Enzymes

10

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Introduction

Enzymes, although minor constituents of many foods, play a major and manifold role in foods. They are highly specialized proteins with the special ability to catalyze specific chemical reactions in biological systems. Although they may undergo change during the catalysis, they are unchanged at the end of the reaction. They are highly selective catalysts, greatly accelerating both the rate and specificity of metabolic reactions from digestion of food to the synthesis of DNA. An enzyme can catalyze only a single reaction of a single compound, called the enzyme's substrate. For example, amylase found in the human digestive tract catalyzes only the hydrolysis of starch to yield glucose; cellulose and other polysaccharides are untouched by amylase. Other enzymes have different specificities. Papain, for example, a globular protein isolated from papaya fruit, catalyzes the hydrolysis of many kinds of peptide bonds, which makes papain useful as a meat tenderizer and a cleaner for contact lenses. Enzymes act only to lower the activation energy for a reaction, thereby making the reaction takes place more rapidly. Starch and water, for example, react very slowly in the absence of a catalyst because the activation energy is too high. When amylase is present, however, the energy barrier is lowered, and the hydrolysis reaction occurs rapidly.

The research on enzymes has immense practical importance in agriculture and food processing. There is not a single food system that does not involve enzyme reactions. Enzymes that are naturally present in foods may change the composition of those foods; in some cases, such changes are desirable but in most instances are undesirable, so the enzymes must be deactivated. The blanching of vegetables is an example of enzyme deactivation to prevent undesirable change. Some enzymes are used as indicators in analytical methods; phosphatase, for instance, is used in the phosphatase test of pasteurization of milk. Enzymes are also used as processing aids in food manufacturing. For example, rennin, contained in extract of calves' stomachs, is used as a coagulant for milk in the production of cheese.

Recent advances in biotechnology offer a new promise for tailoring enzymes for specific functions of particular applications and their production in quantities for industrial uses. By manipulating the genetic code of an enzyme, it is now possible to target a specific amino acid residue at any location for modification. During the last three decades, the use of cellulases, hemicellulases and pectinase which account for approximately 20% of the world enzyme market for food, brewery, wine, and textile industries has increased considerably and many commercial enzyme producers are marketing tailor-made enzyme preparations suitable for specific purposes (Wong 1995; Bhat 2000).

J.M. deMan et al., *Principles of Food Chemistry*, Food Science Text Series,
https://doi.org/10.1007/978-3-319-63607-8_10

Food science's emphasis in the study of enzymes differs from that in biochemistry. The former deals mostly with decomposition reactions, hydrolysis, and oxidation; the latter is more concerned with synthetic mechanisms. Whitaker (1972) has prepared an extensive listing of the uses of enzymes in food processing (Table 10.1) and this gives a good summary of the many and varied possible applications of enzymes.

Table 10.1 Some uses and suggested uses of enzymes in foods and food processing

Enzyme	Food	Purpose or action
Amylases	Baked goods	Increase sugar content for yeast fermentation
	Brewing	Conversion of starch to maltose for fermentation; removal of starch turbidities
	Cereals	Conversion of starch to dextrins, sugar; increase water absorption
	Chocolate-cocoa	Liquidification of starches for free flow
	Confectionery	Recovery of sugar from candy scraps
	Fruit juices	Remove starches to increase sparkling properties
	Jellies	Remove starches to increase sparkling properties
	Pectin	An aid in preparation of pectin from apple pomace
	Syrups and sugars	Conversion of starches to low molecular weight dextrins (corn syrup)
	Vegetables	Hydrolysis of starch as in tenderization of peas
Cellulase	Brewing	Hydrolysis of complex carbohydrate cell walls
	Coffee	Hydrolysis of cellulose during drying of beans
	Fruits	Removal of graininess of pears; peeling of apricots, tomatoes
Dextran-sucrase	Sugar syrups	Thickening of syrup
	Ice cream	Thickening agent, body
Invertase	Artificial honey	Conversion of sucrose to glucose and fructose
	Candy	Manufacture of chocolate-coated, soft, cream candies
Lactase	Ice cream	Prevent crystallization of lactose, which results in grainy, sandy texture
	Feeds	Conversion of lactose to galactose and glucose
	Milk	Stabilization of milk proteins in frozen milk by removal of lactose
Tannase	Brewing	Removal of polyphenolic compounds
Pentosanase	Milling	Recovery of starch from wheat flour
Naringinase	Citrus	Debittering citrus pectin juice by hydrolysis of the glucoside, naringin
Pectic enzymes (useful)	Chocolate-cocoa	Hydrolytic activity during fermentation of cocoa
	Coffee	Hydrolysis of gelatinous coating during fermentation of beans
	Fruits	Softening
	Fruit juices	Improve yield of press juices, prevent cloudiness, improve concentration processes
	Olives	Extraction of oil
	Wines	Clarification
Pectic enzymes (deteriorative)	Citrus juice	Destruction and separation of pectic substances of juices
	Fruits	Excessive softening action

(continued)

Table 10.1 (continued)

Enzyme	Food	Purpose or action
Proteases (useful)	Baked goods	Softening action in doughs; cut mixing time, increase extensibility of doughs; improvement in grain, texture, loaf volume; liberate β-amylase
	Brewing	Body, flavor and nutrients development during fermentation; aid in filtration and clarification, chill-proofing
	Cereals	Modify proteins to increase drying rate, improve product handling characteristics; manufacture of miso and tofu
	Cheese	Casein coagulation; characteristic flavors during aging
	Chocolate-cocoa	Action on beans during fermentation
	Eggs, egg products	Improve drying properties
	Feeds	Use in treatment of waste products for conversion to feeds
	Meats and fish	Tenderization; recovery of protein from bones, trash fish; liberation of oils
	Milk	In preparation of soybean milk
	Protein hydrolysates	Condiments such as soy sauce and tamar sauce; specific diets; bouillon, dehydrated soups, gravy powders, processed meats
	Wines	Clarification
Proteases (deteriorative)	Eggs	Shelf life of fresh and dried whole eggs
	Crab, lobster	Overtenderization if not inactivated rapidly
	Flour	Influence on loaf volume, texture if too active
Lipase (useful)	Cheese	Aging, ripening, and general flavor characteristics
	Oils	Conversion of lipids to glycerol and fatty acids
	Milk	Production of milk with slightly cured flavor for use in milk chocolate
Lipase (deteriorative)	Cereals	Overbrowning of oat cakes; brown discoloration of wheat bran
	Milk and dairy products	Hydrolytic rancidity
	Oils	Hydrolytic rancidity
Phosphatases	Baby foods	Increase available phosphate
	Brewing	Hydrolysis of phosphate compounds
	Milk	Detection of effectiveness of pasteurization
Nucleases	Flavor enhancers	Production of nucleotides and nucleosides
Peroxidases (useful)	Vegetables	Detection of effectiveness of blanching
	Glucose determinations	In combination with glucose oxidase
Peroxidases (deteriorative)	Vegetables	Off-flavors
	Fruits	Contribution to browning action
Catalase	Milk	Destruction of H_2O_2 in cold pasteurization
	Variety of products	To remove glucose and/or oxygen to prevent browning and/or oxidation; used in conjunction with glucose oxidase
Glucose oxidase	Variety of products	Removal of oxygen and/or glucose from products such as beer, cheese, carbonated beverages, dried eggs, fruit juices, meat and fish, milk powder, wine to prevent oxidation and/or browning; used in conjunction with catalase
	Glucose determination	Specific determination of glucose; used in conjunction with peroxidase
Polyphenol oxidase (useful)	Tea, coffee, tobacco	Development of browning during ripening, fermentation, and/or aging process
Polyphenol oxidase (deteriorative)	Fruits, vegetables	Browning, off-flavor development, loss of vitamins
Lipoxygenase	Vegetables	Destruction of essential fatty acids and vitamin A; development of off-flavors
Ascorbic acid oxidase	Vegetables, fruits	Destruction of vitamin C (ascorbic acid)
Thiaminase	Meats, fish	Destruction of thiamine

Source: Reprinted with permission from Whitaker, J. R. (1972). *Principles of enzymology for the food sciences*, by courtesy of Marcel Dekker, Inc

Nature and Kinetics of Enzymes

Nature of Enzymes

The catalytic properties of enzymes are quite specific, which makes enzymes useful in analytical studies. Some enzymes consist only of protein, but most enzymes contain additional nonprotein components such as carbohydrates, lipids, metals, phosphates, or some other organic moiety. The complete enzyme is called *holoenzyme*; the protein part, *apoenzyme*; and the nonprotein part, *cofactor.* The compound that is being converted in an enzymic reaction is called *substrate.* In an enzyme reaction, the substrate combines with the holoenzyme and is released in a modified form, as indicated in Fig. 10.1.

Kinetics of Enzymes

Enzyme activity can be controlled in a number of ways that are very important to food chemists. The velocity of an enzyme-catalyzed reaction is usually proportional to the active enzyme concentration, and dependent on substrate, inhibitor, and cofactor concentration, and on temperature and pH.

Enzyme Concentration: The relationship between reaction velocity and enzyme concentration can be illustrated as the following Fig. 10.2.

In the first part of the curve, a straight line plot results when the amount of enzyme is increased in the presence of an excess of substrate that shows the concentration of an enzyme is directly proportional to the rate of the reaction. In these conditions, doubling the amount of enzyme will double the reaction velocity. In the second part of the line, at higher levels of enzyme concentration, the amount of substrate becomes the limiting factor and the linear relationship cannot be maintained, causing the line to flatten out. Under these conditions, adding more enzyme has no effect on reaction rate.

Substrate Concentration: For a given amount of enzyme under standard conditions, the initial reaction velocity (V_0) varies with an increase of initial substrate concentration (S). Figure 10.3

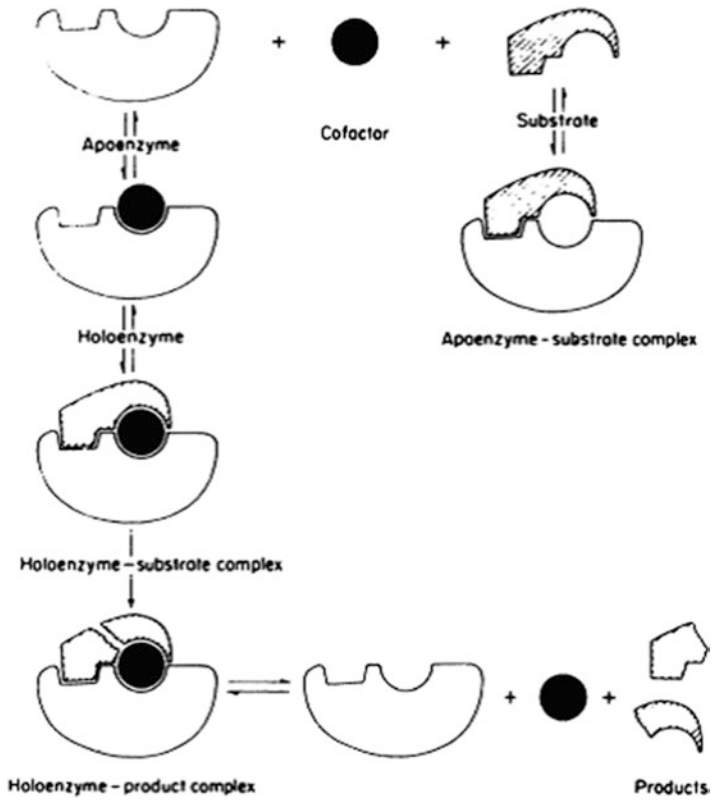


Fig. 10.1 The nature of enzymes—substrate reactions

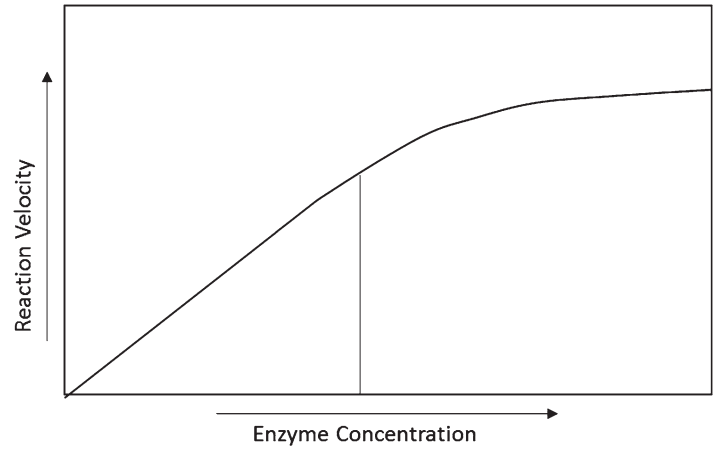


Fig. 10.2 Effect of enzyme concentration on reaction velocity

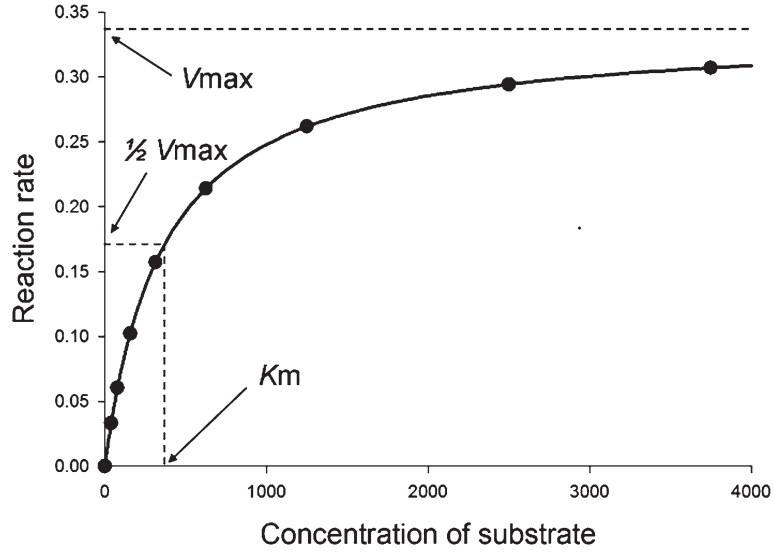


Fig. 10.3 Effect of substrate concentration on the initial velocity of an enzyme catalyzed reaction

shows the effect on V_0 of varying (S) when the enzyme concentration is held constant. When substrate concentrations are low compare to available enzyme, V_0 is nearly linearly related to (S) because not all of the enzyme molecules are combined with substrate. Therefore, substrate concentration is the limiting factor accounting for the lower reaction rate. At high substrate concentration, V_0 increases by smaller amounts in response to increases in (S) and almost unaffected by change in substrate concentration. This plateau is called the maximum velocity (V_{max}), as shown in Fig. 10.3.

This hyperbolic shape of the curve may be explained in terms of the following equation with the formation of an enzyme-substrate complex (E-S) explained by German scientists, Leonor Michaelis and Maud Menten in 1913. They postulated that enzyme first combines reversibly with its substrate to form an enzyme-substrate complex in a fast reversible step, then breaks down in a slower second step to yield the free enzyme (E) and the reaction product (P). The second step is slower and limits the rate of the overall reaction.

$$E + S \underset{k_2}{\overset{k_1}{\Leftrightarrow}} E - S \tag{10.1}$$

$$E - S \xrightarrow{k_3} E + P \tag{10.2}$$

At any given instant in an enzyme-catalyzed reaction, the enzyme exists in two forms, the free form, E and the combined form, ES. At low (S), most of the enzyme will be in the E form and the rate will be proportional to (S) because of the equilibrium will be pushed toward formation of more E–S as (S) is increased. The maximum initial rate of the catalyzed reaction (V_{max}) is observed when all of enzyme is present as E–S. At this point the enzyme is saturated with its substrate, so that further increases in (S) have no effect on rate. The saturation effect is a distinguishing characteristic of enzyme catalysis and responsible for the plateau observed in Fig. 10.3. After the E–S breaks down to yield the product P, the enzyme is free to catalyze another reaction (Eq. 10.2).

