1/2).

into contact with different volumes of the oil under study to give a cumulative exposure of the enzyme to a large amount of oil, thus simulating the conditions in a packed bed reactor. Cowan and Willits[9] quantified this and demonstrated that $PV > 4$ had a damaging effect on enzyme stability (Fig. 15.5). In this study, the amount of oil in sequential contact with the enzyme that causes a reduction in activity to 50% of the starting value (V1/2) is determined for oils of differing starting PV values. Low PV contents in the oil are associated with high V1/2 values and if three half-lives are used as an assessment of productivity, an estimate of potential working lifetime of the enzyme can be made.

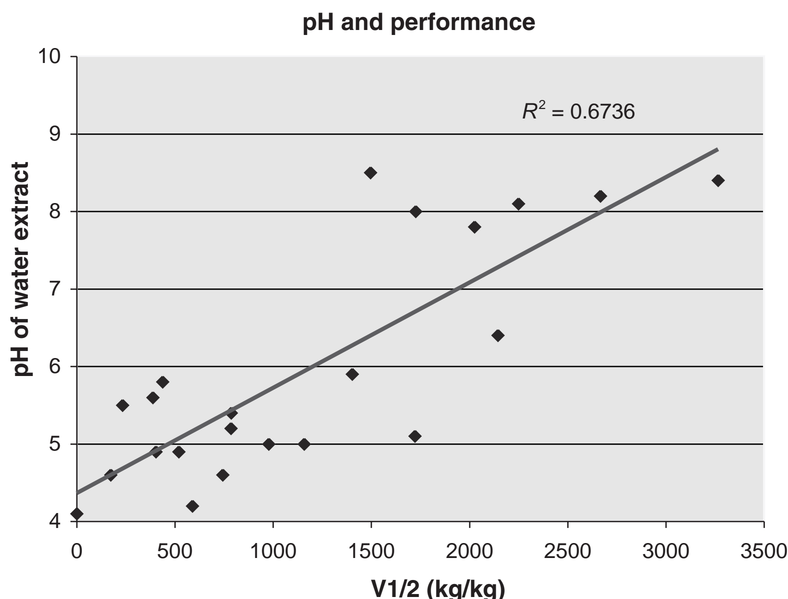


Fig. 15.6 Influence of water extract pH of oil on enzyme productivity.

The other main group of chemical compounds that can affect enzyme activity are acids coming from degumming and/or bleaching processes. Degumming of oils is the process of removing phosphatides (see next section), and for palm oil it is often carried out with phosphoric acid. The removal of colour, oxidation compounds and residual phosphatides is normally carried out by a bleaching process using absorption onto bleaching earth.

Acid-activated bleaching earths are more efficient at removal of these substances and hence tend to be used within the industry.

Unfortunately, both treatments can lead to low levels of mineral acids being extracted into the oil because the oil is never totally water free. The acids dissolve in the water phase and when this oil is passed down a column of immobilized enzyme, the acids can pass into the enzyme particle and reduce the pH of its micro-environment. This moves the pH away from the optimum pH of the enzyme and activity is reduced. Also, as enzymes are most stable around their pH optimum, activity loss may also be irreversible. Similar determinations to those above have also been conducted for acid content and demonstrate the negative effect of high mineral acidity levels (Fig. 15.6).

Mineral acid residues in oil can be extracted by vigorously mixing equal amounts of oil and deionized water, allowing the two phases to separate and then measuring the pH of the water portion. It is important not to use a buffer system in the extraction process as the amounts of acid are relatively low and would not change the pH of buffered water. The influence of the acid is exerted by it accumulating in the enzyme particle as more and more oil passes down the column, gradually lowering the micro pH within the granule.

15.5.2 Improving oil quality

Control of PV and AV is best made within the oil production process (refining). By good degumming, control of oxygen access and the removal of pro-oxidants (bleaching and

deodorization), these values can be kept at a constant low level. In cases where the levels of these components are too high, reprocessing may be required if a satisfactory enzyme working life is to be achieved. Additives such as silica have been suggested as absorbents of polar compounds. Lee and Sleeter[13] proposed the use of a number of different pretreatment materials to be used in a column including activated carbon, spent enzyme kieselguhr and silica. They reported that for a soya bean oil-based blend, the flow rate required for full conversion was 10% of the starting level after 40 days for the standard blend. For the reactor in which the oil had received silica pretreatment, the flow was 18% of the starting level, indicating almost double the residual enzyme activity at this time. Based on the type of materials being used, it would seem that it was primarily oxidation compounds that were being removed. Ibrahim *et al.*[14] also focused on oxidation compounds and proposed the use of spent enzyme catalyst as a means of removing enzyme-inactivating species. Using molecular sieves, activated carbon and deactivated but unused enzyme, they increased productivity (kg oil converted/kg enzyme) by a factor of 3.1, 7.4 and 4.1 fold, respectively. From this they concluded that spent enzyme might be an effective purifying material but this assumes that the sites of absorption for enzyme-poisoning compounds have not all been occupied during the normal use of the enzyme product.

Elimination of acids can be achieved by not using phosphoric acid in degumming and ensuring that a bleaching earth, which has been fully washed to remove residual acid, is employed. Where this is not possible or where the oils are produced at another location, alkali treatment of the oil is a possibility. Sodium carbonate and potassium hydroxide have both been used to remove residual acidity and cause an increase in enzyme productivity.[13]

15.5.3 Practical operation of EIE

In factory operations there are two parameters that also need to be controlled. The fat melting profile or SFC needs to match that of the desired specification, and the quality (measured as crystallization characteristics – relative content of different crystalline forms; colour – predominately red and yellow; and odour) of the produced fat needs to be at least as good as that produced by the alternative technology.

The SFC is a measure of the amount of fat solid at a particular temperature and it is this which controls the melting properties. The SFC curve shown in Fig. 15.1 does not totally match the one for CIE. In practice, batch reactions with differing proportions of the two components of the blend fats would be conducted in order to find the exact mix that gave the desired results. This is illustrated in Fig. 15.7, which compares the SFC curve for EIE of three blends of palm stearine and sunflower oil with a product obtained from CIE of a 30:70 blend of the same fats. The curve for the same proportion blend made by EIE is slightly below that of the chemical process. By adjusting the blend used for EIE towards a 32:68 proportion, an exact match can be achieved. Similarly to replace partly hydrogenated fats, a similar approach can be followed but a wider range of fat blend compositions may be required in order to fully match the properties of the originally hydrogenated fat. For example, partially hydrogenated soya bean oil can be substituted by the interesterification of fully hydrogenated soya bean oil with liquid soya bean oil. This produces the desired physical properties that does not lead to the formation of trans fats nor increase overall the level of saturated fats.

The composition that provides the desired SFC values is then used for continuous interesterification in the series reactor system. The batch interesterification is carried out to

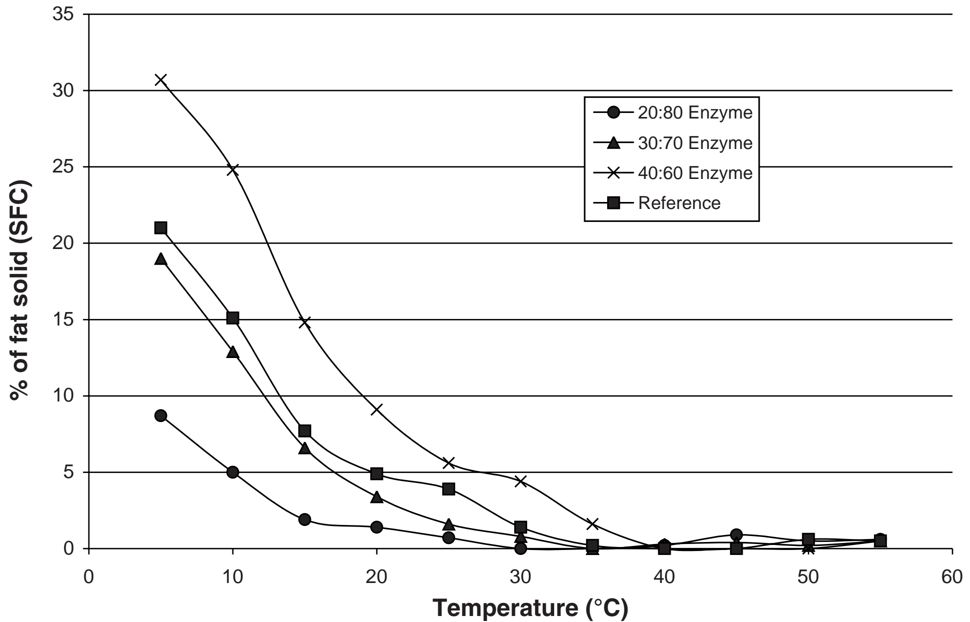


Fig. 15.7 Optimization of blend composition to match the SFC curve of a CIE fat.

