HANDBOOK OF FOOD ENZYMOCOLOGY

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Significance of Indigenous Enzymes in Milk and Dairy Products

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1. INTRODUCTION

About 60 indigenous enzymes have been reported in normal bovine milk (1); with the exception of α-lactalbumin, most of these have no obvious physiological role. The indigenous enzymes are constituents of the milk as excreted and arise from three principal sources: (a) the blood via defective mammary cell membranes; (b) secretory cell cytoplasm, some of which is occasionally entrapped within fat globules by the encircling fat globule membrane (MFGM); and (c) the MFGM itself, the outer layers of which are derived from the apical membrane of the secretory cell, which in turn originates from the Golgi membranes; this is probably the principal source of the enzymes. Thus, most enzymes enter milk owing to peculiarities of the mechanism by which milk constituents, especially the fat globules, are excreted from the secretory cells. Milk does not contain substrates for many of the enzymes present, while others are inactive in milk owing to unsuitable environmental conditions such as pH.

Many indigenous milk enzymes are technologically significant from five viewpoints:
1. Deterioration (lipase [potentially the most significant enzyme in milk], proteinase, acid phosphatase and xanthine oxidase) or preservation (lactoperoxidase, sulfhydryl oxidase, superoxide dismutase) of milk quality.
2. As indices of the thermal history of milk; these include alkaline phosphatase, γ-glutamyl transpeptidase, lactoperoxidase, and perhaps others.
3. As indices of mastitic infection; the concentration of several enzymes increases on mastitic infection, especially catalase, N-acetyl-α-glucosaminidase and acid phosphatase.
4. Antimicrobial activity, such as lysozyme and lactoperoxidase (which is exploited as a component of the lactoperoxidase–H2O2–thiocyanate system for the cold pasteurization of milk).
5. As commercial source of enzymes; these include ribonuclease and lactoperoxidase.

With a few exceptions (e.g., lysozyme and lactoperoxidase), the indigenous milk enzymes do not have a beneficial effect on the nutritional or organoleptic attributes of milk, and hence their destruction by heat is one of the objectives of many dairy processes.

The following abbreviations are used: MFGM, milk fat globule membrane; β-CN, β-casein; αs2-CN, αs2-casein; LPL, lipoprotein lipase; UHT, ultra high temperature; HTST, high temperature short time; LTLT, low temperature long time; PE, pasteurization equivalent; HML, human milk lysozyme; BML, bovine milk lysozyme; EWL, egg white lysozyme; NAGase, N-acetyl-β-D-glucosaminidase; GGTP, γ-glutamyl transpeptidase; LPO, lactoperoxidase; XO, xanthine oxidase; SO, sulfhydryl oxidase; SOD, superoxide dismutase.

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activities that have been detected in milk but which have not been isolated and have no known significance in milk are listed in Table 3; it is possible that some of these enzymes are secreted by contaminating bacteria in milk.

The indigenous enzymes in milk have attracted the attention of researchers for > 100 years, mainly because of their potential to cause defects in milk and dairy products, especially lipase, and their usefulness as indicators of the thermal treatment of milk. More recently, they have assumed importance as indices of animal health and of the mechanisms involved in the synthesis and secretion of milk. A very extensive literature has accumulated. The general topic has been the subject of several general reviews (1–10); in addition, the literature on the principal technologically significant enzymes has been reviewed separately (see below).

In this chapter the occurrence, distribution, isolation, and characterization of the principal indigenous enzymes in bovine milk will be discussed, with emphasis on their commercial significance in milk and dairy

Table 2  Other Enzymes That Have Been Isolated From Milk and Partially Characterized but Which Are of No Known Significance in Milk

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Reaction catalyzed</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutathione peroxidase</td>
<td>EC 1.11.1.92 GSH + H_2O → GSSH</td>
<td>Contains Se</td>
</tr>
<tr>
<td>Ribonuclease</td>
<td>EC 3.1.27.5 Hydrolysis of RNA</td>
<td>Milk is a very rich source; similar to pancreatic RNase</td>
</tr>
<tr>
<td>α-Amylase</td>
<td>EC 3.2.1.1 Hydrolysis of starch</td>
<td></td>
</tr>
<tr>
<td>β-Amylase</td>
<td>EC 3.2.1.2 Hydrolysis of starch</td>
<td></td>
</tr>
<tr>
<td>α-Mannosidase</td>
<td>EC 3.2.1.24 Hydrolysis of mannan</td>
<td></td>
</tr>
<tr>
<td>β-Glucuronidase</td>
<td>EC 3.2.1.31 Hydrolysis of glucuronides</td>
<td>Contains Zn^{2+}</td>
</tr>
<tr>
<td>5'-Nucleotidase</td>
<td>EC 3.1.3.5 5'-Nucleotides + H_2O → ribonucleosides + P_1</td>
<td>Diagnostic test for mastitis</td>
</tr>
<tr>
<td>Adenosine triphosphatase</td>
<td>EC 3.6.1.3 ATP + H_2O → ADP + P_1</td>
<td></td>
</tr>
<tr>
<td>Aldolase</td>
<td>EC 4.1.2.13 Fructose, 1,6 diP = glyceraldehyde-3-P + dihydroxyacetone-P</td>
<td></td>
</tr>
</tbody>
</table>

Source: Ref. 70.
products. The available information indicates that the milks of other species have an enzyme profile similar to bovine milk, although very considerable interspecies differences exist in the level of certain enzymes, e.g., the very high level of lysozyme in human and equine milks. Human milk and that of other primates contain a bile salts-activated lipase, in addition to the ubiquitous lipoprotein lipase, which is not present in the milk of other species. The indigenous enzymes in human milk have been described by Hamos et al. (11) and Hernell and Lonnerdal (12).

II. PROTEINASES (EC 3.4.–.–)

The presence of an indigenous proteinase in milk was suggested by Babcock and Russel in 1897 but because it occurred at a low concentration or had low activity in milk, it was believed until the 1960s that the protease in milk might have been of microbial origin. Recent changes in the dairy industry, e.g., improved hygiene in milk production, extended storage of milk at a low temperature at the farm and/or factory, and altered product profile—e.g., UHT processing of milk—have increased the significance of indigenous milk proteinase which has, consequently, been the focus of considerable research.

Milk contains at least two proteinases, plasmin (alkaline milk proteinase) and cathepsin D (acid milk proteinase) and possibly several other proteolytic enzymes, e.g., two thiol proteinases, thrombin, and an aminopeptidase. In terms of activity and technological significance, plasmin is the most important of the indigenous proteolytic enzymes and has been the subject of most attention. The relevant literature has been reviewed by Grufferty and Fox (13) and Bastian and Brown (14).

A. Plasmin (EC 3.4.21.7)

The physiological function of plasmin (fibrinolysin) is to dissolve blood clots. It is part of a complex system consisting of plasmin, its zymogen (plasminogen), plasminogen activators, plasmin inhibitors, and inhibitors of plasminogen activators (Fig. 1). In milk, there is about four times as much plasminogen as plasmin, and both, as well as plasminogen activators, are associated with the casein micelles, from which they dissociate when the pH is decreased to 4.6; the inhibitors of plasmin and of plasminogen activators are in the milk serum. It has been reported that there is a low level of plasmin activity in the milk fat globule membrane but this appears to be due to the adsorption of plasmin to casein micelles which are adsorbed on the membrane (15). The concentration of plasmin and plasminogen in milk increase with advancing lactation, mastitic infection, and number of lactations. The conversion of plasminogen to plasmin in milk increases with advancing lactation, and there is a positive correlation between plasmin activity and the level of plasminogen activator, which itself is positively correlated with somatic cell count (16). The level of plasmin in milk is also affected by diet and management practices (16). No activation of plasminogen to plasmin is reported (17) to occur during storage of milk at 4°C for 6 days; in fact, plasmin and potential plasmin (plasminogen) activity decreased under these conditions.