The above curve is described by the Michaelis-Menten equation:

$$V_0 = \frac{V_{max}(S)}{K_m + (S)} \qquad (10.3)$$

where

V_0 = initial reaction velocity,

(S) = initial substrate concentration,

V_{max} = maximum reaction velocity, attained when E–S is at its maximum value,

K_m = Michaelis-Menten constant, the substrate concentration where one-half of the maximum reaction velocity is attained.

This equation shows the quantitative relationship between the initial velocity V_0, the maximum initial velocity V_{max}, and the initial substrate concentration (S), all related through the Michaelis-Menten constant K_m. The effects of enzyme concentration and of substrate concentration on the reaction velocity are often described in terms of the order of the reaction that expresses the reaction rate as a function of the concentration of one or more of the substances present. An enzymatic reaction follows first-order kinetics whenever the substrate concentration is much less than the K_m value for that particular reaction. When the substrate concentration is much greater than the K_m value, the reaction is zero-order with respect to substrate concentration, since all the enzyme molecules are fully saturated with substrate molecules.

This Michaelis-Menten eq. (10.3) can be algebraically transformed into forms that are useful in the practical determination of K_m and V_{max}.

By taking the reciprocal of both sides of the Michaelis-Menten equation:

$$\frac{1}{V_0} = \frac{K_m + (S)}{V_{max}(S)} \text{ and } \frac{1}{V_0} = \frac{K_m}{V_{max}(S)} + \frac{(S)}{V_{max}(S)}$$

and simplifies to

$$\frac{1}{V_0} = \frac{K_m}{V_{max}}\frac{1}{(S)} + \frac{1}{V_{max}}$$

This equation is a transform of the Michaelis-Menten equation called the Lineweaver-Burk equation. For enzymes obeying the Michaelis-Menten relationship, a plot of $1/V_0$ versus 1/(S) yields a straight line as shown in Fig. 10.4.

This double-reciprocal presentation is also called a Lineweaver-Burk plot. The straight line will have a slop of K_m/V_{max}, an intercept of $1/V_{max}$ on the $1/V_0$ axis, and an intercept of $-1/K_m$ on the 1/(S) axis. The K_m value which is sometimes used as an indication of the affinity of an enzyme for its substrate can vary greatly from enzyme to enzyme, therefore, useful for the study and comparison of different enzymes. V_{max} also varies greatly from one enzyme to the next.

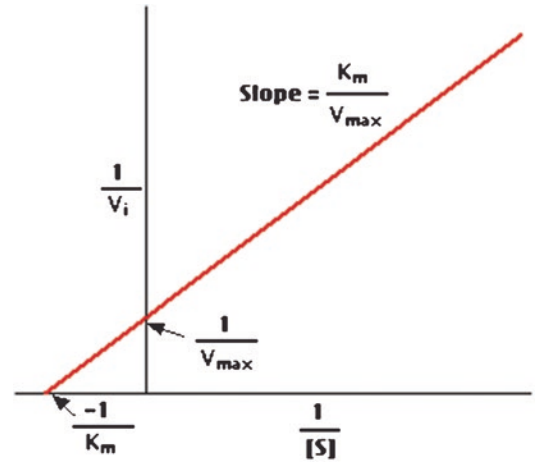
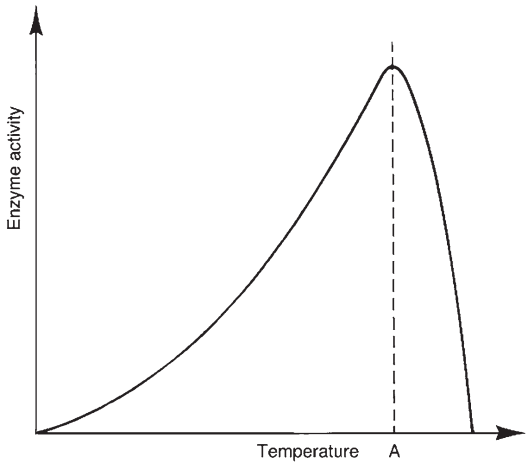


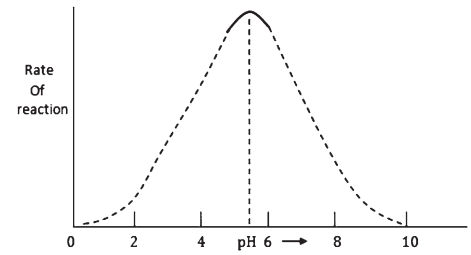
Fig. 10.4 Lineweaver-Burk (or double-reciprocal) plot

Effect of Temperature: Changes in temperature affect the rate of enzyme reaction. It is well known that enzyme-catalyzed reaction occur more slowly when a food is placed in a refrigerator, but the reactions do not stop at 0–4 °C. Most enzyme-catalyzed reactions decrease 1.4–2 time per 10 °C decrease in temperature. The usual effect of temperature on enzymatic reactions is in two stages: (1) the rate of the reaction increases with increasing temperature up to maximum (see the following graph); (2) above this temperature followed by decreasing activity at higher temperatures, due to denaturation of the enzyme.



The increasing temperature at the start produces greater molecular activity, which increases the rate of reaction. The enzyme activity increases with temperature increase such that the reaction velocity is approximately doubled for every 10 °C rise up to 45–50 °C for most enzymes. The temperature coefficient, Q_{10}, is used as an expression of the change in rate of reaction for a 10 °C change in temperature. The value of Q_{10} is determined by dividing the reaction rate at a given temperature plus 10 °C by the reaction rate at that given temperature. At higher temperature, the enzyme begins to denature (loss of activity), followed by rapid thermal inactivation at above 60–75 °C. Some enzymes, such as peroxidase, are much more thermostable than others. The criterion of heat stability is very valuable in characterizing an enzyme and is more pertinent in food processing. Heat-stability studies determine the temperature-time combination required for the inactivation of undesirable enzymes in foods. Some enzymes are known to regenerate when they are cooled following denaturation by heat. Peroxidase in vegetables which has been inactivated by scalding (blanching) can recover at least part of its enzyme activity during frozen storage.

Effect of pH: The hydrogen ion concentration of the medium in which the enzyme works affects greatly the activity of the enzyme because each enzyme works within a small pH range. Therefore, changing the pH of the system by 1 or 2 pH units from the optimum pH, can decrease enzyme velocity to 0.5 or 0.1, respectively, of that at the pH optimum. The greatest reaction velocity is attained at the optimal pH of the enzyme. Most enzymes pH optima lie often within the pH range of 4.5–8.0: β-amylase, 4.8; invertase, 5.0, and pectin methylesterase, 6.5–8.0. Denaturation of the enzyme by high or low pH can result in inactivation. Thus, it is important that pH must be controlled for two main reasons: (1) the enzyme reaction proceeds at a maximum rate at a specific pH and (2) the range of maximum stability of an enzyme also occurs at a definite pH. In food industry, the pH is controlled either to inhibit the enzyme activity or to produce the maximum activity. In fruit and vegetable products, the pH is lowered by the addition of compounds such as citric or phosphoric acids. As shown in the following figure, when the activity of an enzyme is plotted against varying pH values the result is ordinarily a bell-shaped.



Effect of Inhibitors: Some substances reduce or even stop the catalytic activity of enzymes in biochemical reactions. They block or distort the active site. Inhibitors that occupy the active site

and prevent a substrate molecule from binding to the enzyme are said to be active site-directed. It is called competitive inhibitor, as they 'compete' with the substrate for the active site. Competitive inhibitors are often compounds that resemble the substrate and combine with the enzyme. Inhibitors that attach to other parts of the enzyme molecule, perhaps distorting its shape, are said to be non-active site-directed are called noncompetitive inhibitors.

Practical methods of enzyme inhibition that may be used in the food industry are very limited due to problems associated with sensory quality and economic feasibility. In practice, the food industry is limited in enzyme inhibition or control to inactivation by heat, pH, dehydration, high pressure processing or the use of chemicals such as sulfur dioxide and phenolic antioxidants.

Specificity

The nature of the enzyme-substrate reaction as explained in Fig. 10.1 requires that each enzyme reaction is highly specific. The shape and size of the active site of the enzyme, as well as the substrate, are important. But this complementarity may be even further expanded to cover amino acid residues in the vicinity of the active site, hydrophobic areas near the active site, or the presence of a positive electrical charge near the active site (Parkin 1993). Types of specificity may include group, bond, stereo, and absolute specificity, or some combination of these. An example of the specificity of enzymes is given in Fig. 10.5, which illustrates the specificity of proline-specific peptidases (Habibi-Najafi and Lee 1996). The amino acid composition of casein is high in proline, and the location of this amino acid in the protein chain is inaccessible to common aminopeptidases and the di- and tripeptidases with broad specificity. Hydrolysis of the proline bonds requires proline-specific peptidases, including several exopeptidases and an endopeptidase. Figure 10.5 illustrates that this type of specificity is related to the type of amino acid in a protein as well as its location in the chain. Neighboring amino acids also determine the type of peptidase required to hydrolyze a particular peptide bond.

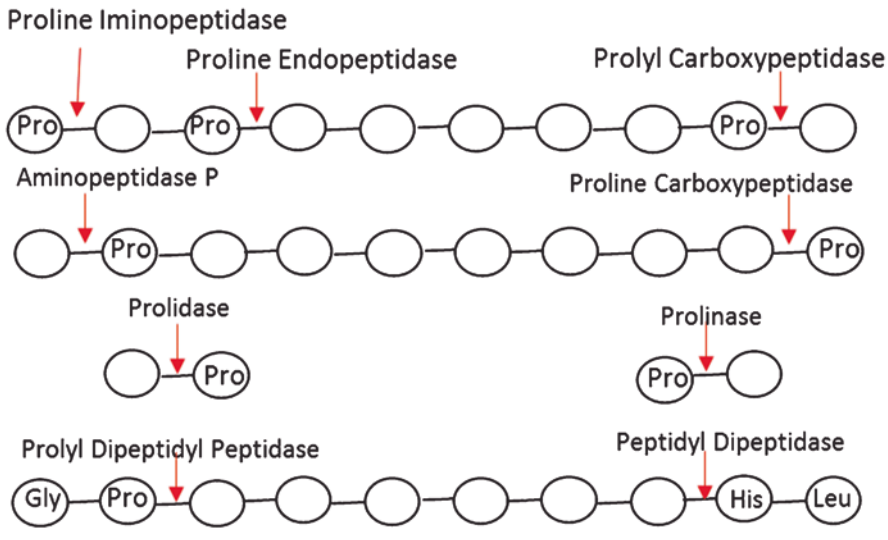


Fig. 10.5 Mode of action of proline-specific peptidases. Adopted from from Habibi-Najafi, M. B., & Lee, B. H. Bitterness in cheese: A review. *Critical Reviews in Food Science and Nutrition*, *36*(5), 408

Classification

Enzymes are classified by the Commission on Enzymes of the International Union of Biochemistry. The basis for the classification is the division of enzymes into groups according to the type of reaction catalyzed. This, together with the name or names of substrate(s), is used to name individual enzymes. Each well-defined enzyme can be described in three ways—by a systematic name, by a trivial name, and by a number of the Enzyme Commission (EC). Thus, the enzyme α-amylase (trivial name) has the systematic name α-l,4-glucan-4-glucanohydrolase, and the number EC 3.2.1.1. The system of nomenclature has been described by Whitaker (1972, 1974) and Parkin (1993).

Enzyme Production

Some of the traditionally used industrial enzymes (e.g., rennet and papain) are prepared from animal and plant sources. Recent developments in industrial enzyme production have emphasized the microbial enzymes (Frost 1986). Microbial enzymes are very heat stable and have a broader pH optimum. Most of these enzymes are made by submerged cultivation of highly developed strains of microorganisms. Developments in biotechnology will make it possible to transfer genes for the elaboration of specific enzymes to different organisms. The major industrial enzyme processes are listed in Table 10.2.

Hydrolases

The hydrolases as a group include all enzymes that involve water in the formation of their products. For a substrate *AB*, the reaction can be represented as follows:

$$AB + HOH \rightarrow HA + BOH$$

The hydrolases are classified on the basis of the type of bond hydrolyzed. The most important are those that act on ester bonds, glycosyl bonds, peptide bonds, and C–N bonds other than peptides.

Esterases

The esterases are involved in the hydrolysis of ester linkages of various types. The products formed are acid and alcohol. These enzymes may hydrolyze triglycerides and include several lipases; for instance, phospholipids are hydrolyzed by phospholipases, and cholesterol esters are hydrolyzed by cholesterol esterase. The carboxylesterases are enzymes that hydrolyze triglycerides such as tributyrin. They can be distinguished from lipases because they hydrolyze soluble substrates, whereas lipases only act at the water-lipid interfaces of emulsions. Therefore, any condition that results in increased surface area of the water-lipid interface will increase the activity of the enzyme. This is the reason that lipase activity is much greater in homogenized (not pasteurized) milk than in the non-homogenized product. Most of the lipolytic enzymes are specific for either the acid or the alcohol moiety of the substrate, and, in the case of esters of polyhydric alcohols, there may also be a positional specificity.

Lipases are produced by microorganisms such as bacteria and molds; are produced by plants; are present in animals, especially in the pancreas; and are present in milk. Lipases may cause spoilage of food because the free fatty acids formed cause rancidity. In other cases, the action of lipases is desirable and is produced intentionally. The boundary between flavor and off-flavor is often a very narrow range. For instance, hydrolysis of milk fat in milk leads to very unpleasant off-flavors at very low free fatty acid concentration. The hydrolysis of milk fat in cheese contributes to the desirable flavor. These differences are probably related to the

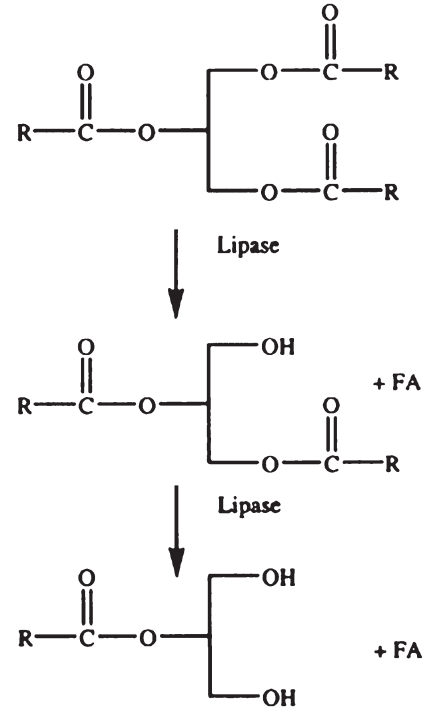
Table 10.2 Major industrial enzymes and the process used for their production

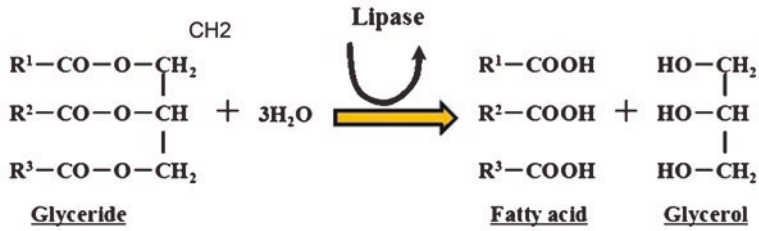
Enzyme	Source	Submerged fermentation	Surface fermentation	Intracellular	Extracellular	Concentration	Precipitation	Drying	Pelleting	Further purification	Solid product	Solution product	Immobilized product
Proteases													
Rennet	Calf stomach	–	–	–	✓	–	–	✓	–	–	✓	✓	–
Trypsin	Animal pancreas	–	–	–	✓	✓	✓	✓	–	✓	✓	–	–
Papain	Carica papaya fruit	–	–	–	✓✓	–	–	✓	–	–	✓	✓	–
Fungal	*Aspergillus oryzae*	✓	✓	–	✓	✓	✓	✓	–	–	✓	–	–
Fungal (rennins)	*Mucor* spp.	✓	✓	–	✓	✓	–	✓	–	–	✓	✓	–
Bacterial	*Bacillus* spp.	✓	–	–	✓	✓	✓	✓	✓	–	✓	✓	–
Glycosidases													
Bacterial α-amylase	*Bacillus* spp.	✓	–	–	✓	✓	–	✓	–	–	✓	✓	–
Fungal α-amylase	*Aspergillus oryzae*	✓	–	–	✓	✓	–	✓	–	–	✓	✓	–
β-amylase	Barley	–	–	–	✓	✓	✓	✓	–	–	✓	–	–
Amyloglucosidase	*Aspergillus niger*	✓	–	–	✓	✓	–	–	–	–	–	✓	–
Pectinase	*Aspergillus niger*	–	✓	–	✓	✓	–	✓	–	–	✓	✓	–
Cellulase	Molds	✓	–	–	✓	✓	✓	–	–	✓	✓	–	–
Yeast lactase	*Kluyveromyces* spp.	✓	–	✓	–	–	✓	✓	–	–	✓	✓	–
Mold lactase	*Aspergillus* spp.	✓	✓	–	✓	–	✓	✓	–	–	✓	–	✓
Others													
Glucose isomerase	Various microbial sources	✓	–	✓	–	✓	✓	✓	–	–	–	–	✓
Glucose oxidase	*Aspergillus niger*	✓	–	✓	–	–	✓	–	–	✓	✓	✓	–
Mold catalase	*Aspergillus niger*	✓	–	✓	–	–	✓	–	–	–	–	✓	–
Animal catalase	Liver	–	–	✓	–	✓	✓	✓	–	✓	✓	✓	–
Lipase	Molds	✓	–	–	✓	✓	✓	✓	–	–	✓	–	–

Source: From Frost, G. M. (1986). Commercial production of enzymes. In B. J. F. Hudson (Ed.), *Developments in food proteins.* Elsevier Applied Science Publishers Ltd

background upon which these fatty acids are superimposed and to the specificity for particular groups of fatty acids of each enzyme. In seeds, lipases may cause fat hydrolysis unless the enzymes are destroyed by heat. Palm oil produced by primitive methods in Africa used to consist of more than 10% of free fatty acids. Such spoilage problems are also encountered in grains and flour. The activity of lipase in wheat and other grains is highly dependent on water content. In wheat, for example, the activity of lipase is five times higher at 15.1% than at 8.8% moisture. The lipolytic activity of oats is higher than that of most other grains.