provide a full conversion of the fats. The flow rate through the series reactors is adjusted to give the same conversion, ensuring the same quality of product is constantly produced. This is only possible where several reactors are connected in series because with a single reactor, reduction in flow rate would be required to compensate for loss of enzyme activity. With a series configuration, no single reactor is responsible for the total conversion and hence flow can be kept constant, whilst at the same time all the enzyme activity is utilized.

The resulting modified fat will be incorporated into a margarine, shortening or other food product. It is essential that the product quality of this modified fat is at least as good as that produced by the alternative technology. EIE is a milder process than either CIE or hydrogenation and results in better retention of natural anti-oxidants (tocopherols, etc). This by itself could be expected to improve oxidative stability of the resulting fats but it is normally the case that additional anti-oxidants are added which may blur this distinction. However, some studies have reported lower PVs and better colour for margarines produced with enzymatically modified fats[15] and no observations of poorer quality have been recorded.

Baking quality of the produced margarines is also a critical parameter and a number of investigations into this area have been made. Kirkeby[16] made baking tests to produce puff pastry and commented that the margarine from enzymatically interesterified hardstock gave superior results to that from CIE. Cowan *et al.*[17] focused on margarine for bread production and showed that equal bread volume and other characteristics could be obtained for a shortening made by EIE compared to a commercial product. Finally, Siew *et al.*[18] interesterified different blends of hard palm stearin with canola oil to produce a range of different margarine and fat products. They observed that by varying the proportions between the two starting materials, they could produce modified fats with physical properties suitable for the production of stick margarine, shortenings, puff pastry margarine and vanaspati.

15.5.4 Future directions for EIE

The EIE process is now well on the way to becoming the process of choice for fat modification within the oils and fats industry. Many full-scale installations have been completed and some have run for a number of years. Now that the main factors influencing enzyme activity and productivity have been determined, research can focus on mitigating the effect of these on enzyme working life. Protein engineering is capable of modifying the sensitivity of enzymes to both oxidative compounds and altering their pH optima. Thus, we can expect that new lipases will be developed with reduced or eliminated sensitivities to these factors. Also, as the immobilization system could in principle be extended to other lipases, then different reactions could be carried out to produce fats of different physical or nutritional qualities.

15.6 ENZYMATIC DEGUMMING

In edible oil refining, it is necessary to remove impurities that affect the taste, smell, visual appearance and storage stability of the oil. There are a number of different types of impurities (metal ions, free fatty acids, suspended solids, waxes, etc.) and the refining industry has developed a range of different processes to deal with them. One important class of undesirable compounds are the phosphatides (commonly known as lecithin's), derived from the seeds and present as hydratable and non-hydratable gums.

A number of different methods have been developed to remove these gums and they can be broadly grouped as physical or chemical methods. Chemical refining of vegetable oils is still the most common refining process. In this process, alkali (sodium hydroxide) is added to neutralize the free fatty acids to soaps, followed by a washing process that removes the entrained phosphatides and other contaminants. However, physical refining is increasingly taking over, due to cost and environmental benefits. In one form of physical refining, water is used to remove the hydratable gum portion. Acids such as phosphoric are added to convert the remaining gums to a hydratable form which can then be removed by further water washing and centrifugation. The fatty acids are not eliminated by this treatment but are subsequently removed by the deodorization process.

Enzymatic degumming is a form of physical refining in which a phospholipase is used to convert the non-hydratable phosphatides into a hydratable form, allowing them to be removed by a one-step centrifugation process.

All degumming methods must, to be successful, reduce the phosphorus level in oil to <10 ppm. Phosphorus content of oils is the accepted measure of phospholipid content and is routinely determined by inductively coupled plasma mass spectrometer (ICP-MS) or if this is not available, by a less reliable colorimetric method. The drawback of the older method is that the reaction is less sensitive for phosphorus levels of 0–20 ppm and errors in the ashing process for sample preparation can further reduce the accuracy of the method. Both methods assume that all phosphorus in the oil is associated with phospholipid but some may result from phosphoric acid if this is used in the degumming process.

15.6.1 Phospholipid structure and phospholipases

Vegetable oil lecithin has a similar structure to a triglyceride where the fatty acid at the sn-3 position is replaced by a functional phosphatide group (Fig. 15.8).

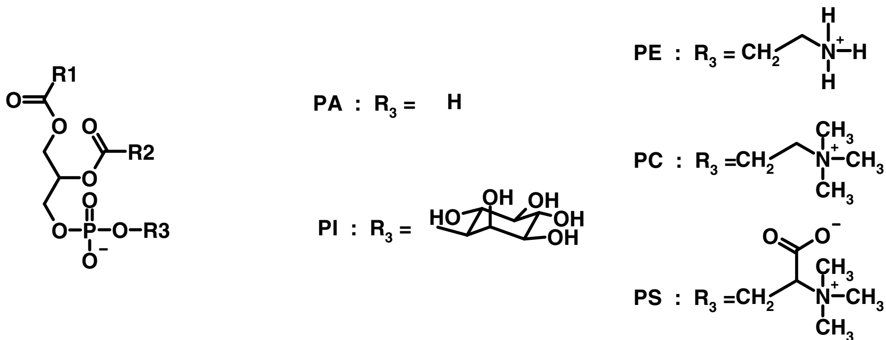


Fig. 15.8 Structure of the most common phospholipids: R_1, R_2: fatty acid residues, PA: phosphatidic acid, PI: phosphatidylinositol, PE: phosphatidylethanolamine, PC: phosphatidylcholine and PS: phosphatidylserine.

Phospholipases are classified according to which part of the phospholipid molecule they hydrolyze (Fig. 15.9). Phospholipase A_1 and A_2 remove a fatty acid at the sn-1 or sn-2 position, respectively. The formed lyso-phospholipid is water hydratable and can then be removed by centrifugation with the small amount of water used in the hydrolysis reaction.

Phospholipase D is mainly found in the oil seeds themselves and can produce increased levels of phosphatidic acid if seeds are not correctly stored. Phospholipase C produces a diglyceride rather than a lyso-phospholipid and is so far only used on a limited scale in combination with phospholipase A_1.[19]

15.6.2 Mechanism of enzymatic degumming

Phosphatides in vegetable oils are present as water hydratable or non-hydratable. Seghers[20] demonstrated that the rate of hydration was the highest with phosphatidylcholine and the lowest with phosphatidic acid (Fig. 15.10).

The content of the different phosphatides varies somewhat with source and also with harvest and storage conditions; in particular activation of phospholipase D in the seed can

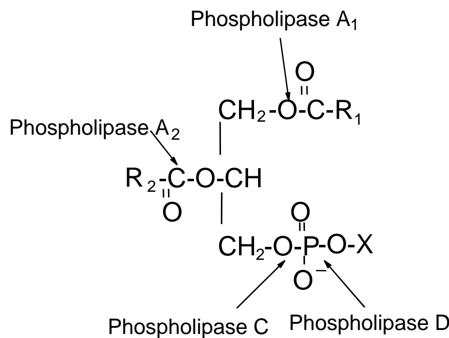


Fig. 15.9 Mode of action of phospholipases.