Bovine plasminogen contains 786 amino acids with a mass of 88,092 kDa. Its primary structure is arranged in five loops (called kringsles), each stabilized by three intramolecular disulfide bonds. Plasminogen is activated in a two-step process: it is first cleaved at Arg557-Ile558 (bovine) by plasmin (a trace of which occurs in blood) to yield Lys-plasminogen which is inactive but undergoes a conformational change...
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Reaction catalyzed</th>
<th>Source</th>
<th>Distribution in milk</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC 1.1.1.1 Alcohol dehydrogenase</td>
<td>Ethanol + NAD$^+$ $\rightarrow$ acetaldehyde + NADH + H$^+$</td>
<td>—</td>
<td>SM</td>
</tr>
<tr>
<td>EC 1.1.1.14 L-Iditol dehydrogenase</td>
<td>L-Iditol + NAD$^+$ $\rightarrow$ L-sorbose + NADH + H$^+$</td>
<td>—</td>
<td>SM</td>
</tr>
<tr>
<td>EC 1.1.1.27 Lactate dehydrogenase</td>
<td>Lactic acid + NAD$^+$ $\rightarrow$ pyruvate acid + NADH + H$^+$</td>
<td>—</td>
<td>SM</td>
</tr>
<tr>
<td>EC 1.1.1.37 Malate dehydrogenase</td>
<td>Malate + NAD$^+$ $\rightarrow$ oxaloacetate + NADH + H$^+$</td>
<td>Mammary gland</td>
<td>SM</td>
</tr>
<tr>
<td>EC 1.1.1.40 Malic enzyme</td>
<td>Malate + NADP$^+$ $\rightarrow$ pyruvate + CO$_2$+NADH + H$^+$</td>
<td>Mammary gland</td>
<td>SM</td>
</tr>
<tr>
<td>EC 1.1.1.42 Isocitrate dehydrogenase</td>
<td>Isocitrate + NADP$^+$ $\rightarrow$ 2-oxoglutarate + CO$_2$ + NADH + H$^+$</td>
<td>Mammary gland</td>
<td>SM</td>
</tr>
<tr>
<td>EC 1.1.1.44 Phosphogluconate dehydrogenase</td>
<td>6-Phospho-D-gluconate + NADP$^+$ $\rightarrow$ D-ribose-5-P + CO$_2$ + NADPH + H$^+$</td>
<td>Mammary gland</td>
<td>SM</td>
</tr>
<tr>
<td>EC 1.1.1.49 Glucose-6-phosphate dehydrogenase</td>
<td>D-Glucose-6-P + NADP$^+$ $\rightarrow$ D-glucono-1,5-lactone-6-P + NADPH + H$^+$</td>
<td>Mammary gland</td>
<td>SM</td>
</tr>
<tr>
<td>EC 1.4.3.6 Amine oxidase (Cu-containing)</td>
<td>RCH$_2$NH$_2$ + H$_2$O + O$_2$ $\rightarrow$ RCHO + NH$_3$ + H$_2$O</td>
<td>—</td>
<td>SM</td>
</tr>
<tr>
<td>— Polyamine oxidase</td>
<td>Spermine$^{10}$, spermidine$^{12}$, putrescine</td>
<td>—</td>
<td>SM</td>
</tr>
<tr>
<td>— Fucosyltransferase</td>
<td>Catalyzes the transfer of fucose from GDP L-fucose to specific oligosaccharides and glycoproteins</td>
<td>—</td>
<td>SM</td>
</tr>
<tr>
<td>EC 1.6.99.3 NADH dehydrogenase</td>
<td>NADH + acceptor $\rightarrow$ NAD$^+$ + reduced acceptor</td>
<td>—</td>
<td>FGM</td>
</tr>
<tr>
<td>EC 1.8.1.4 Dihydrolipoamide dehydrogenase (diaphorase)</td>
<td>Dihydrolipoamide + NAD$^+$ $\rightarrow$ lipoamide + NADH</td>
<td>—</td>
<td>SM/FGM</td>
</tr>
<tr>
<td>EC 2.4.1.22 Lactose synthetase (A protein: UDP-galactose; D-glucose, 1-galactosyltransferase; B protein: $\alpha$-lactalbumin)</td>
<td>UDP galactose + D-glucose $\rightarrow$ UDP + lactose</td>
<td>Golgi apparatus</td>
<td>SM</td>
</tr>
<tr>
<td>EC 2.4.1.38 Glycoprotein +$\beta$-galactosyltransferase</td>
<td>UDP galactose+$N$-acytel D-glucosaminyl gycopeptide $\rightarrow$ UDP + 4, $\beta$-D-galactosyl-$N$-acytel-D-glucosaminyl gycopptide</td>
<td>—</td>
<td>FGM</td>
</tr>
<tr>
<td>EC 2.4.1.90 $N$-Acetylglucosamin synthase</td>
<td>UDP galactose+$N$-acytel-D-glucosamine $\rightarrow$ UDP</td>
<td>Golgi apparatus</td>
<td>—</td>
</tr>
<tr>
<td>EC 2.4.99.6 CMP-$N$-acytel-$N$-acytel-lactosaminide $\alpha$-2,3-sialyltransferase</td>
<td>CMP-$N$-acetyneuraminylide + $\beta$-D-galactosyl-$1,4$-$N$-acytel-D-glucosaminyl glycoprotein $\rightarrow$ CMP+$\alpha$-$N$-acetyneuraminylide 1-2, 3-$\beta$-D-galactosyl-$1,4$-$N$-acytel-D-glucosaminyl glycoprotein</td>
<td>—</td>
<td>SM</td>
</tr>
<tr>
<td>EC Code</td>
<td>Enzyme Name</td>
<td>Substrate/Reaction</td>
<td>Source</td>
</tr>
<tr>
<td>---------</td>
<td>-------------------------------------------------</td>
<td>----------------------------------------------------------------------------------</td>
<td>---------</td>
</tr>
<tr>
<td>2.5.1.3</td>
<td>Thiamine-phosphate pyrophosphorylase</td>
<td>2-Methyl-4-amino-5-hydroxymethyl/pyrimidine diphosphate + 4-methyl-5-(2-phosphonoxyethyl)-thiazole (\rightleftharpoons) pyrophosphate + thiamine monophosphate</td>
<td>—</td>
</tr>
<tr>
<td>2.6.1.1</td>
<td>Aspartate aminotransferase</td>
<td>L-aspartate + 2-oxoglutarate (\rightleftharpoons) oxaloacetate + L-glutamate</td>
<td>Blood</td>
</tr>
<tr>
<td>2.6.1.2</td>
<td>Alanine aminotransferase</td>
<td>L-alanine + 2-oxoglutarate (\rightleftharpoons) pyruvate + L-glutamate</td>
<td>Blood</td>
</tr>
<tr>
<td>2.7.7.49</td>
<td>RNA-directed DNA polymerase</td>
<td>(n) Deoxyribonucleoside triphosphate (\rightleftharpoons) (n) pyrophosphate + DNA(_{n})</td>
<td>—</td>
</tr>
<tr>
<td>2.8.1.1</td>
<td>Thiosulfate sulfur transferase</td>
<td>Thiosulfate + cyanide (\rightleftharpoons) sulfate + thiocyanate</td>
<td>—</td>
</tr>
<tr>
<td>3.1.1.8</td>
<td>Cholinesterase</td>
<td>Acylcholine + H(_{2})O (\rightleftharpoons) choline + carboxylic acid anion</td>
<td>Blood</td>
</tr>
<tr>
<td>3.1.3.9</td>
<td>Glucose-6-phosphatase</td>
<td>D-Glucose 6-P + H(<em>{2})O (\rightleftharpoons) D-glucose + P(</em>{1})</td>
<td>—</td>
</tr>
<tr>
<td>3.1.4.1</td>
<td>Phosphodiesterase</td>
<td>Phosphodiester + H(_{2})O (\rightleftharpoons) phosphomonoester+ alcohol</td>
<td>—</td>
</tr>
<tr>
<td>3.1.6.1</td>
<td>Arylsulfatase</td>
<td>Phenol sulfate + H(_{2})O (\rightleftharpoons) phenol + sulfate</td>
<td>—</td>
</tr>
<tr>
<td>3.2.1.21</td>
<td>(\beta)-Glucosidase</td>
<td>Hydrolysis of terminal non-reducing (\beta)-D-glucose residues</td>
<td>Lysosomes</td>
</tr>
<tr>
<td>3.2.1.23</td>
<td>(\beta)-Galactosidase</td>
<td>Hydrolysis of terminal nonreducing (\beta)-D-galactose residues in (\beta)-D-galactosides</td>
<td>Lysosomes</td>
</tr>
<tr>
<td>3.2.1.51</td>
<td>(\alpha)-Fucosidase</td>
<td>An (\alpha)-L-fucose + H(_{2})O (\rightleftharpoons) an alcohol + L-fucose</td>
<td>Lysosomes</td>
</tr>
<tr>
<td>3.4.11.1</td>
<td>Cytosol aminopeptidase (leucine aminopeptidase)</td>
<td>Aminoacyl-peptide + H(_{2})O (\rightleftharpoons) amino acid + peptide</td>
<td>—</td>
</tr>
<tr>
<td>3.4.11.3</td>
<td>Cystyl-aminopeptidase (Oxytocinase)</td>
<td>Cystyl-peptides + H(_{2})O (\rightleftharpoons) amino acid + peptide</td>
<td>—</td>
</tr>
<tr>
<td>3.4.21.4</td>
<td>Trypsin</td>
<td>Hydrolyzes peptide bonds, preferentially Lys-X, Arg-X</td>
<td>—</td>
</tr>
<tr>
<td>3.6.1.1</td>
<td>Inorganic pyrophosphatase</td>
<td>Pyrophosphate + H(_{2})O (\rightleftharpoons) 2 orthophosphate</td>
<td>—</td>
</tr>
<tr>
<td>3.6.1.9</td>
<td>Nucleotide pyrophosphatase</td>
<td>A dinucleotide + H(_{2})O (\rightleftharpoons) 2 mononucleotides</td>
<td>—</td>
</tr>
<tr>
<td>4.2.1.11</td>
<td>Carbonate dehydratase</td>
<td>H(<em>{2})CO(</em>{3}) (\rightleftharpoons) CO(<em>{2}) + H(</em>{2})O</td>
<td>—</td>
</tr>
<tr>
<td>5.3.1.9</td>
<td>Glucose-6-phosphate isomerase</td>
<td>D-glucose-6-P (\rightleftharpoons) fructose-6-P</td>
<td>—</td>
</tr>
<tr>
<td>6.4.1.2</td>
<td>Acetyl-CoA carboxylase</td>
<td>ATP + acetyl-CoA (\rightleftharpoons) ADP + orthophosphate + malonyl-CoA</td>
<td>—</td>
</tr>
</tbody>
</table>