Lipases can be divided into those that have a positional specificity and those that do not. The former preferentially hydrolyze the ester bonds of the primary ester positions. This results in the formation of mono- and diglycerides, as represented by the following reaction:





During the progress of the reaction, the concentration of diglycerides and monoglycerides increases, as is shown in Fig. 10.6. The β-monoglycerides formed are resistant to further hydrolysis. This pattem is characteristic of pancreatic lipase and has been used to study the triglyceride structure of many fats and oils.

The hydrolysis of triglycerides in cheese is an example of a desirable flavor-producing process. The extent of free fatty acid formation is much higher in blue cheese than in Cheddar cheese, as is shown in Table 10.3. This is most likely the result of lipases elaborated by organisms growing in the blue cheese, such as *P. roqueforti, P. camemberti,* and others. The extent of lipolysis increases with age, as is demonstrated by the increasing content of partial glycerides during the aging of cheese (Table 10.4). In many cases, lipolysis is induced by the addition of lipolytic enzymes. In the North American chocolate industry, it is customary to induce some lipolysis in chocolate by means of lipase. In the production of Italian cheeses, lipolysis is induced by the use of pregastric esterases. These are lipolytic enzymes obtained from the oral glands located at the base of the tongue in calves, lambs, or kids.

Specificity for certain fatty acids by some lipolytic enzymes has been demonstrated. Pancreatic lipase and milk lipase are broad-spectrum enzymes and show no specificity for

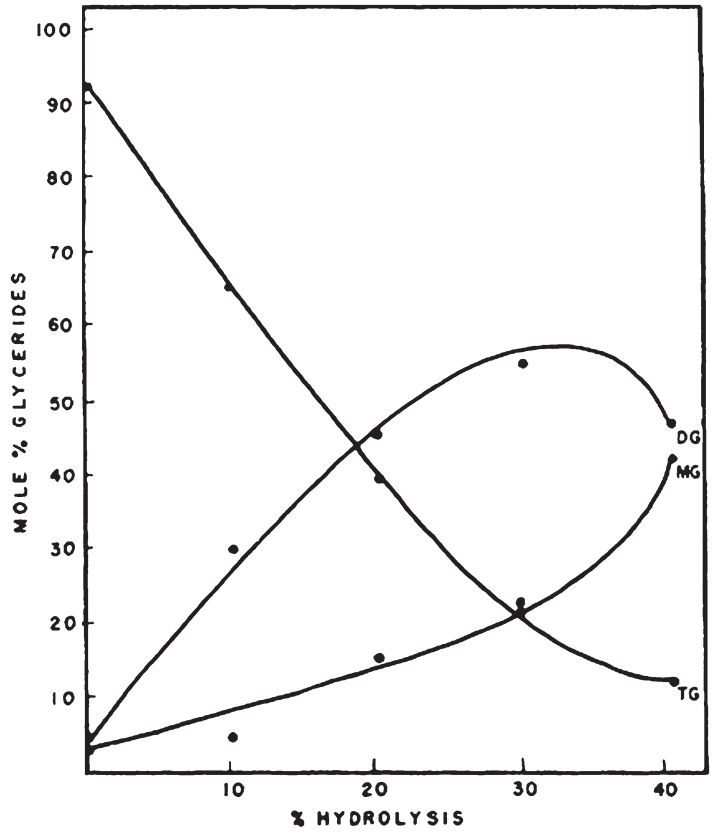


Fig. 10.6 The course of pancreatic lipase hydrolysis of tricaprylin. *MG* monoglycerides, *DG* diglycerides, *TG* triglycerides. *Source*: From Boudreau, A., & deMan, J. M. (1965). The mode of action of pancreatic lipase on milkfat glycerides. *Canadian Journal of Biochemistry*, *43*, 1799–1805

Table 10.3 Free fatty acids in some dairy products

Product	Free fatty acids (mg/kg)
Fresh milk	415
Moderately rancid cream	1027
Butter	2733
Cheddar cheese	1793 (avg. of 12 samples)
Blue cheese	23,500–66,700 (range 3 samples)

Source: From Day, E. A. (1966). Role of milk lipids in flavors of dairy products. In R. F. Gould (Ed.), *Flavor chemistry*. American Chemical Society

Table 10.4 Formation of partial glycerides in cheddar cheese

Product type	Diglycerides (wt %)	Mono-glycerides (wt %)
Mild	7.4–7.6	1.0–2.0
Medium	7.6–9.7	0.5–1.4
Old	11.9–15.6	1.1–3.2

any of the fatty acids found in fats. Instead, the fatty acids that are released from the glycerides occur in about the same ratio as they are present in the original fat. Specificity was shown by Nelson (1972) in calf esterase and in a mixed pancreatin-esterase preparation (Table 10.5). Pregastric esterases and lipase from *Aspergillus* species primarily hydrolyze shorter chain-length fatty acids (Arnold et al. 1975).

Specificity of lipases may be expressed in a number of different ways—substrate specific, regiospecific, nonspecific, fatty acyl specific, and stereospecific. Examples of these specificities have been presented by Villeneuve and Foglia (1997) (Table 10.6).

Substrate specificity is the ability to hydrolyze a particular glycerol ester, such as when a lipase can rapidly hydrolyze a triacylglycerol, but acts on a monoacylglycerol only slowly. Regiospecificity involves a specific action on

Table 10.5 Free fatty acids released from milkfat by several lipolytic enzymes

Fatty acid	Milk lipase	Steapsin	Pancreatic lipase	Calf esterase	Esterase pancreatin
4:0	13.9	10.7	14.4	35.00	15.85
6:0	2.1	2.9	2.1	2.5	3.6
8:0	1.8	1.5	1.4	1.3	3.0
10:0	3.0	3.7	3.3	3.1	5.5
12:0	2.7	4.0	3.8	5.1	4.4
14:0	7.7	10.7	10.1	13.2	8.5
16:0	21.6	21.6	24.0	15.9	19.3
18:1 and 18:2	29.2	24.3	25.5	14.2	21.1
18:0	10.5	13.4	9.7	3.2	10.1

Source: From Nelson, J. H. (1972). Enzymatically produced flavors for fatty systems. Journal of the American Oil Chemists' Society, *49*, 559–562

Table 10.6 Examples of lipase specificities

Specificity	Lipase
Substrate specific	
Monoacylglyercols	Rat adipose tissue
Mono- and diacylglycerols	*Penicillium camembertii*
Triacylglycerols	*Penicillium* sp.
Regiospecific	
1,3-regioselective	*Aspergilllus niger*
	Rhizopus arrhizus
	Mucor miehei
sn-2-regioselective	*Candida antarctica* A
Nonspecific	*Penicillium expansum*
	Aspergillus sp.
	Pseudomonas cepacia
Fatty acylspecific	
Short-chain fatty acid (FA)	*Penicillium roqueforti*
	Premature infant gastric
cis-9 unsaturated FA	*Geotrichum candidum*
Long-chain unsaturated FA	*Botrytis cinerea*
Stereospecific	
sn-1 stereospecific	*Humicola lanuginosa*
	Pseudomonas aeruginosa
sn-3 stereospecific	*Fusarium solani* cutinase
	Rabbit gastric

Source: Reprinted with permission from Villeneuve, R., & Foglia, T. A. Lipase specificities: Potential application in lipid bioconversions. Journal of the American Oil Chemists' Society, *8*, 641, © 1997, AOCS Press

either the sn-1 and sn-3 positions or reaction with only the sn-2 position. The 1,3-specific enzymes have been researched extensively, because it is now recognized that lipases in addition to hydrolysis can catalyze the reverse reaction, esterification or transesterification. This has opened up the possibility of tailor-making triacylglycerols with a specific structure, and this is especially important for producing high-value fats such as cocoa butter equivalents. The catalytic activity of lipases is reversible and depends on the water content of the reaction mixture. At high water levels, the hydrolytic reaction prevails, whereas at low water levels the synthetic reaction is favored. A number of lipase catalyzed reactions are possible, and these have been summarized in Fig. 10.7 (Villeneuve and Foglia 1997). Most of the lipases used for industrial processes have been developed from microbes because these usually exhibit high temperature tolerance. Lipases from *Mucor miehei* and *Candida antarctica* have been cloned and expressed in industry-friendly organisms. Lipases from genetically engineered strains will likely be of major industrial importance in the future (Godtfredsen 1993). Fatty acid–specific lipases react with either short-chain fatty acids (*Penicillium roqueforti*) or some long-chain fatty acids such as *cis*-9-unsaturated fatty acids (*Geotrichum candidum*). Stereospecific lipases react with only fatty acids at the sn-1 or sn-3 position.

The applications of microbial lipases in the food industry involve the hydrolytic as well as the synthetic capabilities of these enzymes and have been summarized by Godtfredsen (1993) in Table 10.7.

The lipase-catalyzed interesterification process can be used for the production of triacylglycerols with specific physical properties, and it also opens up possibilities for making so-called

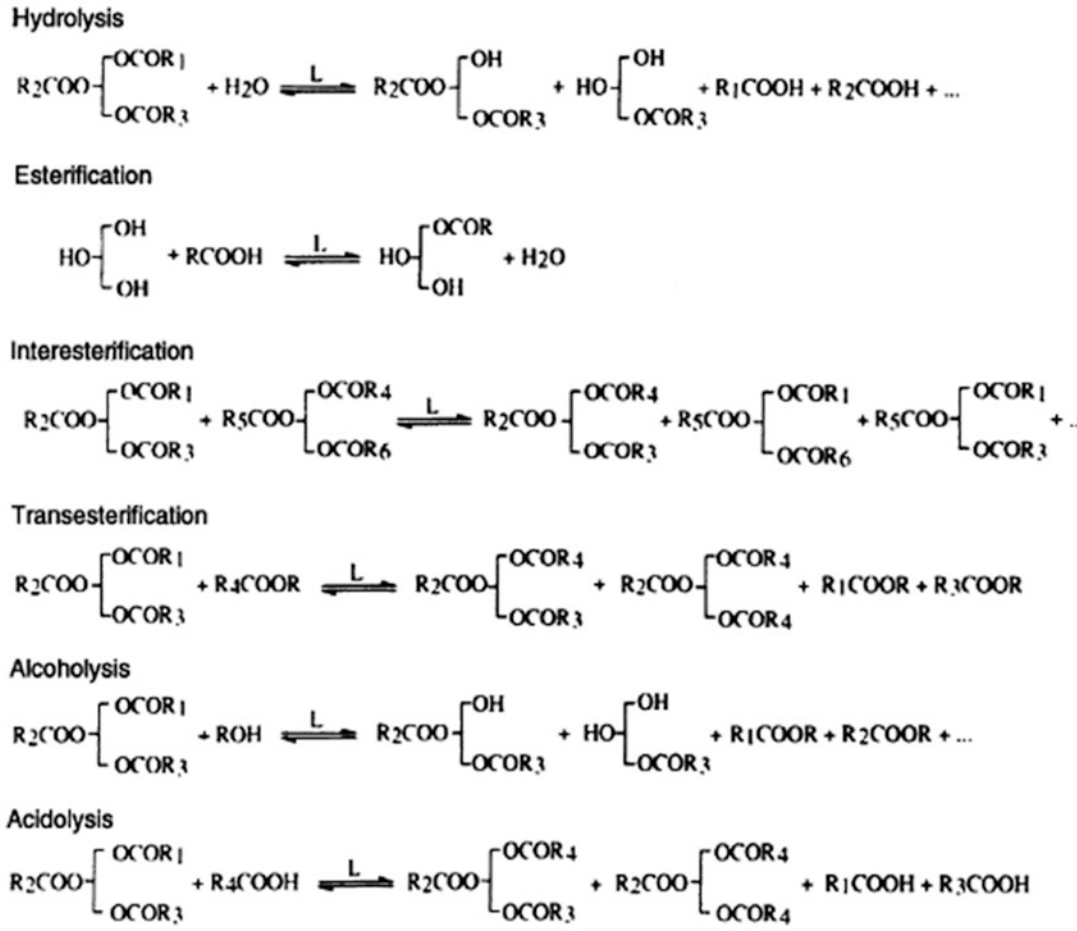


Fig. 10.7 Lipase catalyzed reactions used in oil and fat modification. *Source:* Reprinted with permission from Villeneuve, R., & T. A. Foglia. Lipase specificities: Potential application in lipid bioconversions. *Journal of the American Oil Chemists' Society*, *8*, 642, © 1997, AOCS Press

Table 10.7 Application of microbial lipases in the food industry

Industry	Effect	Product
Dairy	Hydrolysis of milk fat	Flavor agents
	Cheese ripening	Cheese
	Modification of butter fat	Butter
Bakery	Flavor improvement and shelf-life prolongation	Bakery products
Beverage	Improved aroma	Beverages
Food dressing	Quality improvement	Mayonnaise, dressing, and whipped toppings
Health food	Transesterification	Health foods
Meat and fish	Flavor development and fat removal	Meat and fish products
Fat and oil	Transesterification	Cocoa butter, margarine
	Hydrolysis	Fatty acids, glycerol, mono- and diglycerides

Source: Reprinted with permission from Godtfredsen, S. E. Lipases, enzymes in food processing. T. Nagodawithana and G. Reed (Eds.), p. 210, © 1993, Academic Press

structured lipids (Akoh 1997). An example is a triacylglycerol that carries an essential fatty acid (e.g., DHA-docosahexaenoic acid) in the sn-2 position and short-chain fatty acids in the sn-1 and sn-3 positions. Such a structural triacylglycerol would rapidly be hydrolyzed in the digestive tract and provide an easily absorbed monoacylglycerol that carries the essential fatty acid (Godtfredsen 1993).