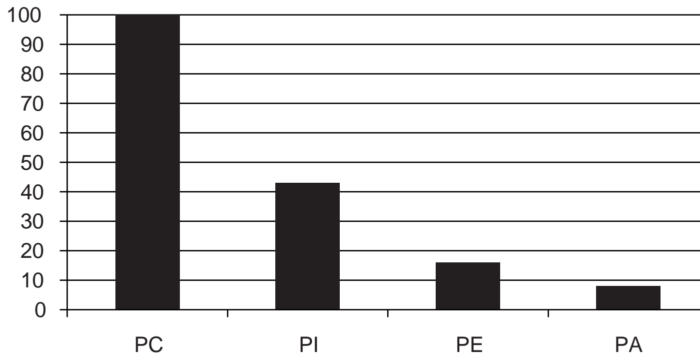


Fig. 15.10 Rate of hydration of different phosphatides PC, phosphatidyl choline; PI, phosphatidyl inositol; PE, phosphatidyl ethanolamine; PA, phosphatidic acid; HPLC, high performance liquid chromatography.

lead to elevated PA levels. Analysis of five rapeseed oil samples containing on average 450 ppm of phosphorus gave the distribution of the four phospholipid types, shown in Fig. 15.11. Water degumming of this oil could not bring the phosphorus content to <10 ppm due to the relatively high content of non-hydratable phosphatides and enzymatic degumming is required.[21]

The process of degumming oils with Lecitase Ultra consists of three steps:

1. Bringing the lecithin to an oil/water interface to allow the enzyme to react
   a. Mixing with citric acid to chelate the metal ions (Ca^{++}, Fe^{++}, etc.) which normally hold the lecithin within aggregates in the crude oil.
   b. Applying high shear mixing to provide a large surface area for the lecithin through emulsification.
2. Reacting the lecithin with enzyme
   a. The phospholipase converts the aggregated lecithin to lyso-lecithin.
3. Separation
   a. Centrifugation in one step, which efficiently removes the water phase, which contains all the lecithin. The phosphorus content in the oil should be <10 ppm after centrifugation.

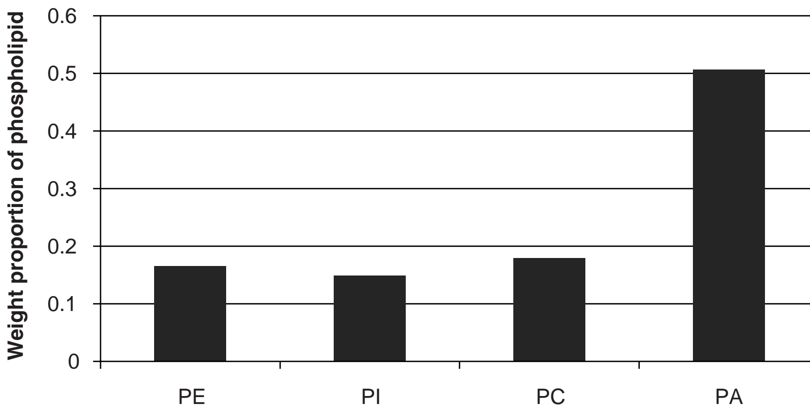


Fig. 15.11 Phosphatide content of rapeseed oil determined by HPLC analysis.

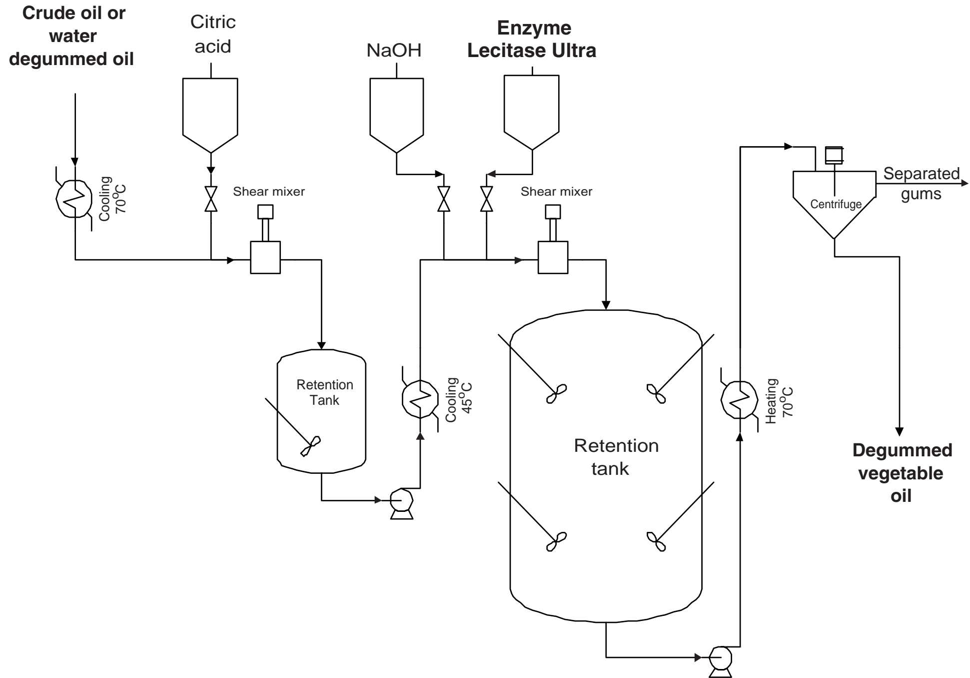


Fig. 15.12 Equipment layout for enzymatic degumming.

A schematic description of the process is given in Fig. 15.12.

Following the three steps outlined above, it is clear that the first part of the process is the citric acid step, where a small amount of citric acid (0.04–0.1%) is added to the oil as a concentrated solution (45–50%). The citric acid is distributed with a high shear mixer, and allowed to react in a holding tank with a retention time of 10–30 min. NaOH is added to adjust the pH of the water phase. The amount of NaOH is balanced with the citric acid. The optimum amount is 1.5 mol NaOH for each mol of citric acid. Water (up to 1.5–2.5% by weight of oil water) is added together with the enzyme. Enzyme dosage is approximately 30 ppm (30 g/1000 kg oil). A second high shear mixer is assuring complete distribution of the ingredients and creation of a water-in-oil emulsion with the phospholipids distributed at the water/oil interface.

The second phase of the process is to allow time enough for the enzyme to react. This is done in a continuous stirred tank system. In the first generation of enzymatic degumming, the typical tank volume is four to six times the hourly production capacity of the production line.

After the reaction, the phospholipids/gums are separated from the oil in the third phase of the process. As all the gums are now hydratable they will be eliminated with the water phase by the centrifugation.

15.6.3 Industrial experience with enzymatic degumming

Although the use of this degumming process is quite widespread, the published data on its efficiency is limited. However, the published data that is available confirms that the process

Table 15.3 Degumming operations with soya bean oil.

	Crude oil		Water degummed oil	
	Caustic refining	**Enzymatic refining**	**Caustic refining**	**Enzymatic refining**
P level in oil	525 ppm	525 ppm	150 ppm	150 ppm
P level after centrifuge	2 ppm	2 ppm	2 ppm	2 ppm
Soapstock (%)	3.19	1.7	1.51	0.5
Refining loss (%)	3.08	1.57	1.42	0.45
Yield of oil (%)	96.6	97.8	98.3	99

can produce the lowest oil yields of all the degumming methods. An enzymatic degumming installation for soya bean oil capable of running with either crude or water degummed oil was described by Dayton.[22] Using Lecitase Ultra, they were able, by use of meticulous mass balance studies, to document their oil recovery over an extended period. Table 15.3 summarizes the results that were obtained and proves that yield loss was virtually eliminated by the enzymatic process.

Yang *et al.*,[23,24] report two separate studies of the enzymatic degumming of rapeseed oil and soya bean oil. In both cases, using two, three stage CSTR reaction tanks, the resulting phosphorus level in the oil was below 10 ppm before bleaching and deodorization. They also noted that the resulting gums were more fluid than those from normal acid degumming which improved their handling properties.