Source: Ref. 70.

SM = skin milk; FGM = fat globule membrane; P = phosphate.
which exposes the bond Lys$_{57}$-Arg$_{78}$ to hydrolysis by urokinase or tissue-type plasminogen activator. Hydrolysis of this bond yields a mature enzyme of $\sim$81 kDa, consisting of two polypeptide chains held together by a single disulfide bond. The five kringsles of plasminogen are retained in plasin; they are required for activity and are conserved in plasmins from different species. Bovine plasmin is also cleaved at Arg$_{342}$-Met$_{343}$ to yield midi plasmin (15). Bovine plasminogen cDNA has been cloned (18).

Plasmin is a serine proteinase (inhibited by di-isopropylfluorophosphate, phenylmethyl sulfonl fluoride, and trypsin inhibitors) with a high specificity for peptide bonds to which lysine or arginine supplies the carboxyl group. The active site is in the smaller of the two chains and consists of His$_{598}$, Asp$_{641}$, and Ser$_{736}$. It is optimally active at $\sim$ pH 7.5 and $\sim$ 35°C; it exhibits $\sim$ 20% of maximum activity at 5°C and is stable over the pH range 4–9.

Plasmin is usually extracted from casein by washing with water at pH 3.5 and purified by precipitation with (NH$_4$)$_2$SO$_4$ and various forms of chromatography, including affinity chromatography. Plasmin is quite heat stable; it is partially inactivated by heating at 72°C for 15 sec, but its activity in milk increases following HTST pasteurization, probably owing to inactivation of the indigenous inhibitors of plasmin or, more likely, inhibitors of plasminogen activators. It partly survives UHT sterilization and is inactivated by heating at 80°C for 10 min at pH 6.8; its thermal stability decreases with increasing pH in the range 3.5–9.2.

The inactivation of plasmin in milk follows first-order kinetics. Arrhenius plots show that inactivation is not linear with increasing temperature. In the temperature range 63–110°C, $E_a$ for inactivation is 52.75 and 74.44 kJ mol$^{-1}$ for low and high somatic cell count (SCC) milk, respectively, while in the range 100–130°C, $E_a$ is 22.03 and 24.70 kJ mol$^{-1}$ for low and high SCC milk, respectively (19). Plasmin is more heat stable to low-temperature treatments, e.g., thermization and HTST pasteurization, in high than in low SCC milk but the reverse is true in the UHT range (19). Milk with a high SCC has high plasminogen activator activity (19).

1. Assay of Plasmin Activity

Plasmin activity may be assayed on a wide range of substrates, including proteins, but the most widely used substrate is the synthetic fluorogenic peptide, N-succinyl-L-Ala-L-Phe-L-Lys-$\gamma$-amido-4-methyl coumarin; the liberated $\gamma$-amido-4-methyl coumarin is quantified by determining the intensity of fluorescence, with excitation at 380 nm and emission at 460 nm (20). The chromogenic substrate, Val-Leu-Lys-$\gamma$-nitroanilide, is also used (16). Plasminogen activity is measured by assaying plasmin activity before and after activation of indigenous plasminogen by an excess of added urokinase (16). Plasminogen activator activity may be assayed by the ability of an ultracentrifugal casein micelle pellet to activate exogenous (added) plasminogen (16).

2. Activity of Plasmin on Milk Proteins

$\beta$-Casein is the most susceptible milk protein to plasin action; it is hydrolyzed rapidly at Lys$_{28}$-Lys$_{29}$, Lys$_{102}$-His$_{106}$, and Lys$_{107}$-Glu$_{108}$ to yield $\gamma'$ ( $\beta$-CN f29-209), $\gamma^\prime$ ( $\beta$-CN f106-209), and $\gamma^3$ ( $\beta$-CN f108-209) caseins and protease peptone (PP)$_5$ ( $\beta$-CN f1-105/7), and PP8 slowly ( $\beta$-CN f29-105/7) and PP8 fast ( $\beta$-CN f1-29). In vitro (in solution), $\beta$-casein is also hydrolyzed fairly rapidly at Lys$_{113}$-Tyr$_{114}$ and Lys$_{183}$-Asp$_{184}$, but it is not known if these bonds are hydrolyzed in milk. $\gamma$-Caseins normally represent $\sim$ 3% of total N in milk but can be as high as 10% in late-lactation milk; as a percent of total N, the value for protease peptones is about half that of the $\gamma$-caseins.

$\alpha_{S2}$-Casein in solution is also hydrolyzed very rapidly by plasmin at bonds Lys$_{231}$-Gln$_{222}$, Lys$_{247}$-Asn$_{253}$, Arg$_{114}$-Asn$_{115}$, Lys$_{140}$-Lys$_{150}$, Lys$_{150}$-Thr$_{151}$, Lys$_{181}$-Thr$_{182}$, and Lys$_{188}$-Ala$_{189}$ (see 14), but its hydrolysis in milk has not been characterized. Although less susceptible than $\alpha_{S1}$ or $\beta$-caseins, $\alpha_{S1}$-casein in solution is also readily hydrolyzed by plasmin (14) but it does not appear to be hydrolyzed to a significant extent in milk, although it has been suggested that $\lambda$-casein is produced from $\alpha_{S1}$-casein by plasmin. Although $\kappa$-casein contains several Lys and Arg residues, it appears to be quite resistant to plasmin, presumably owing to a relatively high level of secondary and tertiary structures. The whey proteins are quite resistant to plasmin, probably owing to their compact, globular structures; in fact, $\beta$-lactoglobulin, especially when denatured, inhibits plasmin, presumably via sulfhydryl-disulfide interactions which rupture the structurally important kringles.

3. Significance of Plasmin Activity in Milk

There is sufficient plasmin in milk to cause very extensive proteolysis, but this is not realized owing to the presence of inhibitors. If the casein micelles are sedimented by ultracentrifugation and redispersed in buffer, very extensive proteolysis occurs on storage since
the inhibitors are removed in the ultracentrifugal serum. According to Guinot-Thomas et al. (17), microbial proteinases cause more proteolysis in milk than plasmin during storage at 4°C for 6 days.

Plasmin and plasminogen accompany the casein micelles on the rennet coagulation of milk and are concentrated in cheese in which plasmin contributes to primary proteolysis of the caseins, especially in cheeses that are cooked to a high temperature, e.g., Swiss and some Italian varieties, in which the coagulant is totally or extensively inactivated (21). β-Casein is the principal substrate and even in low-cooked cheeses (e.g., Cheddar, Gouda), in which the coagulant is the principal primary proteinase, proteolysis of β-casein is due mainly to plasmin action; some hydrolysis of αs1-casein by plasmin also occurs.

The level of plasmin activity in cheese varies substantially with the variety; Emmental, Parmesan, and Dutch-type cheeses contain about three times as much plasmin activity as Cheddar-type cheeses (22). This is probably due to the greater activation of plasminogen in the former as a result of inactivation of inhibitors of plasminogen activators in high-cooked cheese and their removal from Dutch-type cheese curd on washing (when whey is removed and replaced by water). Plasmin activity is decreased in cheeses made from UF-concentrated milk because of the retention of inhibitors of plasmin and plasminogen activators and β-lactoglobulin in the curd. Proteolysis is less in UF cheeses than in their conventional counterparts; presumably, the lower level of plasmin activity is a contributory factor.