The lipases that have received attention for their ability to synthesize ester bonds have been obtained from yeasts, bacteria, and fungi. Lipases can be classified into three groups according to their specificity (Macrae 1983). The first group contains nonspecific lipases. These show no specificity regarding the position of the ester bond in the glycerol molecule, or the nature of the fatty acid. Examples of enzymes in this group are lipases of *Candida cylindracae, Corynebacterium acnes,* and *Staphylococcus aureus.* The second group contains lipases with position specificity for the 1- and 3-positions of the glycerides. This is common among microbial lipases and is the result of the sterically hindered ester bond of the 2-position's inability to enter the active site of the enzyme. Lipases in this group are obtained from *Aspergillus niger, Mucor javanicus,* and *Rhizopus arrhizus.* The third group of lipases show specificity for particular fatty acids. An example is the lipase from *Geotrichum candidum,* which has a marked specificity for long-chain fatty acids that contain a *cis* double bond in the 2-position. The knowledge of the synthetic ability of lipases has opened a whole new area of study in the modification of fats. The possibility of modifying fats and oils by immobilized lipase technology may result in the production of food fats that have a higher essential fatty acid content and lower *trans* levels than is possible with current methods of hydrogenation.

Amylases

The amylases are the most important enzymes of the group of glycoside hydrolases. These starch-degrading enzymes can be divided into two groups, the so-called debranching enzymes that specifically hydrolyze the 1,6-linkages between chains, and the enzymes that split the 1,4-linkages between glucose units of the straight chains. The latter group consists of endoenzymes that cleave the bonds at random points along the chains and exoenzymes that cleave at specific points near the chain ends. This behavior has been represented by Marshall (1975) as a diagram of the structure of amylopectin (Fig. 10.8). In this molecule, the 1,4-α-glucan chains are interlinked by 1,6-α-glucosidic linkages resulting in a highly branched molecule. The molecule is com posed of three types of chains; the *A* chains carry no substituent, the *B* chains carry other chains linked to a primary hydroxyl group, and the molecule contains only one *C* chain with a free reducing glucose unit. The chains are 25–30 units in length in starch and only 10 units in glycogen.

Alpha-Amylase (α-1,4-Glucan 4-Glucanohydrolase)

This enzyme is distributed widely in the animal and plant kingdoms. The enzyme contains 1 gram-atom of calcium per mole. Alpha-amylase (α-1,4-glucan-4-glucanohydrolase) is an endoenzyme that hydrolyzes the α-l,4-glucosidic bonds in a random fashion along the chain. It hydrolyzes amylopectin to oligosaccharides that contain two to six glucose units. This action, therefore, leads to a rapid decrease in viscosity, but little monosaccharide formation. A mixture of amylose and amylopectin will be hydrolyzed into a mixture of dextrins, maltose, glucose, and oligosaccharides. Amylose is completely hydrolyzed to maltose, although there usually is some maltotriose formed, which hydrolyzes only slowly.

Beta-Amylase (α-1,4-Glucan Maltohydrolase)

This is an exoenzyme and removes successive maltose units from the nonreducing end of the glucosidic chains. The action is stopped at the branch point where the α-1,6 glucosidic linkage cannot be broken by α-amylase. The resulting compound is named *limit dextrin.* Beta-amylase is found only in higher plants. Barley malt, wheat, sweet potatoes, and soybeans are good sources.

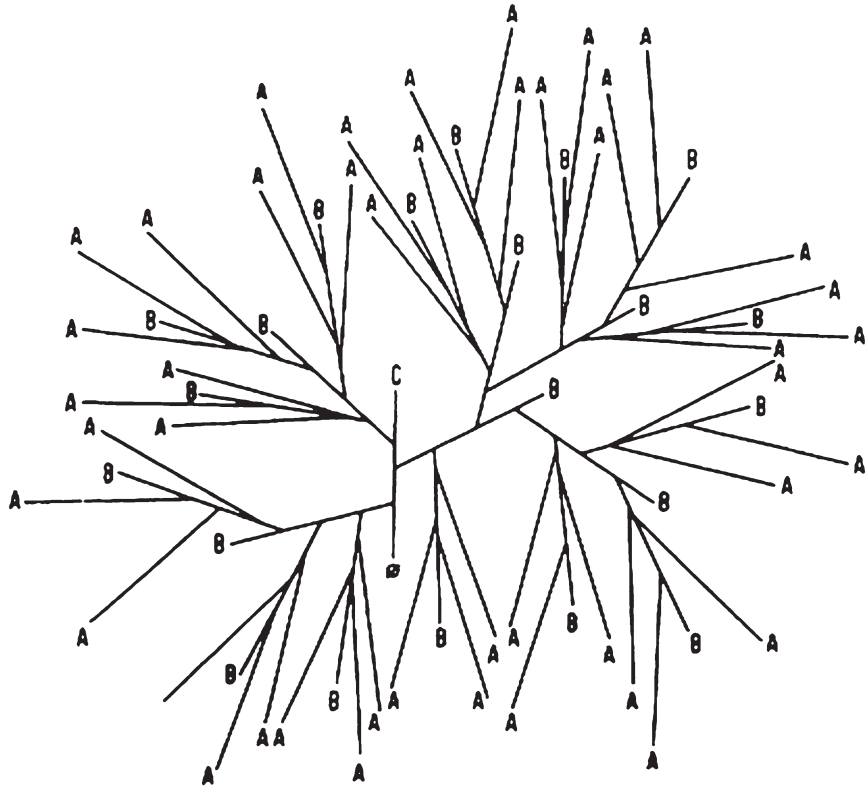


Fig. 10.8 Diagrammatic representation of amylopectin structure. Lines represent α-D-glucan chains linked by 1,4-bonds. The branch points are 1,6-α glucosidic bonds. *Source:* From Marshall, J. J. (1975). Starch degrading enzymes, old and new. *Starke*, *27*, 377–383

Beta-amylase is technologically important in the baking, brewing, and distilling industries, where starch is converted into the fermentable sugar maltose. Yeast ferments maltose, sucrose, invert sugar, and glucose but does not ferment dextrins or oligosaccharides containing more than two hexose units.

Glucoamylase (α-1,4-Glucan Glucohydrolase)

This is an exoenzyme that removes glucose units in a consecutive manner from the nonreducing end of the substrate chain. The product formed is glucose only, and this differentiates this enzyme from α- and β-amylase. In addition to hydrolyzing the α-1,4 linkages, this enzyme can also attack the α-1,6 linkages at the branch point, albeit at a slower rate. This means that starch can be completely degraded to glucose. The enzyme is present in bacteria and molds and is used industrially in the production of corn syrup and glucose.

A problem in the enzymic conversion of corn starch to glucose is the presence of transglucosidase enzyme in preparations of α-amylase and glucoamylase. The transglucosidase catalyzes the formation of oligosaccharides from glucose, thus reducing the yield of glucose.

Nondamaged grains such as wheat and barley contain very little α-amylase but relatively high levels of β-amylase. When these grains germinate, the β-amylase level hardly changes, but the α-amylase content may increase by a factor of 1000. The combined action of α- and β-amylase in the germinated grain greatly increases the production of fermentable sugars. The development

Table 10.8 Development of α-amylase during malting of barley at 20 °C

Days of steeping and germination	α-Amylase (20° dextrose units)
0	0
3	55
5	110
7	130
8	135

Source: From Green, S. R. (1969). New use of enzymes in the brewing industry. *MBAA Technical Quarterly, 6*, 33–39

of α-amylase activity during malting of barley is shown in Table 10.8. In wheat flour, high α-amylase activity is undesirable, because too much carbon dioxide is formed during baking.

Raw, nondamaged, and ungelatinized starch is not susceptible to β-amylase activity. In contrast, α-amylase can slowly attack intact starch granules. This differs with the type of starch; for example, waxy corn starch is more easily attacked than potato starch. In general, extensive hydrolysis of starch requires gelatinization. Damaged starch granules are more easily attacked by amylases, which is important in bread making Alphaamylase can be obtained from malt, from fungi (*Aspergillus oryzae*), or from bacteria (*B. subtilis*). The bacterial amylases have a higher temperature tolerance than the malt amylases.

Beta-Galactosidase (β-D-Galactoside Galactohydrolase)

This enzyme catalyzes the hydrolysis of β-D-galactosides and α-L-arabinosides. It is best known for its action in hydrolyzing lactose and is, therefore, also known as lactase. The enzyme is widely distributed and occurs in higher animals, bacteria, yeasts, and plants. Beta-galactosidase or lactase is found in humans in the cells of the intestinal mucous membrane. A condition that is widespread in non-Caucasian adults is characterized by an absence of lactase. Such individuals are said to have lactose intolerance, which is an inability to digest milk properly.

The presence of galactose inhibits lactose hydrolysis by lactase. Glucose does not have this effect.

Pectic Enzymes

The pectic enzymes are capable of degrading pectic substances and occur in higher plants and in microorganisms. They are not found in higher animals, with the exception of the snail. These enzymes are commercially important for the treatment of fruit juices and beverages to aid in filtration and clarification and increasing yields. The enzymes can also be used for the production of low methoxyl pectins and galacturonic acids. The presence of pectic enzymes in fruits and vegetables can result in excessive softening. In tomato and fruit juices, pectic enzymes may cause "cloud" separation.

There are several groups of pectic enzymes, including pectinesterase, the enzyme that hydrolyzes methoxyl groups, and the depolymerizing enzymes polygalacturonase and pectate lyase.

Pectinesterase (Pectin Pectyl-Hydrolase)

This enzyme removes methoxyl groups from pectin. The enzyme is referred to by several other names, including pectase, pectin methoxylase, pectin methyl esterase, and pectin demethylase. Pectinesterases are found in bacteria, fungi, and higher plants, with very large amounts occurring in citrus fruits and tomatoes. The enzyme is specific for galacturonide esters and will not attack non-galacturonide methyl esters to any large extent. The reaction catalyzed by pectin esterase is presented in Fig. 10.9. It has been suggested that the distribution of methoxyl groups along the chain affects the reaction velocity of the enzyme (MacMillan and Sheiman 1974). Apparently, pectinesterase requires a free carboxyl group next to an esterified group on the galacturonide chain to act, with the pectinesterase moving down the chain linearly until an obstruction is reached.

To maintain cloud stability in fruit juices, high-temperature–short-time (HTST) pasteurization is used to deactivate pectolytic enzymes. Pectin is a protective colloid that helps to keep insoluble particles in suspension. Cloudiness is required in commercial products to provide a desirable appearance. The destruction of the high levels of pectinesterase during the production of

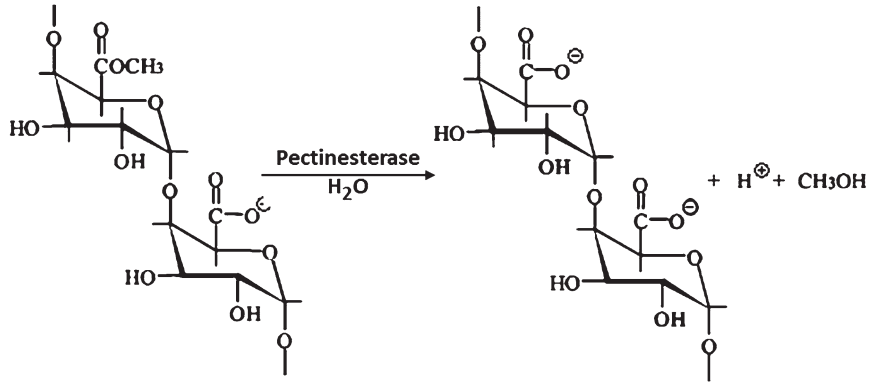


Fig. 10.9 Reaction catalyzed by pectinesterase

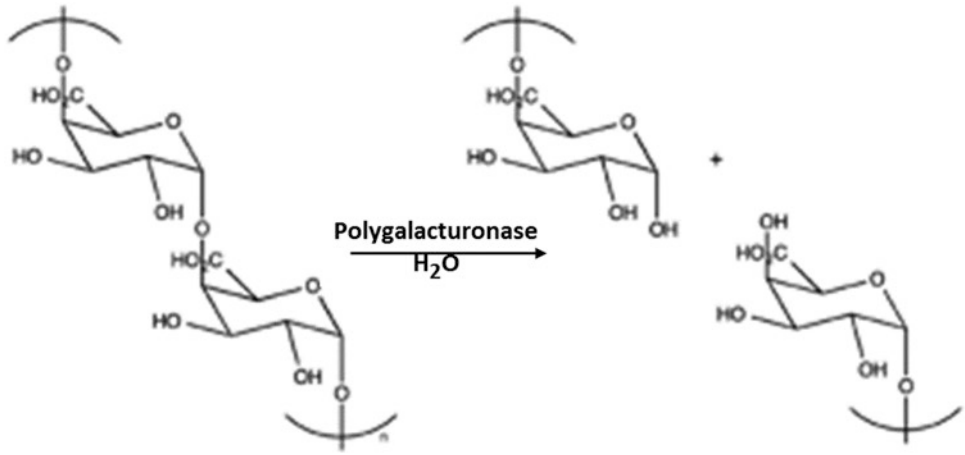


Fig. 10.10 Reaction catalyzed by polygalacturonase

tomato juice and puree is of vital importance. The pectinesterase will act quite rapidly once the tomato is broken. In the so-called hot-break method, the tomatoes are broken up at high temperature so that the pectic enzymes are destroyed instantaneously.

Polygalacturonase (Poly-α-1,4-Galacturonide Glycanohydrolase)

This enzyme is also known as pectinase, and it hydrolyzes the glycosidic linkages in pectic substances according to the reaction pattern shown in Fig. 10.10. The polygalacturonases can be divided into endoenzymes that act within the molecule on α-1,4 linkages and exoenzymes that catalyze the stepwise hydrolysis of galacturonic acid molecules from the nonreducing end of the chain. A further division can be made by the fact that some polygalacturonases act principally on methylated substrates (pectins), whereas others act on substrates with free carboxylic acid groups (pectic acids). These enzymes are named polymethyl galacturonases and polygalacturonases, respectively. The preferential mode of hydrolysis and the preferred substrates are listed in Table 10.9. Endopolygalacturonases occur in fruits and in filamentous fungi, but not in yeast or bacteria. Exopolygalacturonases occur in plants (for example, in carrots and peaches), fungi, and bacteria.

Table 10.9 Action of polygalacturonases

Type of attack	Enzyme	Preferred substrate
Random	Endo-polymethylgalacturonase	Pectin
Random	Endo-polygalacturonase	Pectic acid
Terminal	Exo-polymethylgalacturonase	Pectin
Terminal	Exo-polygalacturonase	Pectic acid

Pectate Lyase (Poly-α-l,4-D-Galacturonide Lyase)

This enzyme is also known as *trans*-eliminase; it splits the glycosidic bonds of a glucuronide chain by *trans* elimination of hydrogen from the 4- and 5-positions of the glucuronide moiety. The reaction pattern is presented in Fig. 10.11. The glycosidic bonds in pectin are highly susceptible to this reaction. The pectin lyases are of the endotype and are obtained exclusively from filamentous fungi, such as *Aspergillus niger*. The purified enzyme has an optimum pH of 5.1 to 5.2 and isoelectric point between 3 and 4 (Albersheim and Kilias 1962).

Commercial Use

Pectic enzymes are used commercially in the clarification of fruit juices and wines and for aiding the disintegration of fruit pulps. By reducing the large pectin molecules into smaller units and eventually into galacturonic acid, the compounds become water soluble and lose their suspending power; also, their viscosity is reduced and the insoluble pulp particles rapidly settle out.

Most microorganisms produce at least one but usually several pectic enzymes. Almost all fungi and many bacteria produce these enzymes, which readily degrade the pectin layers holding plant cells together. This leads to separation and degradation of the cells, and the plant tissue becomes soft. Bacterial degradation of pectin in plant tissues is responsible for the spoilage known as "soft rot" in fruits and vegetables. Commercial food grade pectic enzyme preparations may contain several different pectic enzymes. Usually, one type predominates; this depends on the intended use of the enzyme preparation.