The significant observation in all of these industrial evaluations was not only that phosphorus was reduced to the desired level, but also that the loss of oil in the gums was significantly decreased. With vegetable oil prices almost continually increasing, the value of this recovered oil becomes an increasingly important reason for carrying out this form of degumming.

Using data on oil losses in gums for the different degumming methods and assuming a value of 1200 US$/ton for soya bean oil, the value of the extra oil yield can be calculated (Fig. 15.13).

15.6.4 Process developments in enzymatic degumming

In enzymatic degumming, a long reaction time was initially used because the first generation pancreatic phospholipase had a high cost and required a low dosage to be economic. Developments with microbial phospholipases reduced the dosage cost and hence reaction times could be shortened. By increasing the dosage of Lecitase Ultra from 30 to 60 ppm, the overall reaction time required can be reduced to approximately 1 h (Fig. 15.14).

This has an immediate benefit in that the reactor volume required to degum a given volume of oil can be markedly reduced. This saves cost, in that smaller tanks can be used, but in addition the amount of space required in the factory is reduced. Space for tank installation can be a major hurdle in some refineries and being able to reduce the requirement is a major benefit of short time enzymatic degumming.

A further reduction in required space and tank volume can be achieved in those refineries using a water degumming as a first step followed by caustic neutralization or acid degumming. In these cases, the gums that are removed in the water-washing stage of this process carry a significant amount of oil with them. For each kilogram (dry weight) of gums extracted, 0.9–1.2 kg of oil will be lost. As the gum amount can be between 1.5% and 2.5% by weight of the oil, this loss can be significant. In standard enzymatic degumming of crude vegetable oil,

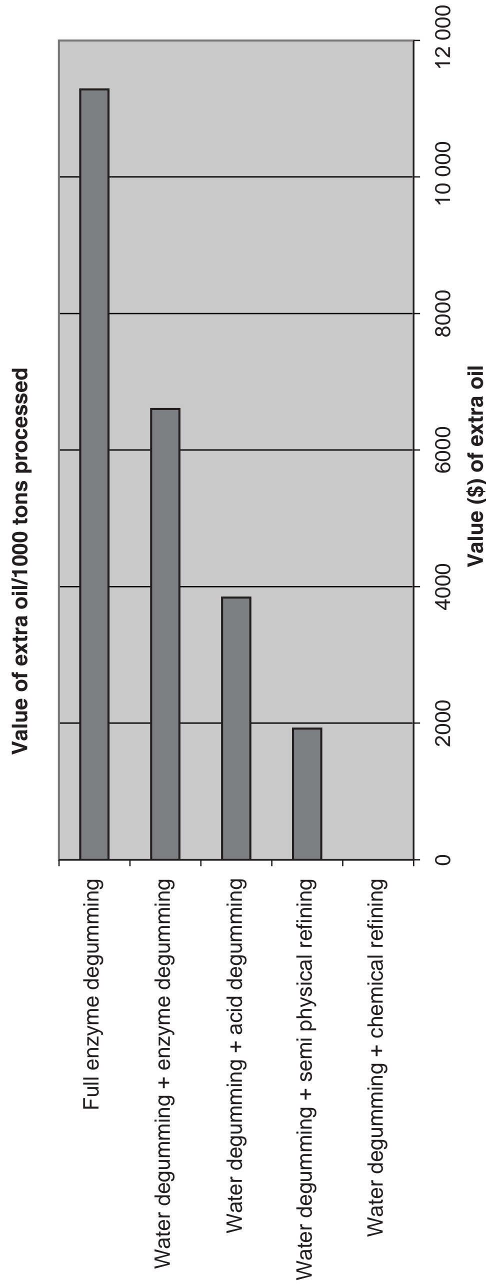


Fig. 15.13 Value of extra oil yield obtained with different degumming processes.

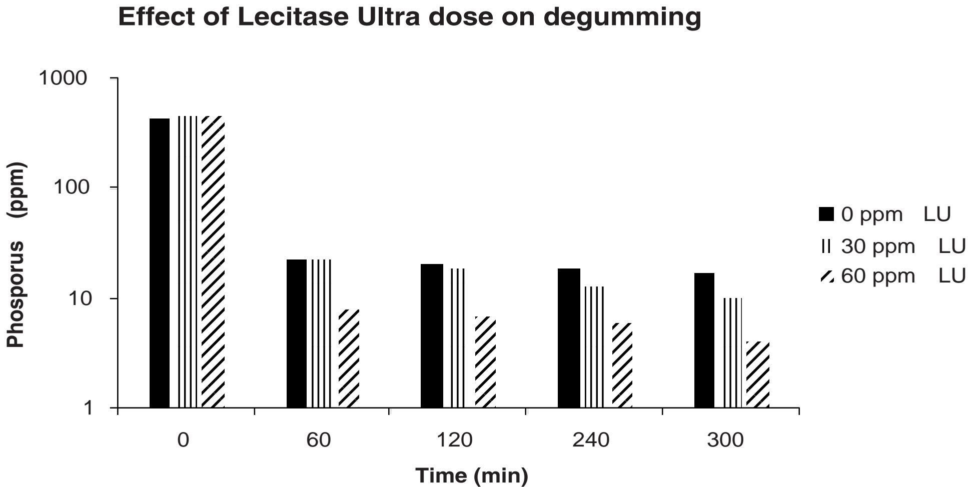


Fig. 15.14 Effect of enzyme dosage on degumming rate.

the oil binding ability of the gums is lost when they are converted to lyso-lecithin. However, as mentioned above, space can be limiting and there may not be the opportunity to install enzymatic degumming, even with the reduced contact times now available.

A process has been developed which operates on the extracted gum itself and permits the recovery of the entrained oil.[25] In this extension of the degumming process, the gums are collected in a stirred reaction vessel at 55°C and Lecitase Ultra (200–300 ppm) is added together with a small amount of citric acid (10 mM calculated on the oil free gum mass). After 2–3 h of reaction, the gums are heated to 80°C to break the emulsion and the oil can then be recovered by centrifugation (Fig. 15.15).

The resulting oil can be blended back into the main oil process where it can be further processed through bleaching and deodorization. The gums resulting from this process are of much lower viscosity, have a reduced oil content and are more readily mixed with the meal left over after oil extraction.

15.6.5 Future developments in enzymatic degumming

Originally, enzymatic degumming was applied mainly to soya bean and rapeseed oils as these were the oil types having the highest phospholipid levels and also believed to be the most appropriate oil types. Enzymatic degumming is now being extended to other types including sunflower, corn, linseed and rice bran oils. Laboratory trials of palm oil degumming have also shown that this oil can be degummed and as more exotic oils, for example jatropha, are developed for biodiesel production, then these will also require enzymatic degumming to maximize yields.

Other phospholipases are also under development and recently other A_1 and A_2 enzymes have been introduced. These products are alternatives to the existing enzyme products and to date have not shown any specific advantages in functionality. However, their introduction is an indication that research is continuing in this field and that more producers regard enzymatic degumming as an interesting opportunity.

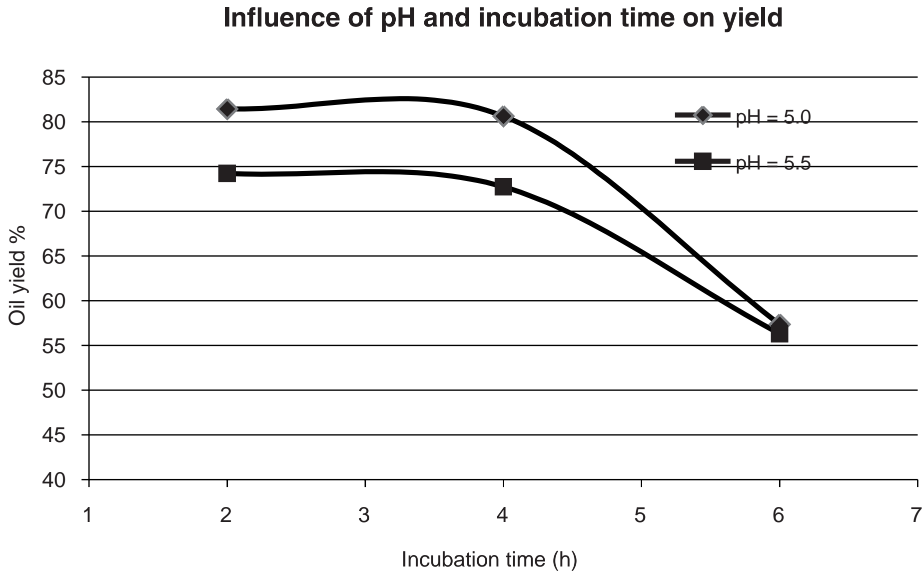


Fig. 15.15 Oil recovery from gums with phospholipase.