It has been suggested that an elevated level of plasmin activity in late-lactation milk contributes to its poor cheese-making properties; however, an elevated level of somatic cells in milk does not appear to lead to defects in cheese (13). The casein micelles in bovine milk are capable of binding ~10 times as much plasmin as occurs naturally in milk. Exogenous plasmin added to milk is incorporated and uniformly distributed in the cheese curd, in which it accelerates proteolysis and maturation (23). When other exogenous proteinases are added to cheese milk, much of the added enzyme is lost in the whey, increasing cost and creating potential problems for whey processors. The yield of cheese may also be decreased owing to early hydrolysis of casein in the vat. For Cheddar-type cheese, exogenous proteinases may be added to the milled curds at salting, but the enzyme is concentrated at the surface of the curd chips. Activation of indigenous plasminogen by added urokinase also accelerates proteolysis (14, 23a).

Cheese analogues are usually produced from rennet casein which may contain active plasmin. Hydrolysis of β-casein and undesirable changes in the rheological properties of cheese analogs have been attributed to plasmin action (14).

Plasmin activity may contribute to the age gelation of UHT milk produced from high-quality raw milk (which contains a low level of Pseudomonas proteinase). The acid precipitability of casein from late-lactation milk is poor, but evidence for the involvement of plasmin is lacking. Reduced yields of cheese and casein can be expected to result from plasmin action in milk since the proteose peptones are, by definition, soluble at pH 4.6 and are not incorporated into acid- or rennet-produced casein curd.

4. Proteinase Inhibitors in Milk

Milk contains several broad-specificity plasma-derived proteinase inhibitors: α1-proteinase inhibitor, α2-antiplasmin, C1 inhibitor, antithrombin-III, α2-macroglobulin, inter-α-trypsin inhibitor, and two inhibitors analogous to human α1-antichymotrypsin which possess inhibitory activity against trypsin and elastase, respectively. Inhibitory activity is highly elevated in mastitic milk owing to increased leakage of blood proteins into milk. It has been proposed that trypsin inhibitory activity in milk may be a useful index of mastitis in cows. Bovine colostrum contains colostrum-specific proteinase inhibitors, including trypsin-inhibitory and thiol proteinase-inhibitory activities, which protect immunoglobulins and other biologically active proteins and peptides against proteolysis by gastrointestinal enzymes in the newborn (24, 25).

Apart from the colostrum-specific inhibitors, the plasma-derived inhibitors are present in milk as a result of membrane leakage (mastitis or late lactation) and therefore might be expected to be without significance. However, they are probably quite significant: (a) The level of plasmin in milk is sufficient to cause very extensive hydrolysis of the caseins, as can be readily demonstrated by dispersing ultracentrifugally sedimented casein micelles in buffer (plasmin and plasminogen accompany the casein micelles while the inhibitors are in the milk serum). Plasmin activity is increased by pasteurization apparently because proteinase inhibitors are inactivated by heating. (b) The inhibitors are retained in cheese made from milk concentrated by ultrafiltration (UF) whereas they are lost in the whey during conventional cheese making. Consequently, proteolysis is retarded in UF cheese (14, 25).
Denatured β-lactoglobulin also inhibits plasmin, apparently owing to sulfhydryl-disulfide interchange reactions. Since β-lactoglobulin is concentrated by UF, it probably contributes to the inhibition of proteolysis in cheese made from UF-concentrated milk during ripening.

5. Plasminogen Activators

There are two types of plasminogen activators, urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA). Both types occur in milk; uPA is confined to cells in milk while tPA is associated with the casein micelles (26, 27). Both activators have been characterized and cloned (28). Lu and Nielsen (29) reported that there are five plasminogen activators in milk, with molecular weights of 93, 57, 42, 35, and 27 kDa; most were uPA-type activators. PA level increases with mastitic infection and advancing lactation (30), which explains the greater conversion of plasminogen to plasmin in such milks.

B. Cathepsin D (EC 3.4.23.5)

It has been known for ∼ 30 years that milk also contains an acid proteinase (optimum pH ∼ 4.0) which is now known to be cathepsin D, a lysosomal enzyme. It is relatively heat labile (inactivated by 70°C for 10 min). Its activity in milk has not been studied extensively, and its significance is unknown. At least some of the indigenous acid proteinase is incorporated into cheese curd; its specificity on 1- and β-caseins is quite similar to that of chymosin, but it has very poor milk clotting activity (31). It may contribute to proteolysis in cheese, but its activity is probably normally overshadowed by chymosin, which is present at a much higher level in cheese.

C. Other Proteinases

The presence of other minor proteolytic enzymes in milk, including thrombin and a lysine aminopeptidase, has been reported (32). In addition to cathepsin D, other proteolytic enzymes from somatic cells are probably present in milk. Verdi and Barbano (33), who studied the degradation of caseins in milk by somatic cells or plasmin, found that somatic cell proteinases and plasmin produced distinctly different peptides, and that the plasmin inhibitor 6-aminohexanoic acid was suitable for studying the action of somatic cell proteinases, without interference from plasmin. Somatic cell proteinases are capable of activating plasminogen (34), and this may influence proteolysis in cheese by elevating plasmin levels. Although leucocyte proteinases were more active on β-casein at pH 6.6 than at pH 5.2, their activity at the lower pH was such as to suggest that they may be active in cheese during ripening (35). Suzuki and Katoh (36) found two cysteine proteinases in milk (45 kDa and > 150 kDa). The authors suggested that these proteinases originated in somatic cells and their level increased during mastitic infection.

Grieve and Kitchen (37) compared the action of leucocyte proteinases, plasmin, and some psychrophilic proteinases on the caseins. Leucocyte extracts hydrolyzed the caseins in order αs1 > β >> κ. Although these authors considered that neutral proteinases from leucocytes (isolated from blood) were unlikely to be important for proteolysis in milk, other authors have found considerably lower proteolytic activity in leucocytes isolated from blood than from milk (34, 35).

Kelly et al. (39), who compared proteolysis in Gouda cheeses made from milks with the same total somatic cell count but different levels of polymorphonuclear (PMN) leucocytes, found more rapid production of αs1-CN f24-199 and total free amino acids in cheese made from milk with high PMN levels (αs1-CN is αs1-casein).

The significance of minor indigenous proteins during mastitic infections was discussed by Fang and Sandholm (40) who investigated the possibility of using specific proteinase inhibitors as therapeutic agents. Although the minor proteinases are probably less significant technologically than plasmin, more work on the subject is warranted.

III. Lipases and Esteras (EC 3.1.1.–)

Lipases catalyze the development of hydrolytic rancidity which is a serious defect in milk and some milk products, and, consequently, lipases and lipolysis in milk have been studied extensively. Milk contains three types of esterase: (a) A-type carboxylic ester hydrolase (arylesterase; EC 3.1.1.2), which hydrolyzes aromatic esters, e.g., phenylacetate. It shows little activity on tributyrin, and is not inhibited by organophosphates. (b) B-type esterase (glycerol tricarboxyl esterase, aliphatic esterase, lipase; EC 3.1.1.3). Such enzymes are most active on aliphatic esters although they show some activity on aromatic esters; they are inhibited by organophosphates. (c) C-type esterase (cholinesterase; EC 3.1.1.7; EC 3.1.1.8). These enzymes are most active on choline esters but hydrolyze some
aromatic and aliphatic esters slowly; they are inhibited by organophosphates.

In normal milk, the ratio of A : B : C types of esterase activity is about 3 : 10 : 1 but the level of A-esterase activity increases considerably on mastitic infection. A and C esterases are of little technological significance in milk. Lipases hydrolyze ester bonds in emulsified esters, i.e., at a water/oil interface, although some may have limited activity on soluble esters. They are usually activated by blood serum albumin and Ca$^{2+}$ which bind free fatty acids, which are inhibitory.

Milk lipase was first isolated and characterized by Fox and Tarassuk (41) and Patel et al. (42). The enzyme was optimally active at pH 9.2 and 37°C and was found to be a serine enzyme (inactivated by organophosphates). A lipoprotein lipase (LPL; activated by lipoprotein cofactors) was demonstrated in milk by Korn in 1962 (42a) and isolated by Egelrud and Olivecrona (43). The lipase isolated by Fox and Tarassuk (41) is, in fact, an LPL which is the principal, probably the only, indigenous lipase in bovine milk. It has been the focus of considerable research and has been characterized at the molecular, genetic, enzymatic, and physiological levels (44).