Proteases

Proteolytic enzymes are important in many industrial food processing procedures. The reaction catalyzed by proteolytic enzymes is the hydrolysis of peptide bonds of proteins; this reaction is shown in Fig. 10.12. Whitaker (1972) has listed the specificity requirements for the hydrolysis of peptide bonds by proteolytic enzymes. These include the nature of R_1, and R_2 groups, configuration of the amino acid, size of substrate molecule, and the nature of the *X* and *Y* groups. A major distinguishing factor of proteolytic enzymes is the effect of R_1, and R_2 groups. The enzyme α-chymotrypsin hydrolyzes peptide bonds readily only when R_1 is part of a tyrosyl, phenylalanyl, or tryptophanyl residue. Trypsin requires R_1 to belong to an arginyl or lysyl residue. Specific requirement for the R_2 groups is exhibited by pepsin and the carboxypeptidases; both require R_2 to belong to a phenylalanyl residue. The enzymes

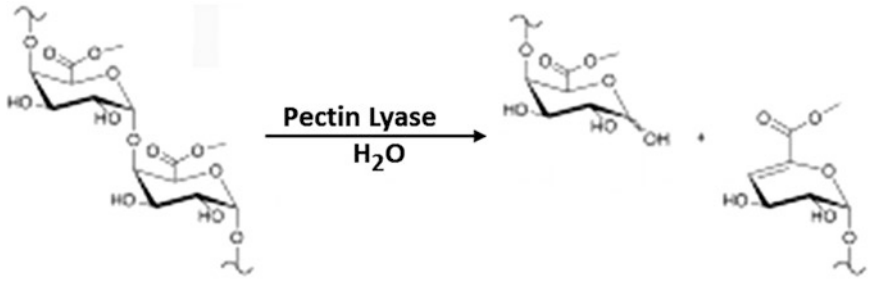


Fig. 10.11 Reaction catalyzed by pectin lyase

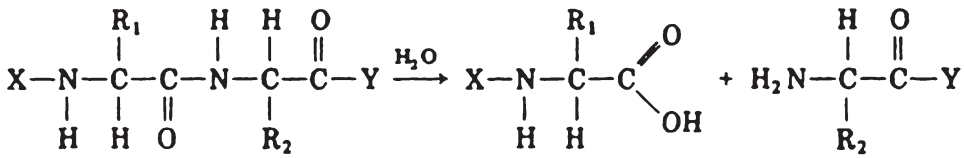


Fig. 10.12 Reaction catalyzed by proteases

require the amino acids of proteins to be in the L-configuration but frequently do not have a strict requirement for molecular size. The nature of *X* and *Y* permits the division of proteases into endopeptidases and exopeptidases. The former split peptide bonds in a random way in the interior of the substrate molecule and show maximum activity when *X* and *Y* are derived. The carboxypeptidases require that *Y* be a hydroxyl group, the aminopeptidases require that *X* be a hydrogen, and the dipeptidases require that *X* and *Y* both be underived.

Proteolytic enzymes can be divided into the following four groups: the acid proteases, the serine proteases, the sulfhydryl proteases, and the metal-containing proteases.

Acid Proteases

This is a group of enzymes with pH optima at low values. Included in this group are pepsin, rennin (chymosin), and a large number of microbial and fungal proteases. Rennin, the pure enzyme contained in rennet, is an extract of calves' stomachs that has been used for thousands of years as a coagulating agent in cheese making. Because of the scarcity of calves' stomachs, rennet substitutes are now widely used, and the coagulants used in cheese making usually contain mixtures of rennin and pepsin and/or microbial proteases. Some of the microbial proteases have been used for centuries in the Far East in the production of fermented foods such as soy sauce.

Rennin is present in the fourth stomach of the suckling calf. It is secreted in an inactive form, a zymogen, named prorennin. The crude extract obtained from the dried stomachs (vells) contains both rennin and prorennin. The conversion of prorennin to rennin can be speeded up by addition of acid. This conversion involves an autocatalytic process, in which a limited proteolysis of the prorennin occurs, thus reducing the molecular weight about 14 percent. The conversion can also be catalyzed by pepsin. The process involves the release of peptides from the N-terminal end of prorennin, which reduces the molecular weight from about 36,000 to about 31,000. The molecule of prorennin consists of a single peptide chain joined internally by three disulfide bridges. After conversion to rennin, the disulfide bridges remain intact. As the calves grow older and start to eat other feeds as well as milk, the stomach starts to produce pepsin instead of rennin. The optimum activity of rennin is at pH 3.5, but it is most stable at pH 5; the clotting of cheese milk is carried out at pH values of 5.5–6.5.

The coagulation or clotting of milk by rennin occurs in two stages. In the first, the enzymic stage, the enzyme acts on κ-casein so that it can no longer stabilize the casein micelle. The second, or nonenzymic stage, involves the clotting of the modified casein micelles by calcium ions. The enzymic stage involves a limited and specific action on the κ-casein, resulting in the formation of insoluble *para*-κ-casein and a soluble macropeptide. The latter has a molecular weight of 6000–8000, is extremely hydrophilic, and contains about 30 percent carbohydrate. The glycomacropeptide contains galactosamine, galactose, and *N*-acetyl neuraminic acid (sialic acid). The splitting of the glycomacropeptide from κ-casein involves the breaking of a phenylalanine-methionine bond in the peptide chain. Other clotting enzymes—including pepsin, chymotrypsin, and microbial proteases—break the same bond and produce the same glycomacropeptide.

Pepsin is elaborated in the mucosa of the stomach lining in the form of pepsinogen. The high acidity of the stomach aids in the autocatalytic conversion into pepsin. This conversion involves splitting several peptide fragments from the N-terminal end of pepsinogen. The fragments consist of one large peptide and several small ones. The large peptide remains associated with pepsinogen by noncovalent bonds and acts as an inhibitor. The inhibitor dissociates from pepsin at a pH of 1–2. In the initial stages of the conversion of pepsinogen to pepsin, six peptide bonds are broken, and continued action on the large peptide (Fig. 10.13) results in three more bonds being hydrolyzed. In this process, the molecular weight changes from 43,000 to 35,000 and the isoelectric point changes from 3.7 to less than 1. The pepsin molecule consists of a single polypeptide chain that contains 321 amino acids. The tertiary structure is stabilized by three disulfide bridges and a phosphate linkage. The phosphate group is

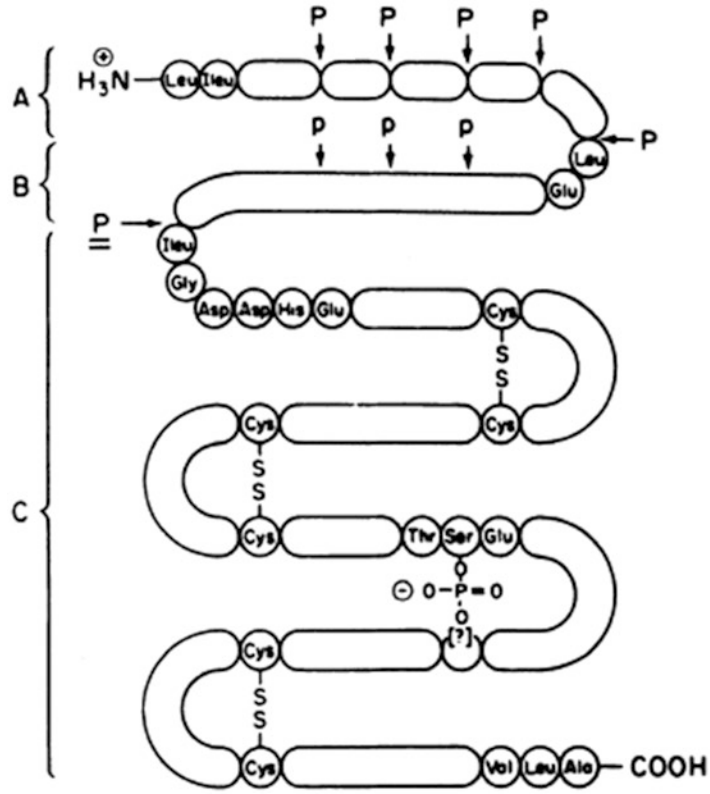


Fig. 10.13 Structure of pepsinogen and its conversion to pepsin. *Source:* From Bovey, F. A., & Yanari, S. S. (1960). Pepsin. In P. D. Boyer et al. (Eds.) *The enzymes* (vol. 4). Academic Press

attached to a seryl residue and is not essential for enzyme activity. The pH optimum of pepsin is pH 2 and the enzyme is stable from pH 2–5. At higher pH values, the enzyme is rapidly denatured and loses its activity. The primary specificity of pepsin is toward the R_2 group (see the equation shown in Fig. 10.12), and it prefers this to be a phenylalanyl, tyrosyl, or tryptophanyl group.

The use of other acid proteases as substitutes for rennin in cheese making is determined by whether bitter peptides are formed during ripening of the cheese and by whether initial rapid hydrolysis causes excessive protein losses in the whey. Some of the acid proteases used in cheese making include preparations obtained from the organisms *Endothia parasitica, Mucor miehei,* and *Mucor pusillus.* Rennin contains the enzyme chymosin, and the scarcity of this natural enzyme preparation for cheese making resulted in the use of pepsin for this purpose. Pepsin and chymosin have primary structures that have about 50% homology and quite similar tertiary structures. The molecular mass of the two enzymes is similar, 35 kDa, but chymosin has a higher pI. Much of the chymosin used in cheese making is now obtained by genetic engineering processes. In the production of soy sauce and other eastern food products, such as miso (an oriental fermented food) and ketjap (Indonesian type soy sauce), the acid proteases of *Aspergillus oryzae* are used. Other products involve the use of the fungus *Rhizopus oligosporus.* Acid proteases also play a role in the ripening process of a variety of soft cheeses. This includes the *Penicillia* used in the blue cheeses, such as Roquefort, Stilton, and Danish blue, and in Camembert and Brie. The molds producing the acid proteases may grow either on the surface of the cheese or throughout the body of the cheese.

Serine Proteases

This group includes the chymotrypsins, trypsin, elastase, thrombin, and subtilisin. The name of this group of enzymes refers to the seryl residue

that is involved in the active site. As a consequence, all of these enzymes are inhibited by diisopropylphosphorofluoridate, which reacts with the hydroxyl group of the seryl residue. They also have an imidazole group as part of the active site and they are all endopeptides. The chymotrypsins, trypsin and elastase, are pancreatic enzymes that carry out their function in the intestinal tract. They are produced as inactive zymogens and are converted into the active form by limited proteolysis.

Sulfhydryl Proteases

These enzymes obtain their name from the fact that a sulfhydryl group in the molecule is essential for their activity. Most of these enzymes are of plant origin and have found widespread use in the food industry. The only sulfhydryl proteases of animal origin are two of the cathepsins, which are present in the tissues as intracellular enzymes. The most important enzymes of this group are papain, ficin, and bromelain. Papain is an enzyme present in the fruit, leaves, and trunk of the papaya tree (*Carica papaya*). The commercial enzyme is obtained by purification of the exudate of full-grown but unripe papaya fruits. The purification involves use of affinity chromatography on a column containing an inhibitor (Liener 1974). This process leads to the full activation of the enzyme, which then contains 1 mole of sulfhydryl per mole of protein. The crude papain is not fully active and contains only 0.5 mole of sulfhydryl per mole of protein. Bromelain is obtained from the fruit or stems of the pineapple plant (*Ananas comosus*). The stems are pressed and the enzyme precipitated from the juice by acetone. Ficin is obtained from the latex of tropical fig trees (*Ficus glabrata*). The enzyme is not homogeneous and contains at least three different proteolytic components.

The active sites of these plant enzymes contain a cysteine and a histidine group that are essential for enzyme activity. The pH optimum is fairly broad and ranges from 6 to 7.5 The enzymes are heat stable up to temperatures in the range of 60–80 °C. The papain molecule consists of a single polypeptide chain of 212 amino acids. The molecular weight is 23,900. Ficin and bromelain contain carbohydrate in the molecule; papain does not. The molecular weights of the enzymes are quite similar; that of ficin is 25,500 and that of bromelain, 20,000–33,200. These enzymes catalyze the hydrolysis of many different compounds, including peptide, ester, and amide bonds. The variety of peptide bonds split by papain appears to indicate a low specificity. This has been attributed (Liener 1974) to the fact that papain has an active site consisting of seven subsites that can accommodate a variety of amino acid sequences in the substrate. The specificity in this case is not determined by the nature of the side chain of the amino acid involved in the susceptible bond but rather by the nature of the adjacent amino acids.

Commercial use of the sulfhydryl proteases includes stabilizing and chill proofing of beer. Relatively large protein fragments remaining after the malting of barley may cause haze in beer when the product is stored at low temperatures. Controlled proteolysis sufficiently decreases the molecular weight of these compounds so that they will remain in solution. Another important use is in the tenderizing of meat. This can be achieved by injecting an enzyme solution into the carcass or by applying the enzyme to smaller cuts of meat. The former method suffers from the difficulty of uneven proteolysis in different parts of the carcass with the risk of overtenderizing some parts of the carcass.

Metal-Containing Proteases

These enzymes require a metal for activity and are inhibited by metal-chelating compounds. They are exopeptidases and include carboxypeptidase A (peptidyl-L-amino-acid hydrolase) and B (peptidyl-L-lysine hydrolase), which remove amino acids from the end of peptide chains that carry a free α-carboxyl group. The aminopeptidases remove amino acids from the free α-amino end of the peptide chain. The metalloexopeptidases require a divalent metal as a cofactor; the carboxypeptidases contain zinc. These enzymes are quite specific in the action; for example, carboxypeptidase B requires the C-terminal amino acid to be either arginine or lysine; the requirement for carboxypeptidase A is phenylalanine, tryptophan, or isoleucine. These specificities are compared with those of some other proteolytic

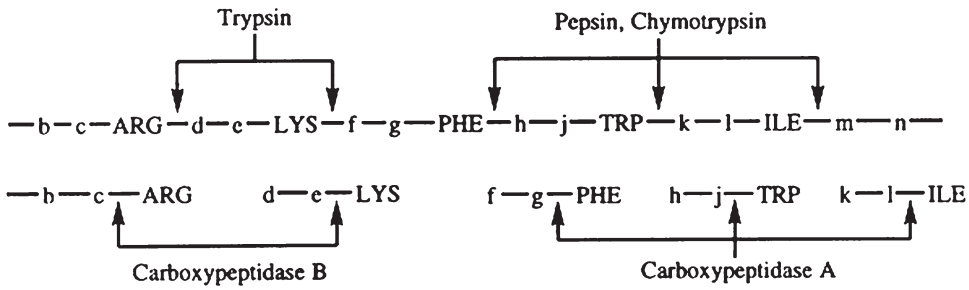


Fig. 10.14 Specificity of some proteolytic enzymes

enzymes in Fig. 10.14. The carboxypeptidases are relatively small molecules; molecular weight of carboxypeptidase A is 34,600. The amino peptidases have molecular weights around 300,000. Although many of the aminopeptidases are found in animal tissues, several are present in microorganisms (Riordan 1974).

Protein Hydrolysates

Protein hydrolysates is the name given to a family of protein breakdown products obtained by the action of enzymes. It is also possible to hydrolyze proteins by chemical means, acids, or alkali, but the enzymatic method is preferred. Many food products such as cheese and soy sauce are obtained by enzymatic hydrolysis. The purpose of the production of protein hydrolysates is to improve nutritional value, cost, taste, antigenicity, solubility, and functionality. The proteins most commonly selected for producing hydrolysates are casein, whey protein, and soy protein (Lahl and Braun 1994). Proteins can be hydrolyzed in steps to yield a series of proteoses, peptones, peptides, and finally amino acids (Table 10.10). These products should not be confused with hydrolyzed vegetable proteins, which are intended as flavoring substances.