Lyso-phospholipases have also been developed in an attempt to further degrade lyso-phospholipids to glycero-phospholipids which would be even more hydrophilic than the lyso form. These enzymes may play a role in oil recovery from gums as they would increase the separation of gum and oil by further reducing the ability of the gum to bind oil.

In enzymatic degumming using the A_1 or A_2 phospholipase there is also a release of fatty acid coming from the hydrolysis of the phospholipid. Stochiometric calculations suggest that a reduction in phosphorus of 100 ppm should be accompanied by the release of 0.1% free fatty acids (FFAs). On that basis degumming of crude oil with ~750 ppm of phosphorus would result in an increase level of FFAs of 0.75%. This would need to be removed in a deodorizer and would increase the load on this equipment. Laboratory and plant-scale tests reveal that the amount of generated FFAs is lower than the amount mentioned because not all the phospholipid needs to be hydrolyzed in order to obtain the desired reduction in phosphorus (Table 15.4).

Phospholipase C has been suggested as an alternative, as this enzyme would produce a diglyceride which would remain in the oil fraction. The first phospholipase C that was introduced could, however, not hydrolyze PA which resulted in the need for a subsequent chemical degumming stage. This reduced markedly the value of this approach. Another solution was to combine the existing phospholipase A_1, which is capable of hydrolyzing all the phosphatides, with phospholipase C. This compromise avoids the need for the chemical

Table 15.4 FFA generation during degumming.

Enzymatic degumming of Canola oil	
Reduction in P (ppm)	175
Calculated increase in FFA	0.175%
Observed increase	0.08%

Table 15.5 Comparison of different degumming methods.

	Caustic refining	PLA enzymatic refining	PLA/PLC enzymatic refining
Starting phos. level in crude oil	500 ppm	500 ppm	500 ppm
Phos. level after centrifuge	2 ppm	2 ppm	2 ppm
Centrifuge discharge (dry %)	3.19	1.13	0.62
Yield of oil (%)	96.5	97.4	98.3

degumming step but still gives an overall good reduction in phosphorus content. Table 15.5 compares the original caustic process with the two enzymatic options and suggests that combined enzyme addition may be a useful route until more efficient phospholipase C enzymes become available.

15.7 ESTER SYNTHESIS

Fatty acid esters are produced from vegetable oil and some are used as ingredients in skin care and other cosmetic products. The conventional production of fatty acid esters is based on catalysis with either tin or acid at high temperature. Novozym 435 is a lipase that is able to catalyze the conversion of vegetable oil to specific fatty acid esters. In this approach, the traditional tin catalyzed process can be substituted with an enzymatic process using Novozym 435 (Fig. 15.16).

The advantages of this approach are considerably lower operating temperatures, a reduced energy demand and less formation of by-products. When operating this type of synthesis, the released water must be removed as this will limit the conversion to the ester. As water levels build up within the system, the synthesis reaction slows and eventually hydrolysis of the ester will occur (Fig. 15.17). In industrial operation this is achieved by operating at reduced pressure or by the addition of molecular sieves (MS) to remove the water.

The purity of the products produced by enzymatic synthesis is much higher than from the conventional chemical process. A comparison of the purity of myristyl myristate produced by tin or enzyme-catalyzed reactions was made by Thum[26] and is shown in Table 15.6.

The product from enzymatic synthesis had the same melting properties as the conventional one but the colour formation was lower and the overall yield higher. The production of cetyl ricinoleate was also considered and an overall yield improvement noted (93% compared to 61%). In addition, not only was the synthesis of dimer reduced but the level of unknown materials as illustrated by the base line on the chromatograph was also lower, indicating an overall purer product (Fig. 15.18).

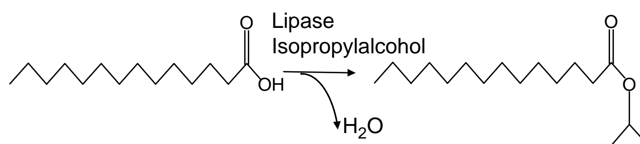


Fig. 15.16 Lipase catalyzed ester synthesis of isopropyl myristate.

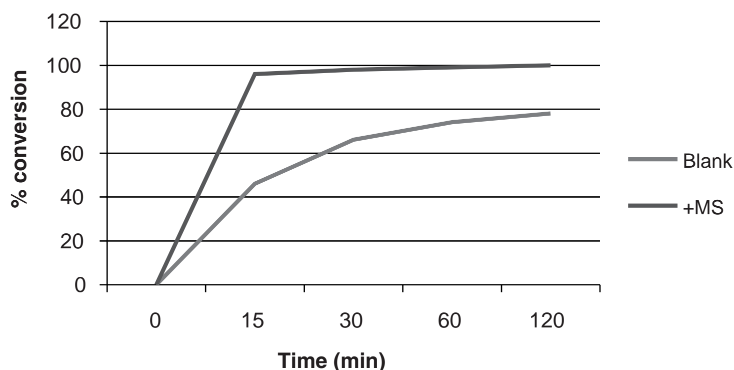


Fig. 15.17 Influence of water on ester synthesis.

15.8 SPECIALITY FATS

The production of fats with particular melting properties or fatty acid compositions has not advanced as far as might be expected when it is recalled that the production of CBEs was the first of the industrial lipase applications. One reason for this was the difficulty of carrying out the reaction using the immobilized lipases available at the time. However, once lipases immobilized onto ion exchange resins were developed, the possibilities inherent with lipase for stereospecific fat modification could be explored. One of the best examples of this is the synthesis of a diglyceride oil from the reaction between glycerol and fatty acids, catalyzed by a 1.3 specific lipase (Lipozyme RM IM).[27] The highest yield of diacetyl glycerol (DAG) with 4 h reaction time was produced using a 1:2 molar ratio of glycerol to FFAs at 50°C and applying a vacuum of 3 mm of mercury. As with the ester synthesis reported above, DAG yield was reduced if the water produced in the reaction was not removed. A number of different water removal possibilities were proposed including nitrogen sparging as well as operation at reduced pressure.

The production of these DAG products is carried out in two stages. First, a fat containing the desired fatty acids is hydrolyzed and the fatty acids are removed by distillation. Glycerol and the wanted fatty acids are then re-combined to form the DAG, through the enzyme catalyzed reaction. These DAG products are without a fatty acid at the sn-2 position and on ingestion are not metabolized as fat by the human body, opening up the possibility of having low calorie fat products for the food industry.

Table 15.6 Comparison of purity of myristyl myristate made by two routes.