Under optimum conditions, the $k_{cat}$ for milk LPL is $\sim 3000 \text{s}^{-1}$ and milk contains sufficient enzymes (1–2 mg/L; i.e., 10–20 nM) to theoretically cause rancidity in 10 sec. However, this does not occur in practice because the triglycerides are protected by the MFGM while the lipase is naturally associated with the casein micelles. Also, environmental conditions, e.g., pH, are not optimal. However, if the MFGM is damaged by agitation (e.g., by milking machines, bulk tanks, pumps, etc.), homogenization or temperature fluctuations, lipolysis occurs rapidly and rancidity ensues. Milk LPL appears to be derived from blood plasma and hence any condition that increases the permeability of mammary cell membranes, e.g., physiological stress, mastitic infection, or late lactation, increases the level of LPL in milk and hence the risk of lipolysis. Some individuals produce milk which becomes rancid spontaneously, i.e., without apparent activation. Apparently, spontaneous rancidity occurs when milk contains a high level of lipoprotein (co-lipase) from blood serum which activates the LPL. Normal milk will become spontaneously rancid if blood serum is added, suggesting that “spontaneous milks” contain a higher than normal level of blood serum. Dilution of spontaneous milk with normal milk prevents spontaneous rancidity, which, consequently, is not normally a problem with bulk herd milks. Presumably, dilution with normal milk decreases the lipoprotein content of the bulk milk to below the threshold necessary for lipase activation. Natural variations in the level of free fatty acids in normal milk and the susceptibility of normal milks to induced lipolysis may be due to variations in the level of blood serum components in milk.

In addition to LPL, human milk contains a bile salts–activated lipase, which probably contributes to the metabolism of lipids by breastfed babies who have limited pancreatic lipase activity. Bovine milk and milks from other dairy animals do not contain this enzyme.

**A. Assay of Lipolytic Activity**

Lipase can be assayed by incubating the sample with an emulsified lipid substrate—e.g., milk fat, olive oil, tributyrin, etc.—extraction of liberated fatty acids with diethyl ether-petroleum ether, and titration with ethanolic KOH. This method is rather tedious and tributyrin agar diffusion assays may be used when rapid screening is required. Chromogenic substrates, e.g., β-naphthyl- or p-nitrophenyl derivatives of fatty acids or fluorogenic substrates, e.g., coumarin derivatives of fatty acids, are very sensitive and satisfactory, especially when the enzyme has been at least partially purified.

**B. Significance of Lipase**

Technologically, LPL is, arguably, potentially the most significant indigenous enzyme in milk. Although LPL may play a positive role in cheese ripening, undoubtedly the most industrially important aspect of milk lipase is its role in hydrolytic rancidity which renders liquid milk and dairy products unpalatable and eventually unsaleable. Lipolysis in milk has been reviewed extensively (45). It appears to occur mainly at the farm level and the problem may be minimized by good management practices on the farm:

1. Proper installation, maintenance, and operation of milking machines.
2. Avoidance of excessive agitation by pumps or agitators in bulk tanks or risers in milk pipelines.
3. Avoidance of freezing on the walls of bulk tanks.
4. Avoidance of cooling and warming cycles in the bulk tank.
5. Culling of cows with high somatic cell counts.
The first three factors damage the MFGM, making the core triglycerides accessible to lipase. Some casein micelles probably adsorb on exposed fat surfaces. Pipeline milking machines cause more damage to the MFGM than hand milking or bucket milking machines. Suction of air at teat cups (which causes foaming), risers, and change of dimensions in the pipeline and the hose connecting the clawpiece to the receiving jar are major potential sites for damage. Homogenization causes total replacement of the natural MFGM by a membrane composed of casein micelles or submicelles and whey proteins. Unless indigenous LPL is inactivated by pasteurization before or immediately after homogenization, rancidity will develop very rapidly. Minimal high temperature short time (HTST; 72°C for 15 sec) pasteurization of milk causes extensive inactivation of LPL but pasteurization at 80°C for 10 sec is required for complete inactivation.

Freezing of milk on the walls of bulk tanks will damage the MFGM, inducing lipolysis. Temperature fluctuations, e.g., cooling to ~5°C, rewar ming to ~30°C, and recooling, also activate lipolysis. Such temperature fluctuations may occur if bulk tanks are not completely emptied at each milk collection and warm milk is added at the subsequent milking. The mechanism of thermal activation is not clear, but damage to the MFGM by fat crystals seems likely.

The propensity of milk to lipolysis increases when the producing animals are under any type of stress. The mammary cell membranes become more permeable to blood constituents as a result of mastitic infection in late lactation and as the animal ages. The number of somatic cells in milk is a good index of such stress or damage, and upper limits for somatic cell count (SCC) are now frequently prescribed by milk processors. Although the potential for hydrolytic rancidity always exists in raw milk, the problem can be reduced to insignificant levels by good management practices at the farm.

IV. ALKALINE PHOSPHATASES (EC 3.1.3.1)

Milk contains several phosphatases, the principal ones being alkaline and acid phosphomonoesterases, which are of technological significance, and ribonuclease, which has no known function or significance in milk. The alkaline and acid phosphomonoesterases have been studied extensively (1, 8, 9).

The occurrence of a phosphatase in milk was first recognized in 1925. Subsequently characterized as an alkaline phosphatase, it became significant when it was shown that the time–temperature combinations required for the thermal inactivation of alkaline phosphatase were slightly more severe than those required to kill *Mycobacterium tuberculosis*, then the target microorganism for pasteurization. The enzyme is readily assayed, and a test procedure based on alkaline phosphatase inactivation was developed as a routine quality control test for the HTST pasteurization of milk.

A. Assay Methods

Several major modifications of the original assay have been developed. The usual substrates are phenyl phosphate, *p*-nitrophenyl phosphate, or phenolphthalein phosphate which are hydrolyzed to inorganic phosphate and phenol, *p*-nitrophenol, or phenolphthalein, respectively:

\[
\text{X} - \text{O} - \text{P} - \text{O}^- + \text{H}_2\text{O} \rightarrow \text{HPO}_4^{2-} + \text{XOH}
\]

where \(\text{XOH} = \text{phenol, } p\text{-nitrophenol, or phenolphthalein.}\)

The release of inorganic phosphate may be assayed but the other product is usually determined. Phenol is colorless but forms a colored complex on reaction with one of several reagents, e.g., 2,6-dichloroquinonechlorimide, with which it forms a blue complex. *p*-Nitrophenol is yellow while phenolphthalein is red at the alkaline pH of the assay (~10) and hence these are easily quantified.

A fluorogenic aromatic orthophosphoric monooester, Fluorophos (Advanced Instruments, Needham Heights, MA), has been developed and approved for the determination of alkaline phosphatase in milk and milk products. Hydrolysis of the ester yields a fluorescent compound, Fluoroyellow, the concentration of which is determined fluorometrically (excitation, 439 nm; emission, 560 nm).

Fluorometric methods are about 100–1000 times more sensitive than colorimetric assays. A simple fluorometer has been developed for the analysis (Advanced Instruments). Comparative studies on the fluorometric and the standard colorimetric methods include those of Rocco (46) and Eckner (47).
B. Isolation and Characterization of Alkaline Phosphatase

Alkaline phosphatase is concentrated in the fat globule membrane and hence in cream. The membrane is released into the buttermilk on phase inversion; consequently, buttermilk is the starting material for most published methods for the purification of alkaline phosphatase. Later methods have used chromatography on various media to give a homogeneous preparation; up to ~7500-fold purification with a yield of ~30% have been reported (1). The characteristics of milk alkaline phosphatase are summarized in Table 4. The enzyme appears to be similar to the alkaline phosphatase of mammary tissue, which is the presumed source of the enzyme in milk.

The enzyme is a dimer of two identical 85-kDa subunits. It contains 4 Zn$^{2+}$ per mole which are required for activity; Mg$^{2+}$ are also strong activators, and 1 mM Mg$^{2+}$ is usually added to the assay mixture. The enzyme is strongly but reversibly inhibited by metal chelators; the apoenzyme is reactivated by Zn$^{2+}$, Mg$^{2+}$, and other metal ions. Reaction of apoenzyme may in fact be used as a sensitive assay for available Zn$^{2+}$ in foods. Inorganic orthophosphates are strong competitive inhibitors of the hydrolysis of p-nitrophenylphosphate. Alkaline milk phosphatase can dephosphorylate phosphoproteins, including casein, with a pH optimum of 6.5–7.0; however, it does not appear to do so in milk, probably owing to inhibition by the high level of orthophosphate.