The extent of hydrolysis of protein hydrolysates is measured by the ratio of the amount of amino nitrogen to the total amount of nitrogen present in the raw material (AN/TN ratio). Highly hydrolyzed materials have AN/TN ratios of 0.50–0.60. To obtain the desired level of hydrolysis in a protein, a combination of proteases is selected. Serine protease prepared from *Bacillus licheniformis* has broad specificity and some preference for terminal hydrophobic amino acids. Peptides containing terminal hydrophobic amino acids cause bitterness. Usually a mixture of different proteases is employed. The hydrolysis reaction is terminated by adjusting the pH and increasing the temperature to inactivate the enzymes. The process for producing hydrolysates is shown in Fig. 10.15 (Lahl and Braun 1994). Protein hydrolysates can be used as food ingredients with specific functional properties or for physiological or medical reasons. For example, hydrolyzed proteins may lose allergenic properties by suitably arranged patterns of hydrolysis (Cordle 1994).

Table 10.10 Protein hydrolysate products produced from casein and whey protein concentrate (WPC)

Hydrolysate[a]	Protein source	Average molecular weight[b]	AN/TN[c]
Intact protein	Casein	28,500	0.07
	WPC	25,000	0.06
Proteose	Casein	6000	0.13
	WPC	6800	0.11
Peptone	Casein	2000	0.24
	WPC	1400	0.24
Peptides	Casein	400	0.48
	WPC	375	0.43
Peptides and free amino acids	Casein	260	0.55
	WPC	275	0.58

Source: Reprinted with permission from Lahl, W. J., & S. D. Braun. Enzymatic production of protein hydrolysates for food use. *Food Technology, 48*(10), 69, © 1994, Institute of Food Technologists

[a]Commercial hydrolysates produced by Deltown Specialties, Fraser, NY

[b]Determined by reverse-phase HPLC

[c]Ratio of amino nitrogen present in the hydrolysate to the total amount of nitrogen present in the substrate

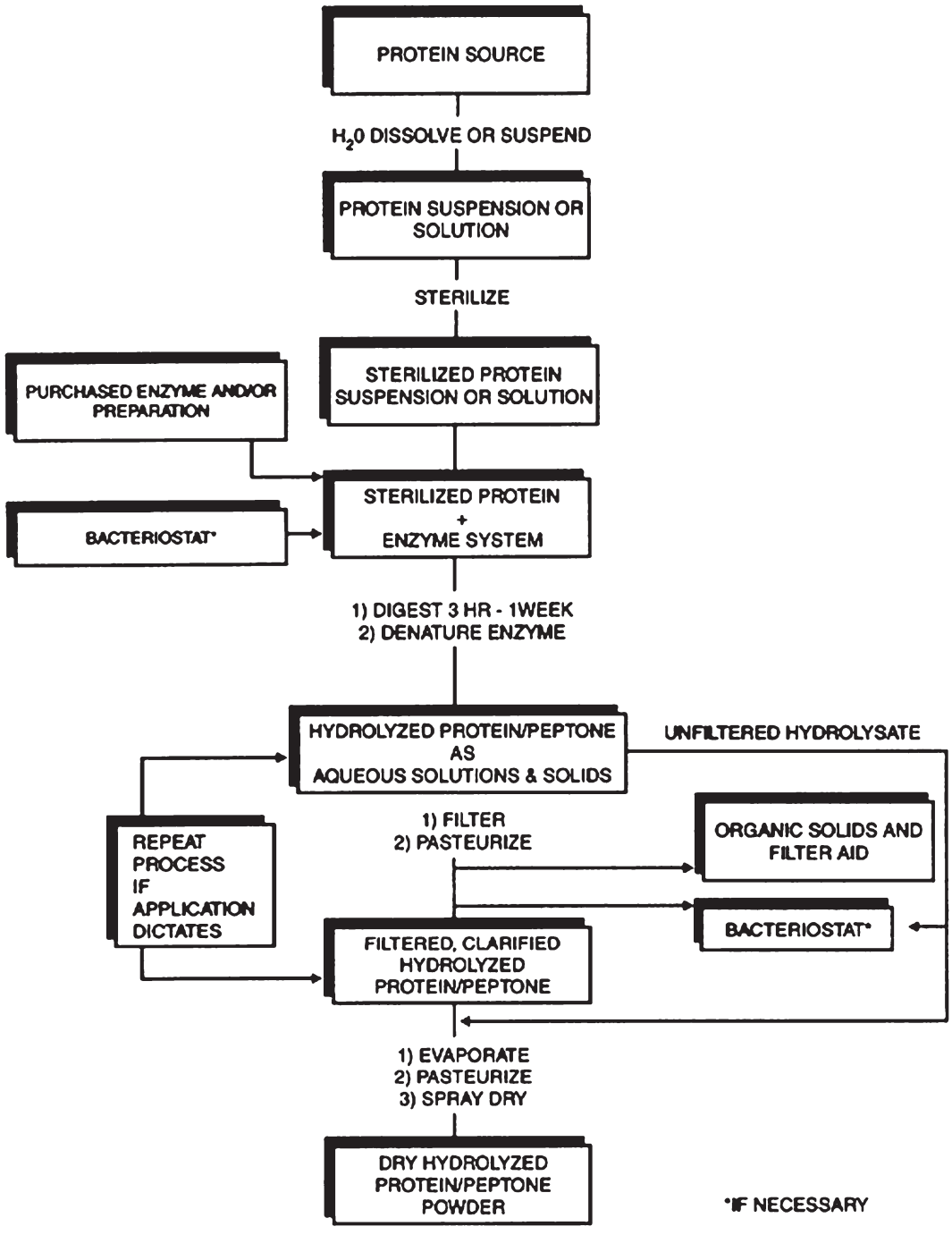


Fig. 10.15 Process for the production of protein hydrolysates. *Source:* Reprinted with permission from Lahl, W. J., & Braun, S. D. Enzymatic production of protein hydrolysates for food use. *Food Technology, 48*(10), 70, © 1994, Institute of Food Technologists

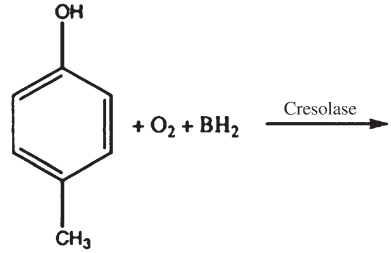
Oxidoreductases

Phenolases

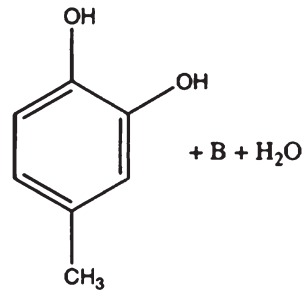
The enzymes involved in enzymic browning are known by the name polyphenoloxidase and are also called polyphenolase, phenolase, tyrosinase, or catechol oxidase. It is generally agreed (Mathew and Parpia 1971) that these terms include all enzymes that have the capacity to oxidize phenolic compounds to *o*-quinones. This can be represented by the conversion of *o*-dihydroxyphenol to *o*-quinone,

The action of polyphenolases is detrimental when it leads to browning in bruised and broken plant tissue but is beneficial in the processing of tea and coffee. The enzyme occurs in almost all plants, but relatively high levels are found in potatoes, mushrooms, apples, peaches, bananas, avocados, tea leaves, and coffee beans.

In addition to changing *o*-diphenols into *o*-quinones, the enzymes also catalyze the conversion of monophenols into *o*-diphenols (called monophenol monooxygenase or cresolase), as follows:



p-cresol



4-methyl-catechol

where BH_2 stands for an *o*-diphenolic compound.

To distinguish this type of activity from the one mentioned earlier, it is described as cresolase activity, whereas the other is referred to as catecholase activity. For both types of activity, the involvement of copper is essential. Copper has been found as a component of all polyphenolases. The activity of cresolase involves three steps, which can be represented by the following overall equation (Mason 1956):

$$\text{Protein} - \text{Cu}_2^{+} - \text{O}_2 + \text{monophenol} \rightarrow \text{Protein} - \text{Cu}_2^{+} + o - \text{quinone} + \text{H}_2\text{O}$$

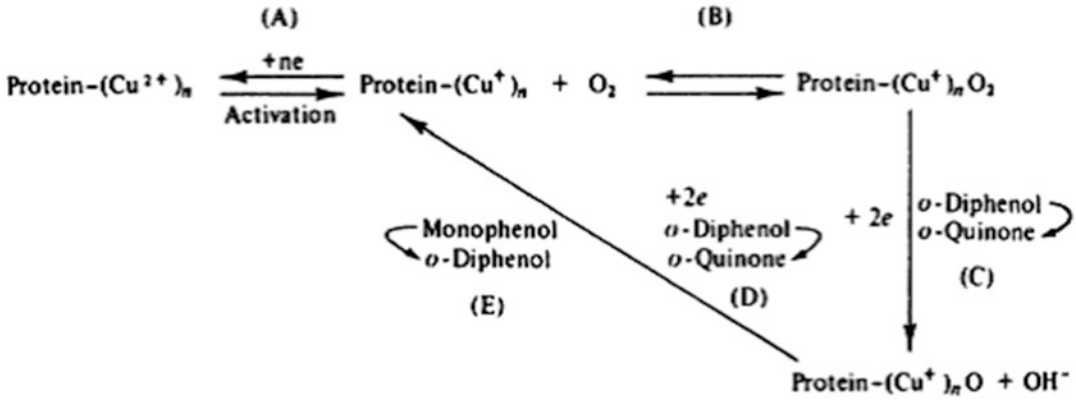


Fig. 10.16 Phenolase catalyzed reactions. (**a**) Activation of phenolase. (**b–d**) Two-step four-electron reduction of oxycuprophenolase, and the associated hydroxylation of monophenols. *Source:* From Mason, H. S. Mechanisms of oxygen metabolism. *Advances in Enzymology, 19*, 79–233, © 1957

The protein copper-oxygen complex is formed by combining one molecule of oxygen with the protein to which two adjacent cuprous atoms are attached.

Catecholase activity involves oxidizing two molecules of *o*-diphenols to two molecules of *o*-quinones, resulting in the reduction of one molecule of oxygen to two molecules of water. The action sequence as presented in Fig. 10.16 has been proposed by Mason (1957). The enzyme-oxygen complex serves as the hydroxylating or dehydroxylating intermediate, and (Cu)*n* represents the actual charge designation of the copper at the active site. In preparations high in cresolase activity, $n = 2$, and in preparations high in catecholase activity, $n = 1$. The overall reaction involves the use of one molecule of oxygen, one atom of which goes into the formation of the diphenol, and the other, which is reduced to water. This can be expressed in the following equation given by Mathew and Parpia (1971):

$$\text{Monophenol} + \text{O}_2 + o-\text{diphenol} \xrightarrow{\text{enzyme}} o-\text{diphenol} + \text{quinone} + \text{H}_2\text{O}$$

The substrates of the polyphenol oxidase enzymes are phenolic compounds present in plant tissues, mainly flavonoids. These include catechins, anthocyanidins, leucoanthocyanidins, flavonols, and cinnamic acid derivatives. Polyphenol oxidases from different sources show distinct differences in their activity for different substrates. Some specific examples of polyphenolase substrates are chlorogenic acid, caffeic acid, dicatechol, protocatechuic acid, tyrosine, catechol, dihydroxyphenylalanine, pyrogallol, and catechins.

To prevent or minimize enzymic browning of damaged plant tissue, several approaches are possible. The first and obvious one, although rarely practical, involves the exclusion of molecular oxygen. Another approach is the addition of reducing agents that can prevent the accumulation of *o*-quinones. Heat treatment is effective in deactivating the enzymes. Metal complexing agents may deactivate the enzyme by making the copper unavailable.

One of the most useful methods involves the use of L-ascorbic acid as a reducing agent. This is practiced extensively in the commercial production of fruit juices and purees. The ascorbic acid reacts with the *o*-quinones and changes them back into *o*-diphenols (Fig. 10.17).

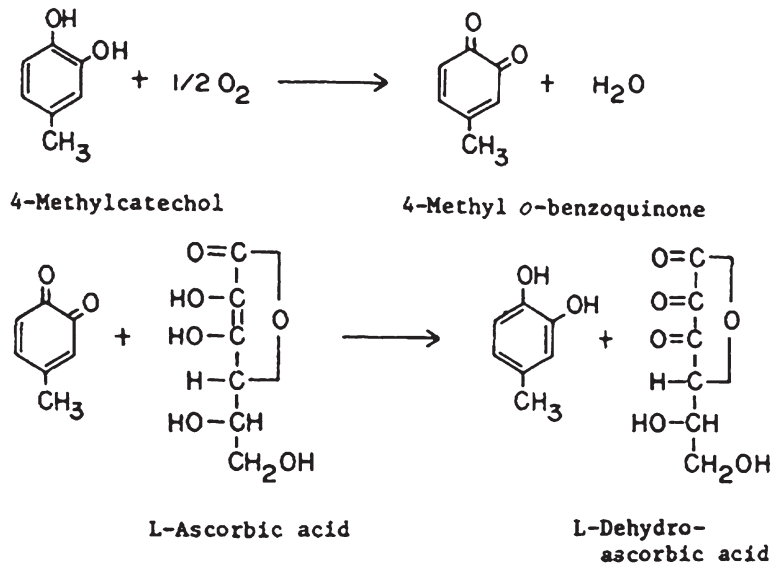


Fig. 10.17 Reaction of L-ascorbic acid with *o*-quinone in the prevention of enzymic browning

Glucose Oxidase (β-D-Glucose: Oxygen Oxidoreductase)

This enzyme catalyzes the oxidation of D-glucose to δ-D-gluconolactone and hydrogen peroxide in the presence of molecular oxygen, as follows:

$$C_6H_{12}O_6 \xrightarrow{\text{enzyme}} C_6H_{10}O_6 + H_2O_2$$

The enzyme is present in many fungi and is highly specific for β-D-glucopyranose. It has been established that the enzyme does not oxidize glucose by direct combination with molecular oxygen. The mechanism as described by Whitaker (1972) involves the oxidized form of the enzyme, flavin adenine dinucleotide (FAD), which serves as a dehydrogenase. Two hydrogen atoms are removed from the glucose to form the reduced state of the enzyme, FADH2, and δ-D-gluconolactone. The enzyme is then reoxidized by molecular oxygen. The gluconolactone is hydrolyzed in the presence of water to form D-gluconic acid.

In food processing, glucose oxidase is used to remove residual oxygen in the head space of bottled or canned products or to remove glucose. Light has a deteriorative effect on citrus beverages. Through the catalytic action of light, peroxides are formed that lead to oxidation of other components, resulting in very unpleasant off-flavors. Removing the oxygen by the use of a mixture of glucose oxidase and catalase will prevent these peroxides from forming. The glucose oxidase promotes the formation of gluconic acid with uptake of one molecule of oxygen. The catalase decomposes the hydrogen peroxide formed into water and one half-molecule of oxygen. The net result is the uptake of one half-molecule of oxygen. The overall reaction can be written as follows:

$$\text{Glucose} + \tfrac{1}{2}O_2 \xrightarrow[\text{catalase}]{\text{glucose oxidase}} \text{gluconic acid}$$

Recent information suggests that this application is not effective because of reversible inhibition of the glucose oxidase by the dyes used in soft drinks below pH 3 (Hammer 1993).

This enzyme mixture can also remove glucose from eggs before drying to prevent Maillard type browning reactions in the dried product.

Catalase (Hydrogen Peroxide: Hydrogen Peroxide Oxidoreductase)

Catalase catalyzes the conversion of two molecules of hydrogen peroxide into water and molecular oxygen as follows:

$$2\ H_2O_2 \xrightarrow{\text{catalase}} 2\ H_2O + O_2$$

This enzyme occurs in plants, animals, and microorganisms. The molecule has four subunits; each of these contains a protohemin group, which forms part of four independent active sites. The molecular weight is 240,000. Catalase is less stable to heat than is peroxidase. At neutral pH, catalase will rapidly lose activity at 35 °C. In addition to catalyzing the reaction shown above (catalatic activity), catalase can also have peroxidatic activity. This occurs at low concentrations of hydrogen peroxide and in the presence of hydrogen donors (e.g., alcohols).

In plants, catalase appears to have two functions. First is the ability to dispose of the excess H_2O_2 produced in oxidative metabolism, and second is the ability to use H_2O_2 in the oxidation of phenols, alcohols, and other hydrogen donors. The difference in heat stability of catalase and peroxidase was demonstrated by Lopez et al. (1959). They found that blanching of southern peas for 1 min in boiling water destroys 70–90% of the peroxidase activity and 80–100% of the catalase activity.