	Enzymatic	Conventional (average of 4)
OH-value	6.0	11.3
Peroxide value	<0.1	0.5
Melting point (°C)	41	40
Colour @ 50°C	28	73
GC: purity (%)	96.4	88.5
GC: C14-OH (%)	1.4	3.5
GC: unknown (%)	0.4	2.1

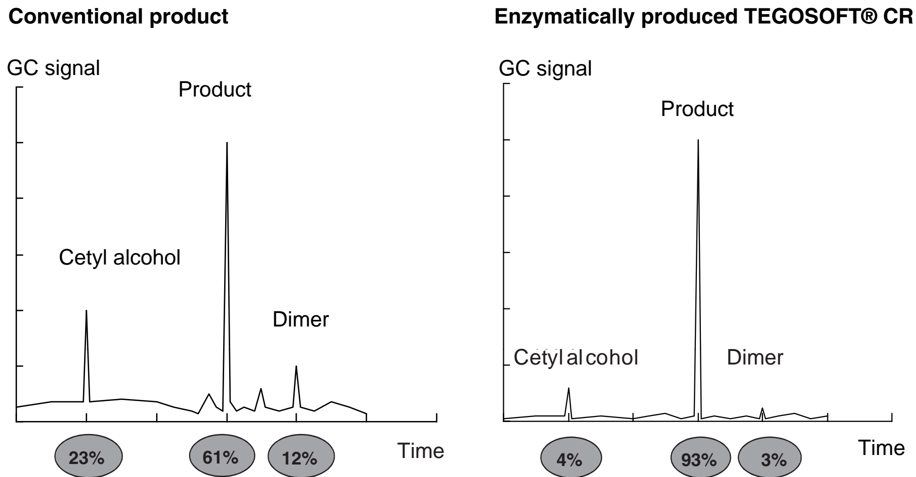


Fig. 15.18 Comparison of product purity for chemically and enzymatically produced cetyl ricinoleate.

Alternative methods of production of these fats are possible, one option being to interesterify a mixture of a triglyceride and glycerol, or by hydrolyzing a fat with an sn-2 specific lipase. Some laboratory evaluations have been made of the first route using specifically produced lipases with the correct regiospecificity but as yet no industrial source of an sn-2 specific lipase is available.

Triglycerides containing elevated levels of essential fatty acids can also be produced by enzymatic synthesis. The essential fatty acids EPA and DHA are found in fish oils but the level is relatively low (Table 15.7).

If an ethanolysis reaction is carried our between the fish oil and ethanol, ethyl esters of the fatty acids can be produced. These have a lower boiling point than the starting fish oil and can be separated by a short path distillation process as described by Kralovec *et al.*[28] The extracted EPA and DHA ethyl esters can then be recombined to form a triglyceride by reaction with an immobilized esterase such as Candida antartica lipase B. The reaction is summarized in Fig. 15.19, and the liberated ethanol can be recycled for further reactions.

The benefit of using the ethyl ester for this reaction is that ethanol has a lower boiling point than water, but has the disadvantage that it will tend to strip away the small amount of water held by the enzyme, thus reducing the catalytic activity. The main reason for this synthesis is to allow for the production of highly concentrated omega 3 supplements and the avoidance of the fishy taste and aroma normally associated with fish oils.

Table 15.7 % EPA and DHA content of fish oils.

	EPA	DHA
Cod liver oil	9.0	9.5
Herring oil	7.1	4.3
Menhaden	12.7	7.9
Salmon	8.8	11.1

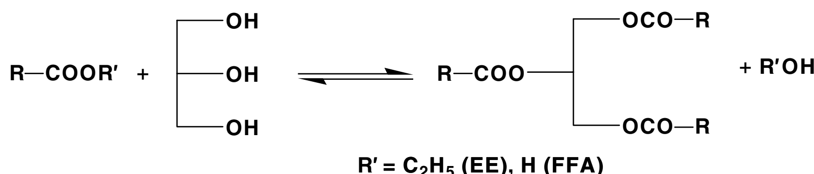


Fig. 15.19 Synthesis of DHA and EPA containing triglycerides.

15.9 ENVIRONMENTAL BENEFITS OF ENZYMATIC PROCESSING

Replacing chemical processing with enzymatic routes clearly involves lower operating temperatures and improved yields of the desired products. Quantification of the environmental benefits demands the use of other assessment systems to determine how the enzymatic process compares to the replaced chemical one.

Life cycle assessment (LCA) is the accepted method for quantifying the environmental impacts of new and existing technologies. The analysis will compare all processes in the product chain – from raw material extraction through production and use to final disposal. LCA is used to compare environmental impacts of two or more alternative processes providing the same benefit to the user. LCA has been applied in three areas (degumming, interesterification and ester synthesis) where lipases can replace chemical processes to examine the environmental impact of the conventional and enzymatic alternative. In each case, inputs and outputs have been quantified and the potential savings have been calculated in terms of energy, global warming contribution, acidification, etc.

For EIE, comparison was made between the chemical and EIE of palm stearin and palm kernel oil to produce a hard stock for margarine production. The analysis compared the parts of the life cycle which will differ, so the production of the oils is not included as this is the same in both cases. For degumming, soya bean oil production was studied and for ester synthesis, the production of the cosmetic wax myristyl myristate.

The LCAs were carried out according to the procedures of Wenzel *et al.*[29] and the assessments are in agreement with ISO 14 040 and are based on the principles described by Hauschild *et al.*,[30] with the modelling carried out in SimaPro 6.0 software. Characterizations of environmental impacts are based on Eco-indicator 95 v2. The assessment covers four environmental indicators: global warming, acidification, nutrient enrichment and photochemical ozone formation. The results of the three studies are summarized in Table 15.8.

Table 15.8 Net savings per 1000 kg product when enzymatic processing replaces chemical.

	Enzymatic degumming	Enzymatic interesterification	Ester synthesis
Fossil energy	400 MJ	280 MJ	2760 MJ
Global warming	44 kg CO_2	23 kg CO_2	187 kg CO_2
Acidification	527 g SO_2	61 g SO_2	2 g SO_2
Nutrient enrichment	375 g PO_4	58 g PO_4	130 g PO_4
Smog formation	18 g C_2H_4	4 g C_2H_4	74 g C_2H_4

In all cases, the replacement of chemical processing by an enzymatic alternative reduces the environmental impact as the net savings also take into account the environmental impact of enzyme production.

15.10 FUTURE DEVELOPMENTS IN LIPASE APPLICATIONS

15.10.1 Biodiesel

Examination of the literature reveals more than 40 papers on the use of lipases in the production of biodiesel (fatty acid methyl ester). Nielsen and Holm[31] have calculated that at current lipase conversion costs, an enzymatic biodiesel route is at least five times more costly than the use of a chemical catalyst. However, they do expect that developments in immobilization technology will bring down this figure and that enzymatic biodiesel production will become economic.

One area that looks of particular promise is the use of second grade oils such as jatropha, which are not intended for human consumption and/or oils with high levels of FFAs. A dual lipase approach in which one lipase is used to convert the FFA to methyl ester, while a second hydrolyzes triglyceride to FFA, providing extra substrate for the first, looks the most likely to succeed.

Methanol poisoning of lipases is also a problem in enzymatic biodiesel production, but a number of options are available to reduce this. Ethanol and propanol, for example, are both much less toxic to the enzyme and can be used as alternatives to methanol. Systems for adding methanol semi-continuously, recycling part of the ester to provide a cosolvent or running the reaction in t-butanol (which is not affected by lipases) have all been proposed as solutions to this issue. With the large amount of research effort being applied to this area, we can expect an enzymatic biodiesel process to be introduced shortly.

15.10.2 Alternative immobilization systems

One of the limiting factors for the production of speciality and structured fats has been the relatively high cost of the immobilization system. The development of more cost effective immobilization processes may make fat modification more widely affordable for synthesis reactions.

Table 15.9 shows the results of an acidolysis reaction carried out with Rhizomucor miehei lipase on two different carriers. The conventional carrier is an ion exchange resin and the other a lower cost alternative. While not producing identical results, much of the stereo specificity of the lipase has been retained using the new immobilization system, opening up the possibility of developing less expensive lipase products.

Table 15.9 Acidolysis reactions with lipase on different carriers at 65°C for 48 h.

TAG type	Palm stearine (%)	After acidolysis with standard enzyme (%)	After acidolysis with enzyme on new carrier (%)
PPP	62.7	1.8	4.5
POP	13.2	17.2	29.1
POO/OPO	4.4	37.8	36.3
OOO	0.6	25.0	11.3

15.10.3 Alternative reaction systems

Many lipase reactions are carried out using immobilized enzymes, partly to be able to reuse the enzyme, but also because it has been assumed that water-soluble enzymes would not function in fats. The use of a water-soluble phospholipase in enzymatic degumming has demonstrated that this form can also be used if the enzyme is well dispersed within the fat in the form of water droplets containing the enzyme.