Table 4 Characteristics of Milk Alkaline Phosphatase

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH optimum</td>
<td>Casein: 6.8 \ p-nitrophenylphosphate: 9.9</td>
</tr>
<tr>
<td>Temperature optimum</td>
<td>37°C</td>
</tr>
<tr>
<td>$K_m$</td>
<td>0.69 mM on \ p-nitrophenylphosphate</td>
</tr>
<tr>
<td>Activators</td>
<td>Ca$^{2+}$, Mn$^{2+}$, Zn$^{2+}$, Co$^{2+}$, Mg$^{2+}$</td>
</tr>
<tr>
<td>Inhibitors</td>
<td>Metal chelators (EDTA, EGTA, etc.); orthophosphates</td>
</tr>
<tr>
<td>Native molecular weights</td>
<td>170–190 kDa</td>
</tr>
<tr>
<td>Quaternary structure</td>
<td>2 subunits each of molecular weights 85 kDa</td>
</tr>
<tr>
<td>Zn content</td>
<td>4 mol mol$^{-1}$ enzyme</td>
</tr>
<tr>
<td>Thermal stability:</td>
<td></td>
</tr>
<tr>
<td>D-value at 60°C, pH 9</td>
<td>27.2 min</td>
</tr>
<tr>
<td>63°C, pH 9</td>
<td>8.3 min</td>
</tr>
</tbody>
</table>

C. Reactivation of Phosphatase

Much work has been focused on a phenomenon known as “phosphatase reactivation,” first recognized by Wright and Tramer in 1953, who observed that UHT-treated milk was phosphatase-negative immediately after processing but became positive on standing; microbial phosphatase was shown not to be responsible. Bulk HTST milk never showed reactivation, although occasional individual cow samples did. HTST pasteurization after UHT treatment usually prevented reactivation which was never observed in in-container sterilized milk. Reactivation can occur following heating at a temperature as low as 84°C for milk or 74°C for cream. The optimum storage temperature for reactivation is 30°C, at which reactivation is detectable after 6 h and may continue for up to 7 days. The greater reactivation in cream than in milk may be due to protection by fat, but this has not been substantiated.

A number of attempts have been made to explain the mechanism of reactivation (8). There is evidence that the form of the enzyme which becomes reactivated is membrane bound, and several factors which influence reactivation have been established. Mg$^{2+}$ and Zn$^{2+}$ strongly promote reactivation; Sn$^{2+}$, Cu$^{2+}$, Co$^{2+}$ and EDTA are inhibitory, while Fe$^{2+}$ has no effect. Sulphydryl (SH) groups appear to be essential for reactivation; perhaps this is why phosphatase becomes reactivated in UHT milk but not in HTST milk. The role of -SH groups, supplied by denatured whey proteins, is considered to be chelation of heavy metals, which would otherwise bind to -SH groups of the enzyme (also activated on denaturation), thus preventing renaturation. It has been proposed that Mg$^{2+}$ and Zn$^{2+}$ cause a conformational change in the denatured enzyme, necessary for renaturation (1).

According to Murthy et al. (48), maximum reactivation occurs in products heated at ~104°C; incubated at 34°C, adjusted to pH 6.5, and containing 0.064 M Mg$^{2+}$; homogenization of products before heat treatment reduces the extent of reactivation. There are reports that raw milk contains three isoenzymes of alkaline phosphatase and that the zymogram patterns of raw and reactivated milk or cream are different (1, 48); however, these different forms probably represent free and bound forms of the enzyme. A mechanism for the reactivation of alkaline phosphatase was proposed by Copius-Peereboom (49); however, this mechanism of reactivation is based on a putative structure of the milk fat globule membrane which is now known to be incorrect. Linden (50)
proposed that reactivation occurs in stages and full reactivation requires Zn$^{2+}$, Mg$^{2+}$, inorganic phosphate (Pi), and substrate (S).

Reactivation of alkaline phosphatase is of considerable practical significance since regulatory tests for pasteurization assume the absence of phosphatase activity. Methods for distinguishing between renatured and residual native alkaline phosphatase are based on the increase in phosphatase activity resulting from addition of Mg$^{2+}$ to the reaction mixture; various versions of the test have been proposed (48, 51, 52). The official AOAC method (53) is based on that of Murthy and Peeler (52). However, difficulties are experienced in the interpretation of this test applied to cream or butter (54, 55).

D. Significance

Alkaline phosphatase in milk is significant mainly because of its use as an index of HTST pasteurization and is used universally for this purpose. However, the enzyme may not be the most appropriate for this purpose (56) becomes (a) reactivation of alkaline phosphatase under certain conditions complicates interpretation of the test; (b) the enzyme appears to be fully inactivated by subpasteurization conditions (70°C for 16 sec); and (c) the relationship between log$_{10}$% initially activity and pasteurization equivalent (PE) is less linear than the relationship of lactoperoxidase or $\gamma$-glutamyl transpeptidase (57).

Although alkaline phosphatase can dephosphorylate casein under suitable conditions, as far as is known, it has no direct technological significance in milk. Perhaps its pH optimum is too far removed from that of milk, especially acid milk products, although the pH optimum on casein is reported to be ~7. It is also inhibited by inorganic phosphate.

Proteolysis is a major contributor to the development of flavor and texture of cheese during ripening (58). Most of the small water-soluble peptides in cheese are from the N-terminal half of $\alpha_{s1}$- or $\beta$-casein; many are phosphorylated but show evidence of phosphatase activity (i.e., they are partially dephosphorylated (59–62). In cheese made from pasteurized milk, both indigenous acid phosphatase and bacterial phosphatase are probably responsible for dephosphorylation (which is the more important is not clear), but in raw milk cheese, e.g., Parmigiano Reggiano or Grana Padano, milk alkaline phosphatase appears to be the most important (63, 64). Further work on the significance of indigenous alkaline and acid phosphatases in the dephosphorylation of phosphopeptides in cheese is warranted.

V. ACID PHOSPHOMONOESTERASE (EC 3.1.3.2)

Milk contains an acid phosphatase which has a pH optimum at 4.0 and is very heat stable. (LTLT pasteurization causes only 10–20% inactivation and 30 min at 88°C is required for full inactivation; when heated in milk at pH 6.7, the enzyme retains significant activity following HTST pasteurization but it does not survive in-bottle sterilization or UHT treatment.) The enzyme is not activated by Mg$^{2+}$ (as is alkaline phosphatase), but it is slightly activated by Mn$^{2+}$ and is very strongly inhibited by fluoride. The level of acid phosphatase activity in milk is only ~2% that of alkaline phosphatase; activity reaches a maximum 5–6 days postpartum, then decreases and remains at a low level to the end of lactation (65).

A. Isolation and Characterization

Acid phosphatase is found free in skim milk, in membrane material in skim milk and in the fat globule membrane. Kitchen (9) appears to believe that a single enzyme is involved and reported that the membrane-bound enzyme is strongly attached and is not released by nonionic detergents. The enzyme has been purified to homogeneity by various forms of chromatography, including affinity chromatography. Purification factors of 10,000 to 1 million have been reported (1). Adsorption onto Amberlite IRC50 resin is a very effective first step in purification. Andrews (65) stated that all the acid phosphatase activity in skim milk is adsorbed by Amberlite IRC50, but this is not indicated in the original papers. In an unpublished study, N.A. Flynn and P.F. Fox found that only ~50% of the total acid phosphatase in skim milk was adsorbed by Amberlite IRC50 even after reextracting the skim milk with fresh batches of Amberlite, suggesting that skim milk may contain two acid phosphatases. About 40% of the acid phosphatase in skim milk partitioned into the whey on rennet coagulation and this enzyme did not adsorb on Amberlite IRC50. The enzyme was partly purified from whey.

Flynn and Fox attempted to purify the alkaline phosphatase from MFGM by gel permeation chromatography; however, sonication and nonionic detergents failed to disassociate the enzyme from the membrane. The MFGM enzyme, which does not adsorb on
amberlite IRC50, was much less heat stable than the acid phosphatase isolated from whey or from skim milk by adsorption on Amberlite IRC50. Attempts were made to confirm that the MFGM, whey, and Amberlite-adsorbed enzymes are different by studying the effects of inhibitors, but the results have been equivocal. Overall, it appears that milk contains more than one acid phosphatase. Using a zymogram technique, Andrews and Alichanidis (66) reported that milk from healthy cows contained one acid phosphatase while that from mastitic cows contained two additional acid phosphatases which were of leucocyte origin. It is unlikely that the heterogeneity observed by Flynn and Fox was due to a high proportion of mastitic milk.

The acid phosphatase isolated from skim milk by adsorption on Amberlite IRC50 has been well characterized. It is a glycoprotein with a molecular weight of ~42 kDa and a pI of 7.9. It is inhibited by many heavy metals, F\(^{-}\), oxidizing agents, orthophosphates, and polyphosphates and is activated by thiol-reducing agents and ascorbic acid. It is not affected by metal chelators. Its amino acid composition indicates a high level of basic amino acids and no methionine (65).