The combination of glucose oxidase and catalase is used in a number of food processing applications, including the removal of trace glucose or oxygen from foods and in the production of gluconic acid from glucose. Greenfield and Lawrence (1975) have studied the use of these enzymes in their immobilized form on an inorganic support.

Peroxidase (Donor: Hydrogen Peroxide Oxidoreductase)

The reaction type catalyzed by peroxidase involves hydrogen peroxide as an acceptor, and a compound AH_2 as a donor of hydrogen atoms, as shown:

$$H_2O_2 + AH_2 \xrightarrow{\text{peroxidase}} 2\ H_2O + A$$

In contrast to the action of catalase, no molecular oxygen is formed.

The peroxidases can be classified into the two groups, iron-containing peroxidases and flavoprotein peroxidases. The former can be further subdivided into ferriprotoporphyrin peroxidases and verdoperoxidases. The first group contains ferriprotoporphyrin III (protohemin) as the prosthetic group (Fig. 10.18). The common plant peroxidases (horseradish, fig, and turnip) are in this group and the enzymes are brown when highly purified. The second group includes the peroxidases of animal tissue and milk (lactoperoxidase). In these enzymes, the prosthetic group is an iron porphyrin nucleus but not protohemin. When highly purified, these enzymes are green in color. Flavoprotein peroxidases occur in microorganisms and animal tissues. The prosthetic group is FAD.

The linkage between the iron-containing prosthetic group and the protein can be stabilized

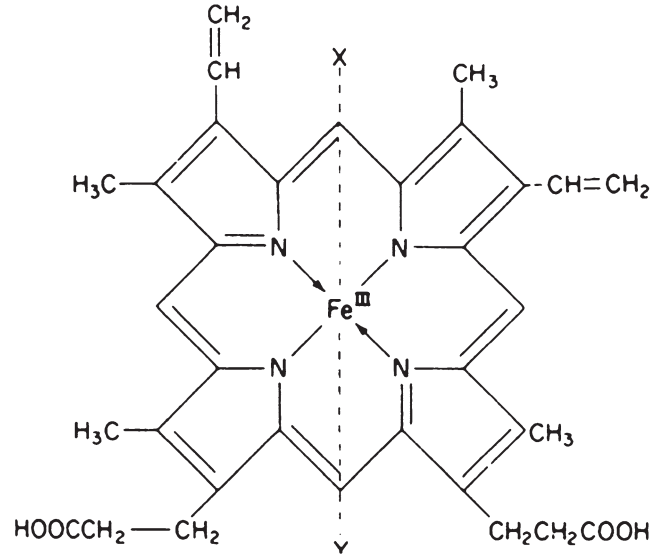


Fig. 10.18 Structural formula of ferriprotoporphyrin III (protohemin). *Source:* From Whitaker, J. R. (1974). *Food related enzymes.* American Chemical Society

by bisulfite (Embs and Markakis 1969). It is suggested that the bisulfite forms a complex with the peroxidase iron, which stabilizes the enzyme.

Because of the widespread occurrence of peroxidase in plant tissues, Nagle and Haard (1975) have suggested that it plays an important role in the development and senescence of plant tissues. It plays a role in biogenesis of ethylene; in regulating ripening and senescence; in the degradation of chlorophyll; and in the oxidation of indole-3-acetic acid.

The enzyme can occur in a variety of multiple molecular forms, named isoenzymes or isozymes. Such isoenzymes have the same enzymatic activity but can be separated by electrophoresis. Nagle and Haard (1975) separated the isoperoxidases of bananas into six anionic and one cationic component by gel electrophoresis. By using other methods of separation, an even greater number of isoenzymes was demonstrated.

Peroxidase has been implicated in the formation of the "grit cells" or "stone cells" of pears (Ranadive and Haard 1972). Bound peroxidase but not total peroxidase activity was higher in the fruit that contained excessive stone cells. The stone cells or sclereids are lignocellulosic in nature. The presence of calcium ions causes the release of wall bound peroxidase and a consequent decrease in the deposition of lignin.

The peroxidase test is used as an indicator of satisfactory blanching of fruits and vegetables. However, it has been found that the enzymes causing off-flavors during frozen storage can, under some conditions, be regenerated. Regeneration of enzymes is a relatively common phenomenon and is more likely to occur the faster the temperature is raised to a given point in the blanching process. The deactivation and 3 reactivation of peroxidase by heat was studied by Lu and Whitaker (1974). The rate of reactivation was at a maximum at pH 9 and the extent of reactivation was increased by addition of hematin.

The deactivation of peroxidase is a function of heating time and temperature. Lactoperoxidase is completely deactivated by heating at 85 °C for 13 s. The effect of heating time at 76 °C on the deactivation of lactoperoxidase is represented in Fig. 10.19, and the effect of heating temperature on the deactivation constant is shown in Fig. 10.20. Lactoperoxidase can be regenerated under conditions of high temperature short time (HTST) pasteurization. Figure 10.21 shows the regeneration of lactoperoxidase activity in milk that is pasteurized for 10 s at 85 °C. The regeneration effect depends greatly on storage temperature; the lower the storage temperature, the smaller the regeneration effect.

Lactoperoxidase is associated with the serum proteins of milk. It has an optimum pH of 6.8 and a molecular weight of 82,000.

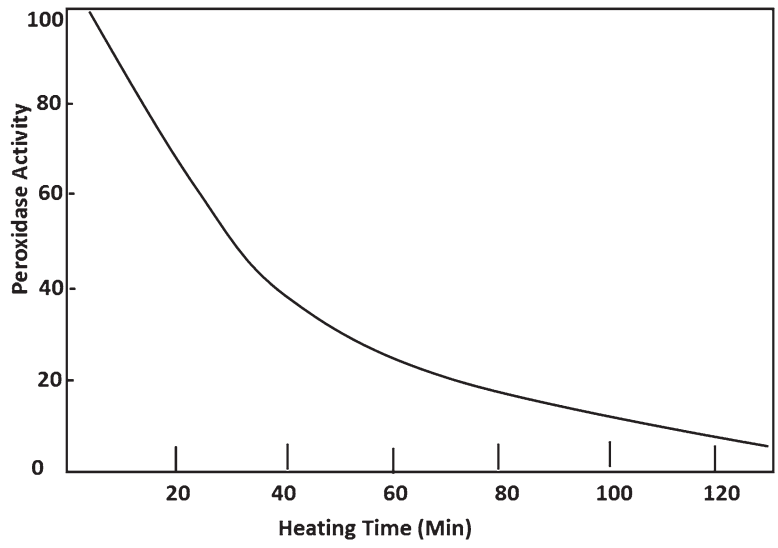


Fig. 10.19 Deactivation of lactoperoxidase as a function of heating time. *Source:* Adapted from Kiermeier, F., & Kayser, C. (1960). Heat inactivation of lactoperoxidase (in German). *Zeitschrift für Lebensmittel-Untersuchung und -Forschung*, *113*

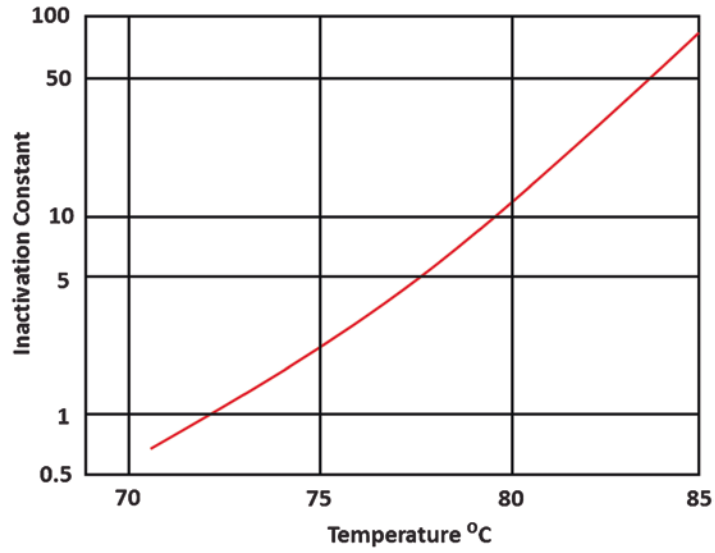


Fig. 10.20 Dependence of the rate constant of heat deactivation of lactoperoxidase on the heating temperature. *Adapted from* Kiermeier, F., & Kayser, C. (1960). Heat inactivation of lactoperoxidase (in German). *Zeitschrift für Lebensmittel-Untersuchung und -Forschung, 113*

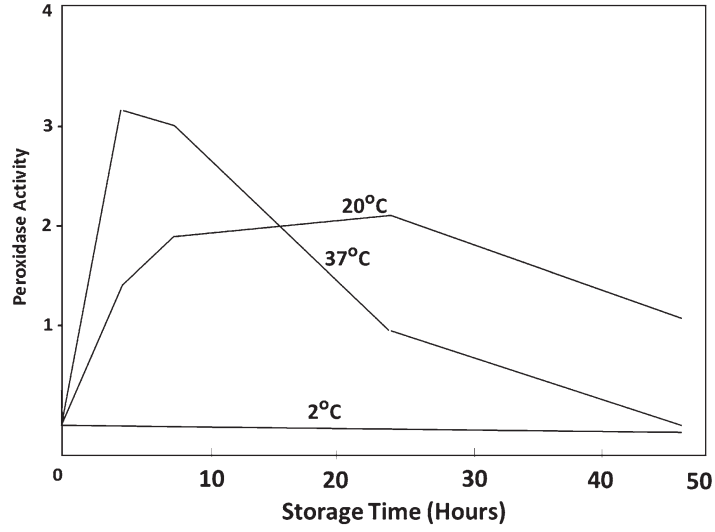


Fig. 10.21 Regeneration in ultra high temperature treated milk as a function of storage temperature. (**a**) 2 °C; (**b**) 20 °C; (**c**) 37 °C. *Adapted from* Kiermeier, F., & Kayser, C. (1960). Heat inactivation of lactoperoxidase (in German). *Zeitschrift für Lebensmittel-Untersuchung und -Forschung*, *113*

Lipoxygenase (Linoleate: Oxygen Oxidoreductase)

This enzyme, formerly named lipoxidase, is present in plants and catalyzes the oxidation of unsaturated fats. The major source of lipoxygenase is legumes, soybeans, and other beans and peas. Smaller amounts are present in peanuts, wheat, potatoes, and radishes. Lipoxygenase is a metallo-protein with an iron atom in its active center. In plants two types of lipoxygenase exist: type I lipoxygenase peroxidizes only free fatty acids with a high stereo- and regioselectivity; type II lipoxygenase is less specific for free linoleic acid and acts as a general catalyst for autoxidation. Type I reacts with fats in a food only after free fatty acids have been formed by lipase action; type II acts directly on triacylglycerols.

Lipoxygenase is highly specific and attacks the *cis-cis*-1,4-pentadiene group contained in the fatty acids linoleic, linolenic, and arachidonic, as follows:

$$-CH=CH-CH_2-CH=CH-$$

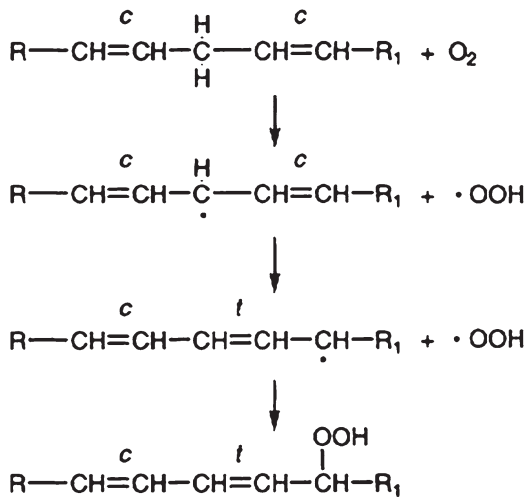


Fig. 10.22 Essential steps in the mechanism of the lipoxygenase-catalyzed oxidation of the 1,4-pentadiene group

The specificity of this enzyme requires that both double bonds are in the *cis* configuration; in addition, there is a requirement that the central methylene group of the 1,4-pentadiene group occupies the ω-8 position on the fatty acid chain and also that the hydrogen to be removed from the central methylene group be in the L-position. Although the exact mechanism of the reaction is still in some doubt, there is agreement that the essential steps are as represented in Fig. 10.22. Initially, a hydrogen atom is abstracted from the ω-8 methylene group to produce a free radical. The free radical isomerizes, causing conjugation of the double bond and isomerization to the *trans* configuration. The free radical then reacts to form the ω-6 hydroperoxide.

Lipoxygenase is reported to have a pH optimum of about 9. However, these values are determined with linoleic acid as substrate, and in natural systems the substrate is usually present in the form of triglycerides. The enzyme has a molecular weight of 102,000 and an isoelectric point of 5.4. The peroxide formation by lipoxygenase is inhibited by the common lipid antioxidants. The antioxidants are thought to react with the free radicals and interrupt the oxidation mechanism.

Most manifestations of lipoxygenase in foods are undesirable. However, it is used in baking to bring about desirable changes. Addition of soybean flour to wheat flour dough results in a bleaching effect, because of oxidation of the xanthophyll pigments. In addition, there is an effect on the rheological and baking properties of the dough. It has been suggested that lipoxygenase acts indirectly in the oxidation of sulfhydryl groups in the gluten proteins to produce disulfide bonds. When raw soybeans are ground with water to produce soy milk, a strong and unpleasant flavor develops that is called painty, green, or beany. Carrying out the grinding in boiling water instantly deactivates the enzyme, and no off-flavor is formed. Blanching of peas and beans is essential in preventing the lipoxygenase-catalyzed development of off-flavor. In addition to the development of off-flavors, the enzyme may be responsible for destruction of carotene and vitamin A, chlorophyll, bixin, and other pigments.

In some cases the action of lipoxygenase leads to development of a characteristic aroma. Galliard et al. (1976) found that the main aroma compounds of cucumber, 2-*trans* hexenol and 2-*trans,* 6-*cis*-nonadienal, are produced by reaction of linolenic acid and lipoxygenase to form hydroperoxide (Fig. 10.23); these are changed into *cis* unsaturated aldehydes by hydroperoxide lyase. The *cis* unsaturated aldehydes are transformed by isomerase into the corresponding *trans* isomers. These same substances in another matrix would be experienced as off-flavors. The use of lipoxygenase as a versatile biocatalyst has been described by Gardner (1996).