A similar approach could also be used for synthesis reactions where a batch conversion is preferred. One model system used to investigate this concept is the removal of FFA from an oil by reaction with glycerol or mono or diglycerides. The reaction takes place under reduced pressure to remove the generated water and with initial dispersion of the enzyme throughout the reactants with the help of a high shear mixer. A distillate from palm oil refining (almost pure FFA) was mixed in a stochiometric ratio with glycerol and liquid Candida antartica B lipase added at 50, 100 or 175 lipase units (LU) g^{-1} glycerol. Following high speed mixing with an Ultra turrax, the reaction was allowed to proceed with stirring under vacuum at 65°C.

Despite relatively high water levels coming from the reaction and the portion introduced together with the enzyme itself, FFA was quickly removed from the reaction (Fig. 15.20). This type of reaction could also be applied for the removal of FFA from oils without yield loss. Oils such as rice bran oil contain quite high FFA levels coming from hydrolysis of the oil on storage of the rice bran. This FFA could be recombined with the mono and diglycerides in the oil to increase the physical yield of the process and avoid losses.

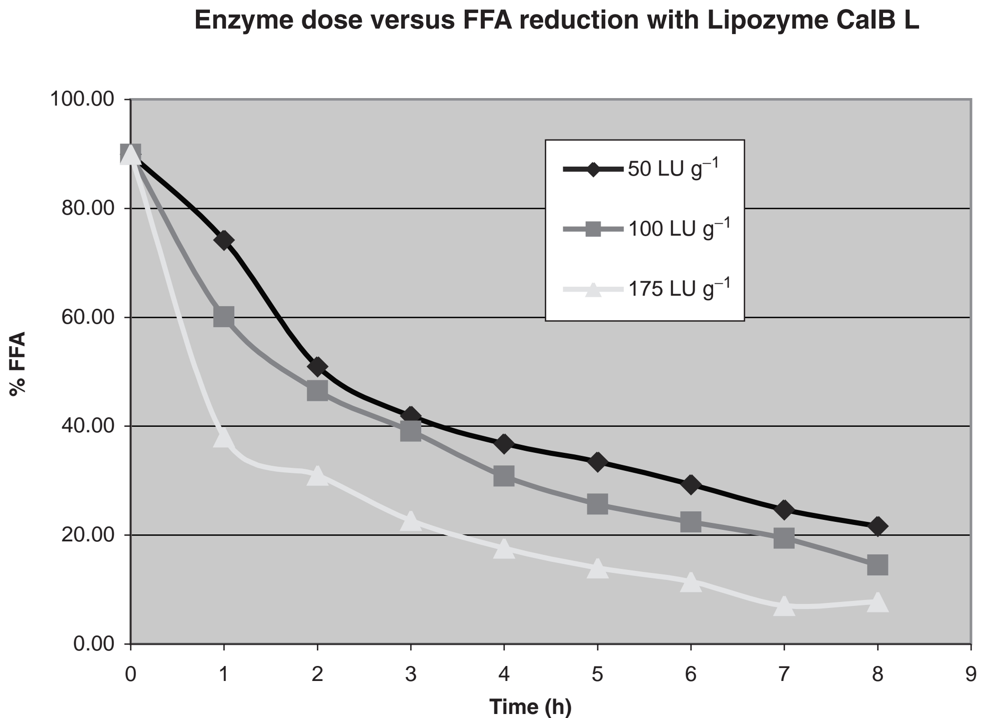


Fig. 15.20 FFA removal using water-soluble lipase.

15.11 CONCLUSIONS

Within the last 25 years, lipases have grown from being one of the smallest application areas of enzymes to a much larger and more important one. From a limited application within the dairy industry, lipases have spread and now offer key technological and environmental benefits for the oils and fats industry. The development of improved production systems giving higher enzyme yields coupled with more cost-effective immobilization systems has made this change possible. However, the growth in applications is far from being complete and it is clear that the benefits of the research and development efforts of the last decades will be new ways of applying lipases and new lipase products of altered properties to supplement those available today.

REFERENCES

1. Godfrey, T. and Reichelt, J. (1983) *Industrial Enzymology: The Application of Enzymes in Industry*, 1st edn. Macmillan Publishers Ltd, Basingstoke, Hants.
2. Zaks, A. and Klibanov, A.M. (1984) Enzymatic catalysis in organic media at 100 degrees C. *Science* **224**(4654), 1249–1251.
3. Coleman, M.H. and Macrae, A.R. (1980) Fat process and composition. UK Patent, 1577933.
4. Matsuo, T., Sawamura, N., Hashimoto, Y. and Hashida, W. (1980) Producing a cocoa butter substitute by transesterification of fats and oils. UK Patent, 2035359A.
5. Macrae, A.R. (1985) Microbial lipases as catalysts for the interesterification of oils and fats. In: *Biotechnology for the Oils and Fats Industry* (eds C. Ratledge, P. Dawson and J. Rattray). AOCS Press, Champaign, IL, pp. 189–198.
6. Eriksen, N. (1996) Detergents. In: *Industrial Enzymology* (eds T. Godfrey and S. West). Stockton, Boston, NY, pp. 187–200.
7. Holm, H.C. and Cowan, D. (2008) The evolution of enzymatic interesterification in the oils and fats industry. *European Journal of Lipid Science and Technology* **110**, 679–691.
8. Gunstone, F.D. (2004) Extraction, refining and processing. In: *The Chemistry of Oils and Fats* (ed. F.D. Gunstone). Blackwell Publishing, Oxford, pp. 42–49.
9. Cowan, W.D., Willits, J. and Pearce, S.W. (2008) Comparison of chemical and enzymatic interesterification. In: Proceedings of the 99th AOCS Conference. Seattle.
10. Ascherio, A., Stampfer, M.J. and Willett, W.C. (2006) Trans fatty acids and coronary heart disease. *The New England Journal of Medicine* **354**(15), 1601–1613.
11. Osório, N.M., da Fonseca, M.M.R. and Ferreira-Dias, S. (2006) Operational stability of *Thermomyces lanuginosa* lipase during interesterification of fat in continuous packed-bed reactors. *European Journal of Lipid Science and Technology* **108**, 545–553.
12. Cowan, W.D., Hemann, J., Holm, H.C. and Yee, H.S. (2007) MPOB International Palm Oil Congress 2007. In: Proceedings of the 2007 PIPOC Conference. Malaysia.
13. Lee, I. and Sleeter, R.T. (2003) Method for producing fats or oils. United States Patent 2003/0054509A.
14. Ibrahim, N.A., Nielsen, S.T., Wigneswaran, V., Zhang, H. and Xu, X. (2008) Online pre-purification for the continuous enzymatic interesterification of bulk fats containing omega-3 oil. *Journal of the American Oil Chemists' Society* **85**, 95–98.
15. Zhang, H., Jacobsen, C. and Adler-Nissen, J. (2005) Storage stability of margarines produced from enzymatically interesterified fats compared to those prepared by conventional methods – chemical properties. *European Journal of Lipid Science and Technology* **107**, 530–539.
16. Kirkeby, P.G. (2003). Experience in margarine processing using enzymatic interesterified hardstock. In: Proceedings of the 94th AOCS Conference. Kansas City.
17. Cowan, W.D., Holm, H.C., Pedersen, L.S., Seng, Y.H. and Pierce, S.W. (2007) Influence of oil type and quality on lipase used for enzymatic interesterification. In: Proceedings of the 98th AOCS Conference. Quebec City.