The enzyme is quite active on phosphoproteins, including caseins. It has been suggested that it is a phosphoprotein phosphatase. Although casein is a substrate for milk acid phosphatase, the major caseins, in the order \(\alpha_\text{S1} > \alpha_\text{S2} > \beta > \kappa\), also act as competitive inhibitors of the enzyme when assayed on \(p\)-nitrophenylphosphate, probably owing to binding of the enzyme to the casein phosphate groups (the effectiveness of the caseins as inhibitors is related to their phosphate content).

B. Assay

Acid phosphatase may be assayed, at pH ~ 5, on the same substrates as used for alkaline phosphatase. If \(p\)-nitrophenyl phosphate or phenolphthalein phosphate is used, the pH must be adjusted to > 8 at the end of the enzymatic reaction in order to induce the color of the product, i.e., \(p\)-nitrophenol or phenolphthalein.

C. Significance

Although acid phosphatase is present in milk at a much lower level than alkaline phosphatase, its greater heat stability and lower pH optimum may make it technologically significant. Dephosphorylation of casein reduces its ability to bind \(\text{Ca}^{2+}\), to react with \(\kappa\)-casein, to form micelles, and its heat stability. As discussed in Section IV.D, several small partially dephosphorylated peptides have been isolated from Cheddar and Parmigiano Reggiano and Grana Padano cheeses. However, it is not known whether indigenous or bacterial acid phosphatase is mainly responsible for dephosphorylation in cheese made from pasteurized milk. It is claimed (62–64) that alkaline phosphatase is mainly responsible for dephosphorylation in raw milk cheese. Dephosphorylation may be rate limiting for proteolysis in cheese ripening since most proteinases and peptidases are inactive on phosphoproteins or phosphopeptides. It has been suggested that phosphatase activity should be included in the criteria for starter selection.

The acid phosphatase activity in milk increases fourfold to 10-fold during mastitic infection. Three isoenzymes are then present, only one of which is indigenous milk acid phosphatase, the other two being of leucocyte origin (66). The latter isoenzymes are more thermostable than the indigenous enzyme and are activated by HTST pasteurization.

The suitability of acid phosphatase as an indicator enzyme for superpasteurization of milk has been assessed (67, 68). It is not as useful for this purpose as some alternatives, e.g., \(\gamma\)-glutamyl transpeptidase or lactoperoxidase.

VI. LYSOZYME (EC 3.2.1.17)

Lysozyme (muramidase, mucopeptide N-acetyl-muramyl hydrolase) is a widely distributed enzyme which lyses certain bacteria by hydrolyzing the \(\beta(1-4)\) linkage between muramic acid and N-acetylglucosamine of mucopolysaccharides in the bacterial cell wall. Lysozyme activity is normally assayed by the lysis of cultures of \(\text{Micrococcus lysodeikticus}\) measured by a decrease in turbidity.

Lysozyme was isolated from human milk by Jolles and Jolles (69), who believed that bovine milk was devoid of lysozyme. Milks of many species, including bovine, have since been shown to contain lysozyme, and several have been isolated and characterized (70). Human and equine milks are exceptionally rich sources, containing 130 mg/L (3000 times the level of bovine milk) and ~ 800 mg/L, respectively.

The pH optima of human milk lysozyme (HML), bovine milk lysozyme (BML), and egg-white lysozyme (EWL) are 7.9, 6.35, and 6.2, respectively (70). BML has a molecular weight of 18 kDa compared with 15 kDa for HML and EWL. The amino acid composition of BML is considerably different from that of HML or EWL. The amino acid sequence of BML is...
highly homologous with that of \( \alpha \)-lactalbumin, a whey protein that is an enzyme modifier in the biosynthesis of lactose. All lysozymes are relatively stable to heat at acid pH values (3–4) but are relatively labile at pH > 7. More than 75% of the lysozyme activity in bovine milk survives heating at 75°C for 15 min or 80°C for 15 sec and is therefore little affected by HTST pasteurization. Low concentrations of reducing agents increase the activity of BML and HML by \( \sim 330\% \) (70).

Presumably, the physiological role of lysozyme is to act as a bacteriocidal agent. In the case of milk, it may simply be a “spillover” enzyme, or it may have a definite protective role. If the latter is true, then the exceptionally high level of lysozyme in human and equine milk may be significant. Breastfed babies generally suffer fewer enteric problems than bottle-fed babies. While there are many major compositional and physicochemical differences between bovine and human milks which may be responsible for the observed nutritional characteristics, the disparity in lysozyme content may be significant. Fortification of bovine milk-based infant formulae with EWL, especially for premature babies, has been recommended but feeding studies are equivocal on the benefits of this practice, and recent trials failed to demonstrate any beneficial effect due to inactivation of EWL in the human stomach (71, 72).

One might expect that, owing to this bacteriocidal effect, indigenous milk lysozyme would have a beneficial effect on the shelf life of milk. Such effects do not appear to have been reported. Exogenous lysozyme may be added to milk for many cheese varieties, e.g., Gouda, Edam, Emmental, and Parmigiano Reggiano, as an alternative to \( \text{KNO}_3 \) to prevent the growth of \( \text{Cl. tyrobutyricum} \) which causes late gas blowing and off-flavors. At present, lysozyme is not widely used in commercial cheesemaking (71, 73). Since indigenous milk lysozyme is in the serum phase, very little is incorporated into cheese.

Addition of lysozyme to milk decreases the heat stability of milk, but the level of indigenous lysozyme is probably too low to contribute to the natural variations in the heat stability of milk.

VII. N-ACETYL-\( \beta \)-D-GLUCOSAMINIDASE (EC 3.2.1.30)

N-Acetyl-\( \beta \)-D-glucosaminidase (NAGase) hydrolyzes terminal, nonreducing N-acetyl-\( \alpha \)-D-glucosamine residues from glycoproteins. It is a lysosomal enzyme which originates principally from mammary gland epithelial cells and, to a lesser extent, from somatic cells. Consequently, NAGase activity correlates highly with the intensity of mastitis. A field test for mastitis based on NAGase activity has been developed, using chromogenic \( p \)-nitrophenyl N-actyl-\( \beta \)-D-glucosamine as substrate. Hydrolysis yields \( p \)-nitrophenol, which is yellow at alkaline pH. NAGase activity is also high in colostrum. NAGase is optimally active at 50°C and pH 4.2 and is inactivated by HTST pasteurization (70–71°C for 15–18 sec). Andrews et al. (68) proposed that NAGase would be a suitable indicator enzyme for assessing heat treatments in the range 65–75°C for 15 sec. NAGase occurs mainly in the whey fraction, from which it has been isolated by various forms of chromatography. Two forms, A and B, differing in molecular weight, 120 and 240 kDa, respectively, and charge were obtained. Each form is dissociated to two dissimilar subunits on treatment with 2-mercaptoethanol and SDS (9, 70).

VIII. \( \gamma \)-GLUTAMYL TRANSEPTIDASE (TRANSFERASE) (EC 2.3.2.2)

\( \gamma \)-Glutamyl transpeptidase (GGTP) catalyzes the transfer of \( \gamma \)-glutamyl residues from \( \gamma \)-glutamyl-containing peptides:

\[
\gamma \text{-glutamyl-peptide} + X \rightarrow \text{peptide} + \gamma \text{-glutamyl-X}
\]

where X is an amino acid.

The enzyme is membrane bound, being found in the membrane material in skim milk (\( \sim 70\% \)) or in the MFGM, from which it can be dissociated by detergents or solvents. The enzyme, which has been purified from the MFGM, has a molecular weight of \( \sim 80 \text{kDa} \) and consists of two subunits of 57 and 25 kDa, both of which are glycoproteins (74, 75). The enzyme is optimally active at pH 8.5–9 and \( \sim 45 \text{C} \) and has an isoelectric point of 3.85. It is strongly inhibited by diisopropylfluorophosphate, iodoacetamide, and metals, e.g., \( \text{Cu}^{2+} \) and \( \text{Fe}^{3+} \) (9, 70).

A. Assay

GGTP is usually assayed using \( \gamma \)-glutamyl-\( p \)-nitroanilide as substrate; the liberated \( p \)-NA can be determined by measuring the absorbance at 410 nm or by reaction with naphthylethlenediamine and measuring the absorbance at 540 nm (76).
B. Significance

GGTP functions in the regulation of cellular glutathione and amino acid transport via the γ-glutamyl cycle; it may be involved in the biosynthesis of milk proteins.