Xanthine Oxidase (Xanthine: Oxygen Oxidoreductase)

This enzyme catalyzes the conversion of xanthine and hypoxanthine to uric acid. The reaction equation is given in Fig. 10.24; heavy arrows indicate the reactions catalyzed by the enzyme and the dashed arrows represent the net result of the catalytic process (Whitaker 1972). Although xanthine oxidase is a nonspecific enzyme and many substances can serve as substrate, the rate

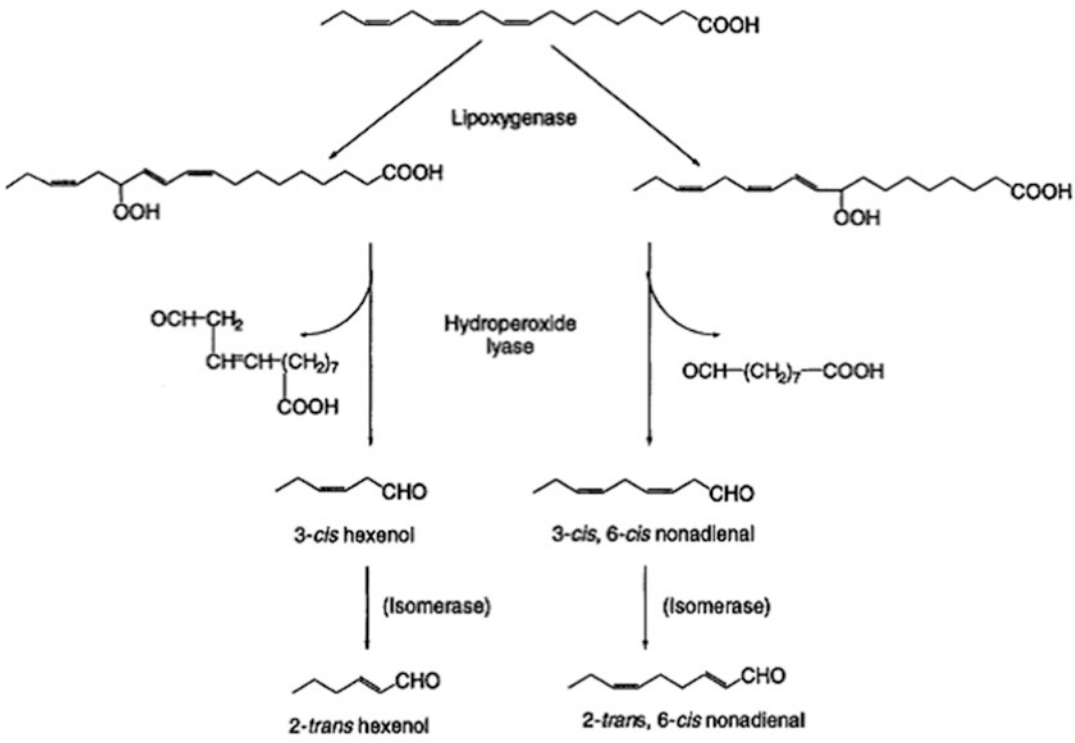


Fig. 10.23 Lipoxygenase catalyzed formation of aroma compounds in cucumber. *Source:* Reprinted from Galliard, T., Phillips, D. R., & Reynolds, J. The formation of *cis*-3-nonenal, *trans*-2-nonenal and hexanol from linoleic acid hydroperoxide isomers by a hydroperoxide cleavage enzyme system in cucumber (Cucumis Sativus) fruits. *Biochimica et Biophysica Acta, 441*, 184, Copyright 1976, with permission from Elsevier Science

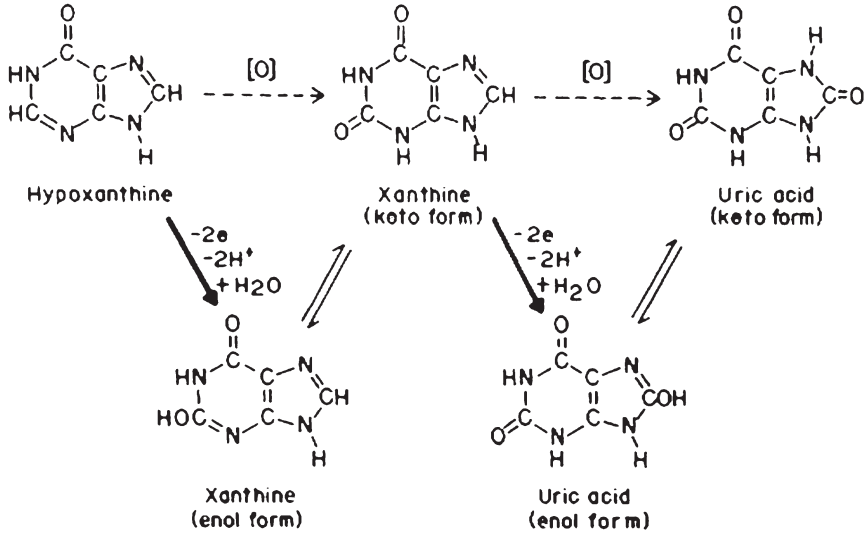


Fig. 10.24 Oxidation of hypoxanthine and xanthine to uric acid by xanthine oxidase. *Source*: From Whitaker, J. R. (1972). *Principles of enzymology for the food sciences.* Marcel Dekker, Inc

of oxidation of xanthine and hypoxanthine is many times greater than that of other substrates.

Xanthine oxidase has been isolated from milk and obtained in the crystalline state. The molecular weight is 275,000. One mole of the protein contains 2 moles of FAD, 2 gram-atoms of molybdenum, 8 gram-atoms of nonheme iron, and eight labile sulfide groups. The eight labile sulfide groups are liberated in the form of H_2S upon acidification or boiling at pH 7. The optimum pH for activity is 8.3. The xanthine oxidase in milk is associated with the fat globules and, therefore, follows the fat into the cream when milk is separated. It seems to be located in small particles (microsomes) that are attached to the fat globules. The microsomes also contain the enzyme alkaline phosphatase. The microsomes can be dislodged from the fat globules by mechanical treatment such as pumping and agitation and by heating and cooling. The enzyme is moderately stable to heat but no less so than peroxidase.

Immobilized Enzymes

One of the most important recent developments in the use of enzymes for industrial food processing is the fixing of enzymes on water-insoluble inert supports. The fixed enzymes retain their activity and can be easily added to or removed from the reaction mixture. The use of immobilized enzymes permits continuous processing and greatly increased use of the enzyme. Various possible methods of immobilizing enzymes have been listed by Weetall (1975) and Hultin (1983). A schematic representation of the available methods is given in Fig. 10.25. The immobilizing methods include adsorption on organic polymers, glass, metal oxides, and siliceous materials such as bentonite and silica; entrapment in natural or synthetic polymers, usually polyacrylamide; microencapsulation in polymer membranes; ion exchange; cross-linking; adsorption and cross-linking combined; copolymerization; and covalent attachment to organic polymers.

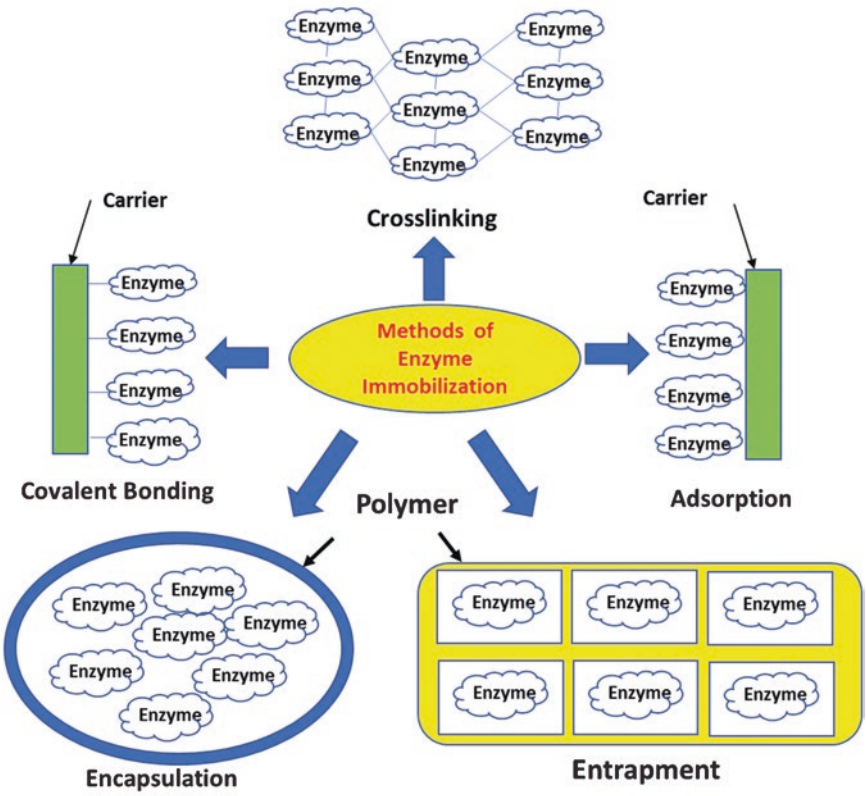


Fig. 10.25 Methods of immobilizing enzymes

The chemistry of immobilizing enzymes has been covered in detail by Stanley and Olson (1974).

A summary of immobilization methods has been provided by Adlercreutz (1993) and is presented in Exhibit 10.1. In membrane reactors, the reaction product is separated from the reaction mixture by a semipermeable membrane. In two-phase systems, a hydrophobic reaction product can be separated from the aqueous reaction mixture by transfer to the organic solvent phase. In aqueous-aqueous systems, two incompatible polymers in aqueous solution form a two-phase system.

Exhibit 10.1Summary of Enzyme Immobilization Methods

1. chemical methods
   - covalent binding
   - cross-linking
2. physical methods
   - adsorption
   - physical deposition
   - entrapment
   - in polymer gels
   - in microcapsules
   - membranes
3. two-phase systems
   - organic-aqueous
   - aqueous-aqueous

Immobilizing enzymes is likely to change their stability, and the method of attachment to the carrier also affects the degree of stability. When a high molecular weight substrate is used, the immobilizing should not be done by entrapment, microencapsulation, or copolymerization, because enzyme and substrate cannot easily get in contact. One of the promising methods appears to be covalent coupling of enzymes to inorganic carriers such as porous silica glass particles. Not all of the immobilized enzyme is active, due to either inactivation or steric hindrance. Usually, only about 30–50% of the bound enzyme is active.

Immobilized enzymes can be used in one of two basic types of reactor systems. The first is the stirred tank reactor where the immobilized enzyme is stirred with the substrate solution. This is a batch system and, after the reaction is complete, the immobilized enzyme is separated from the product. The other system employs continuous flow columns in which the substrate flows through the immobilized enzyme contained in a column or similar device. A simplified flow diagram of such a system is given in Fig. 10.26.

The characteristics of immobilized enzymes are likely to be somewhat different than those of the original enzyme. The pH optimum can be shifted; this depends on the surface charge of the carrier (Fig. 10.27). Another property that can be changed is the Michaelis constant, K_m. This value can become either larger or smaller. Immobilizing may result in increased thermal stability (Fig. 10.28), but in some cases the thermal stability is actually decreased.

Many examples of the use of immobilized enzymes in food processing have been reported. One of the most important of these is the use of immobilized glucose isomerase obtained from

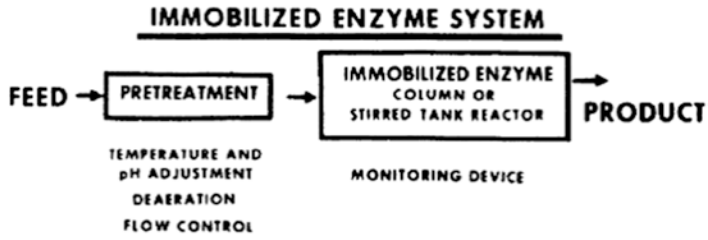


Fig. 10.26 Flow diagram of an immobilized enzyme system (column operation of lactase immobilized on phenol-formaldehyde resin with glutaraldehyde). *Source:* From Stanley, W. L., & Olson, A. C. (1974). The chemistry of immobilizing enzymes. *Journal of Food Science, 39*, 660–666

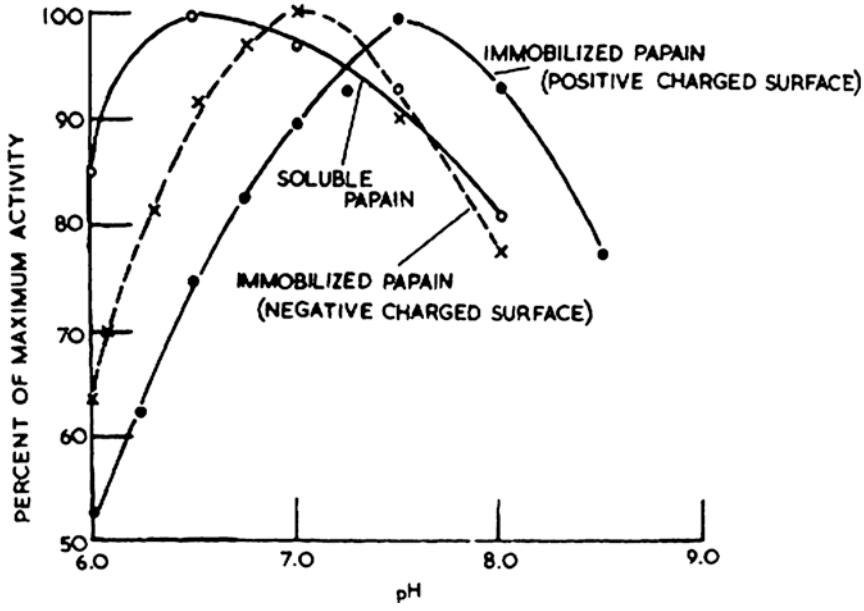


Fig. 10.27 Effect of immobilizing on the pH optimum of papain. *Source:* From Weetall, H. H. (1975). Immobilized enzymes and their application in the food and beverage industry. *Process Biochemistry, 10*, 3–6

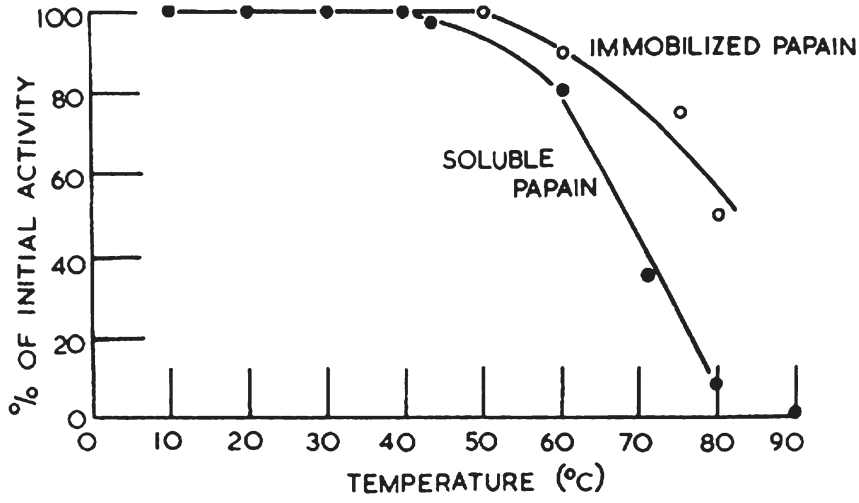


Fig. 10.28 Effect of immobilizing of the thermal stability of papain. *Source*: From Weetall, H. H. (1975). Immobilized enzymes and their application in the food and beverage industry. *Process Biochemistry, 10*, 3–6

Streptomyces for the production of high-fructose com syrup (Mermelstein 1975). In this process, the enzyme is bound to an insoluble carrier such as diethyl amino ethyl cellulose or a slurry of the fixed enzyme coated onto a pressure-leaf filter. The filter then serves as the continuous reactor through which the com syrup flows. The product obtained by this process is a syrup with 71% solids that contains about 42% fructose and 50% glucose; it has high sweetening power, high fermentability, high humectancy, reduced tendency to crystallize, low viscosity, and good flavor.

Examples of the use of immobilized enzymes in food processing and analysis have been listed by Olson and Richardson (1974) and Hultin (1983). L-aspartic acid and L-malic acid are produced by using enzymes contained in whole microorganisms that are immobilized in a polyacrylamide gel. The enzyme aspartase from *Escherichia coli* is used for the production of aspartic acid. Fumarase from *Brevibacterium ammoniagenes* is used for L-malic acid production.

The most widely used immobilized enzyme process involves the use of the enzyme glucose isomerase for the conversion of glucose to fructose in com syrup (Carasik and Carroll 1983). The organism *Bacillus coagulans* has been selected for the production of glucose isomerase. The development of the immobilized cell slurry has not proceeded to the point where half-lives of the enzyme are more than 75 days. A half-life is defined as the time taken for a 50% decrease in activity. Such immobilized enzyme columns can be operated for periods of over three half-lives.

The second important application of immobilized enzymes is the hydrolysis of lactose to glucose and galactose in milk and milk products by lactase (Sprössler and Plainer 1983). Several lactase sources are available; from yeast, *Saccharomyces lactis* and *S. fragilis,* or from fungi, *Aspergillus oryzae* or *A. niger.* The enzymes vary in their optimum pH and optimum temperature, as well as other conditions.

It is to be expected that the use of immobilized enzymes in food processing will continue to grow rapidly in the near future.

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