18. Siew, W.L., Cheah, K.Y. and Tang, W.L. (2007) Physical properties of lipase-catalyzed interesterification of palm stearine with canola oil blends. *European Journal of Lipid Science and Technology* **109**, 97–106.
19. Dayton, C. (2008) Enzymatic degumming of vegetable oils. In: Proceedings of the 99th AOCS Conference. Seattle.
20. Seghers, J. (1990) Degumming – theory and practice. In: World Conference Proceedings, Edible Fats and Oils Processing. AOCS, Champaign, IL, pp. 88–93.
21. Cowan, W.D. (2003) Unpublished results.
22. Dayton, C. (2005) Enzymatic degumming of soybean oil. In: Proceedings of the Nordic Symposium. Enzymes in Lipid Technology. Reykjavik, Iceland.
23. Yang, B., Wang, Y.-H. and Yang, J.-G. (2006) Optimization of enzymatic degumming process for rapeseed oil. *Journal of the American Oil Chemists' Society* **83**, 653–658.
24. Yang, B., Rong, Z., Yang, J.-G., Wang, Y.-H. and Wang, W.-F. (2008) Insight into the enzymatic degumming process of soybean oil. *Journal of the American Oil Chemists' Society* **85**, 421–425.
25. Cowan, W.D. and Holm H.C. (2007) Bioprocessing of vegetable oils. In: Proceedings of the 98th AOCS Conference. Quebec.
26. Thum, O. (2008) Biocatalysis – a tool for sustainable production of emollient esters. In: Proceedings of the 99th AOCS Conference. Seattle.
27. Watanabe, T., Shimizu, M., Sugiura, M., Sato, M., Kohori, J., Yamada, N. and Nakanishi, K. (2003) Optimization of reaction conditions for the production of DAG using immobilized 1,3-regiospecific lipase Lipozyme RM IM. *Journal of the American Oil Chemists' Society* **80**, 1201–1207.
28. Kralovec, J.A., Mugford, P., Wang, W. and Barrow, C.J. (2008) Production of fish oil omega-3 fatty acid concentrates with superior sensory profiles. In: Proceedings of the 6th Euro Fed Lipid Congress. Athens, 7–10 September.
29. Wenzel, H., Hauschild, M. and Alting, L. (1997) Environmental assessment of products. In: *Methodology, Tools and Case Studies in Product Development* (eds M. Hauschild and H. Wenzel), Vol. **1**. Chapman and Hall, London.
30. Hauschild, W., Wenzel, H. and Alting, L. (1997) *Environmental Assessment of Products*, Vol. **1**. Kluwer Academic Publishers, Dordrecht.
31. Nielsen, P.M. and Holm, H.C. (2008) New enzyme process for biodiesel. In: Proceedings of the 99th AOCS Conference. Seattle.

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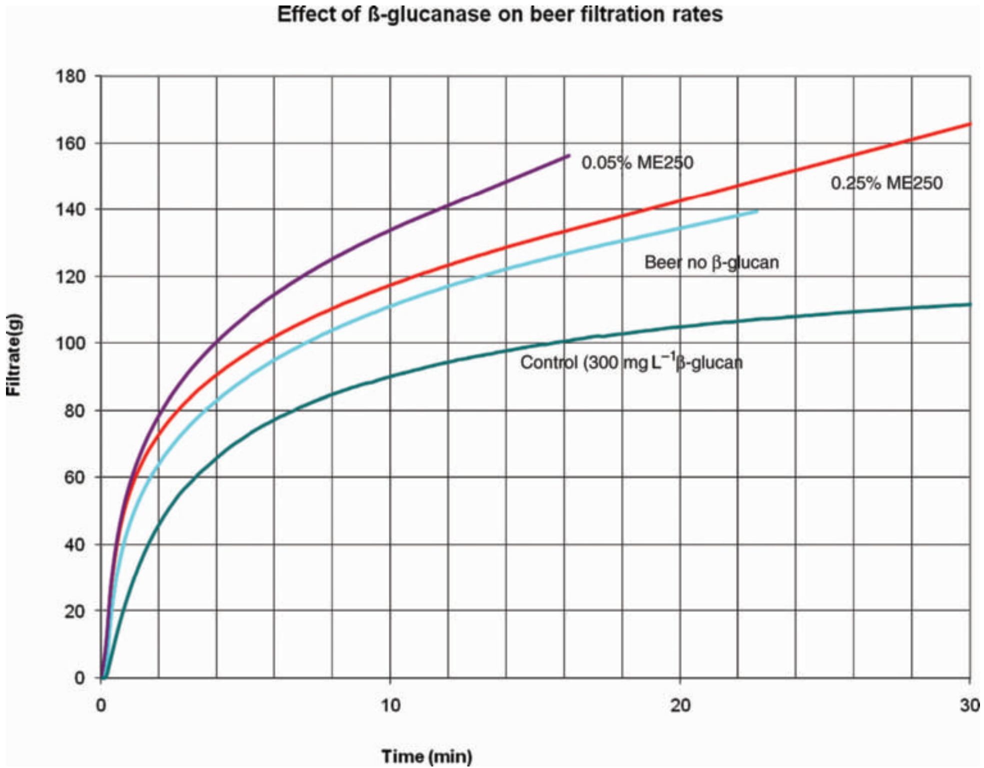
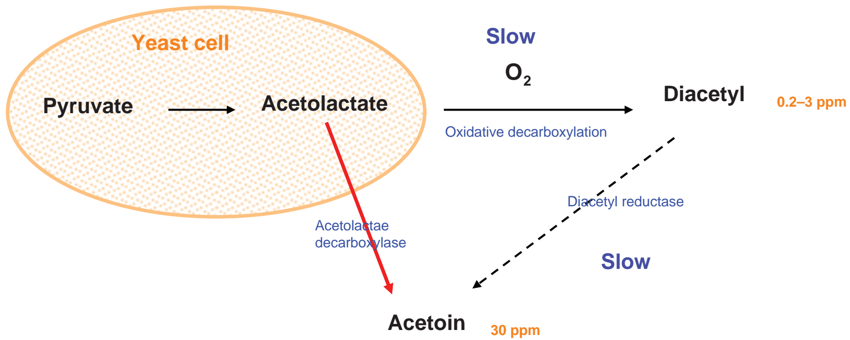
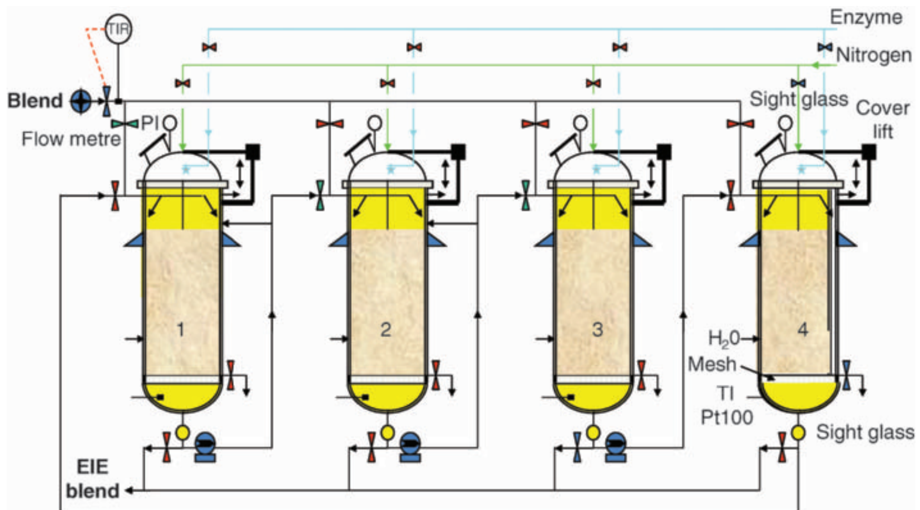


Colour Plate 1 Effect of β-glucanase on beer filtration. (Also see Fig. 8.4.)



Colour Plate 2 Acetolactate-decarboxylase (ALDC) and diacetyl reduction. (Also see Fig. 8.6.)

Colour Plate 3 Cloudy apple juice (left) control (right) process with *Aspergillus niger* pectin methyl esterase. (Also see Fig. 11.5.)



Colour Plate 4 Layout of reactors for EIE. (Also see Fig. 15.4.)