From a dairy technologist’s viewpoint, GGTP is of interest mainly because of its heat stability characteristics. As discussed in Section IV.D, alkaline phosphatase is the test enzyme usually used to evaluate the efficiency of HTST pasteurization; however, as discussed, reactivation of this enzyme in UHT-treated products poses problems with the interpretation of the test. Based on a comparative study on the heat stability characteristics of a number of indigenous enzymes in milk, Andrews et al. (68) concluded that GGTP was appropriate for monitoring heat treatments in the range of 70–80°C for 16 sec. This conclusion has been confirmed in pilot-scale studies (77, 78). In whole or skim milk, GGTP is completely inactivated by heating at 78°C for 15 sec (77) or 77°C for 16 sec (76). No reactivation was found under a variety of conditions, and little seasonal variation occurs. As little as 0.1% raw milk could be detected in skim milk or 0.25% in whole milk (76).

Linear models for the thermal inactivation of GGTP and lactoperoxidase (LPO) in a HTST pasteurizer were developed by McKellar et al. (57). The equation for GGTP was: \( \log_{10}\% \text{ initial activity} = 2.004 - 0.281 \times PE^{0.75} \). For LPO the equation was: \( \log_{10}\% \text{ initial activity} = 2.122 - 0.096 \times PE^{0.75} \).

\[
P E = \frac{t}{t_0} = \int e \left( \frac{E_a}{R} \left( \frac{1}{T} - \frac{1}{T_0} \right) \right) dt
\]

where \( E_a \) = activation energy; \( R = 8.314 \text{ J/mol } \cdot \text{K} \); \( T_0 = 345 \text{ K} \) (72°C reference temperature); \( T = \) experimental temperature; \( t_0 = 15 \text{ sec (reference holding time)} \); \( t = \) experimental holding time, sec; and \( PE = \) pasteurization equivalent (71.6°C for 15 sec).

These equations indicate that 1 log decrease in enzyme activity can be achieved with PE values of 5.46 and 2.65 for GGTP and LPO, respectively, indicating that LPO is considerably more heat stable than GGTP. The assay for LPO (using ABTS; see Sec. XIII.A) is subject to greater variation and interference from milk proteins than the GGTP assay. The relationship between \( \log_{10}\% \text{ initial activity} \) and \( PE \) was more linear than the relationship for alkaline phosphatase, possibly due to more than one form of alkaline phosphatase (56). GGTP was about nine times more stable in ice cream mix than in whole milk, which had a much smaller stabilizing effect on Listeria innocua (79). Thus, it appears that GGTP is a suitable enzyme for estimating the intensity of heat treatment in the range 72–77°C for 15 sec to which milk was subjected.

GGTP is absorbed from the gastrointestinal tract, resulting in high levels of GGTP activity in the blood serum of newborn animals fed colostrum or early breast milk. Since GGTP is inactivated by the heat treatment to which infant formulae are subjected, the level of GGTP activity in infants can be used to distinguish breastfed from formula-fed infants (70).

γ-Glutamyl peptides have been isolated from Comte cheese (80). Since casein contains no γ-glutamyl bonds, the presence of these peptides in cheese suggests GGTP activity in cheese, but there appear to be no data on this.

IX. XANTHINE OXIDASE (EC 1.2.3.2)

It has been recognized for ~90 years that milk contains an enzyme capable of oxidizing aldehydes and purines with the concomitant reduction of \( \text{O}_2 \) to \( \text{H}_2\text{O}_2 \). The enzyme is now generally referred to as xanthine oxidase (XO). Milk is a very good source of XO, at least part of which is transported to the mammary gland via the bloodstream. A similar enzyme is found in various animal tissues and several bacterial species (8, 9, 70).

A. Assay

Xanthine oxidase activity can be assayed manometrically (uptake of \( \text{O}_2 \)), potentiometrically using a platinum electrode, or spectrophotometrically. This last involves the conversion of the xanthine to uric acid which is quantified by measuring absorbance at 290 nm (75).

B. Isolation

The enzyme is concentrated in the MFGM, in which it is one of the principal proteins. Therefore, all isolation methods use cream as starting material, using a dissociating agent to liberate XO from membrane lipoproteins and some form of chromatography for purification.

Milk XO has a molecular weight of ~300 kDa and consists of two identical subunits. The pH optimum is ~8.5 and the enzyme requires flavin adenine dinucleotide (FAD), Fe and Mo cations, and an acid-labile sulfur compound as cofactors. Cows deficient in Mo

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cations have low XO activity. The amino acid composition of XO has been determined by a number of workers; at least five polymorphic forms have been reported (70).

XO can be converted to an NAD-dependent dehydrogenase by treatment with thiol-reducing agents. The enzyme reverts to an oxidase on aerobic storage, treatment with sulfhydryl oxidase, or with sulfhydryl oxidizing agents.

C. Activity in Milk

XO activity in milk varies substantially—2.5-fold (67). Various processing treatments which damage or alter the MFGM affect the XO activity of milk. Activity is increased by ~100% on storage at 4°C for 24 h, by 50–100% on heating at 70°C for 5 min, and by 60–90% on homogenization. These treatments cause the release of XO from the MFGM into the aqueous phase, rendering the enzyme more active. The heat stability of XO is very dependent on whether it is a component of the MFGM or is dissolved in the aqueous phase. Aging and homogenization increase heat susceptibility and explain the inconsistency of early work in which the history of the sample was unknown or unrecorded. XO is most heat stable in cream and least in skim milk. Homogenization of concentrated milk prepared from heated milk (90°C for 15 sec) partially reactivates XO, which persists on drying the concentrate, but no reactivation occurs following more severe heating (93°C for 15 sec). Apparently, homogenization releases potentially active, undenatured XO from the MFGM. All the major milk proteins can act as either activators or inhibitors of XO, depending on their concentration, and may have some significance in the activation, inactivation, and reactivation of the enzyme. Studies on the heat stability of XO have been reviewed by Griffiths (67), who investigated its stability in a pilot scale HTST pasteurizer. The enzyme was not completely inactivated after 120 sec at 80°C, and a Z-value of 6.8 was calculated.

D. Significance of Xanthine Oxidase

1. As an Index of Heat Treatment

The inactivation of XO parallels the conditions necessary for the production of low-heat skim milk powder (82). Andrews et al. (68) considered XO as a suitable indicator of milk heated in the temperature range 80–90°C but Griffiths (67) considered the natural variability in the level of XO activity in milk to be too high for use as a reliable index of heat treatment.

2. Lipid Oxidation

XO, which can excite stable triple oxygen (\(^3\)O\(_2\)) to single oxygen (\(^1\)O\(_2\)), is a pro-oxidant. Some individual-cow milks, which undergo spontaneous oxidative rancidity (i.e., without contamination with metals or exposure to light) contain ~10 times the normal level of XO, and spontaneous oxidation can be induced in normal milk by the addition of XO to about four times normal levels. Heat-denatured or flavin-free enzyme is ineffective in oxidation; the susceptibility of unsaturated fatty acids to oxidation increases with the degree of unsaturation (8).

3. Atherosclerosis

It has been suggested that XO from homogenized milk enters the vascular system and may be involved in atherosclerosis via oxidation of plasmalogen in cell membranes; this aspect of XO attracted considerable attention in the early 1970s (83). However, the experimental evidence in support of this view is very weak and the hypothesis has been disclaimed (8, 70, 84, 85).

4. Reduction of Nitrate in Cheese

Sodium nitrate is added to milk for Dutch, Swiss, and other cheese varieties to prevent the growth of Clostridium tyrobutyricum which causes flavor defects and late gas blowing in these cheeses. Xanthine oxidase reduces nitrate to nitrite which is necessary for the bacteriocidal effect of nitrate.

Various oxidation-reduction reactions occur in milk which affect its flavor. Perhaps XO is significant in these reactions, either directly or indirectly.

5. Production of H\(_2\)O\(_2\)

The H\(_2\)O\(_2\) produced by the action of xanthine oxidase on oxidation of xanthine, hypoxanthine, or other substrates can serve as a substrate for lactoperoxidase in its action as a bacteriocidal agent (see Sec. XIII.B).

X. SULFHYDRYL OXIDASE (EC 1.8.3.–)

Milk contains an enzyme, sulfhydryl oxidase (SO), capable of oxidizing sulfhydryl groups of cysteine, glutathione, and proteins to the corresponding disulfide (for reviews, see 8, 70). The enzyme is an aerobic oxidase which catalyzes the following reaction:

\[ 2\text{RSH} + \text{O}_2 \rightarrow \text{RSSR} + \text{H}_2\text{O}_2 \]  

(5)
It undergoes marked self-association and can be purified readily by chromatography on porous glass. The enzyme has a molecular weight of ~89 kDa, a pH optimum of 6.8–7.0, and a temperature optimum of 35°C. Its amino acid composition, its requirement for iron but not for molybdenum and FAD, and the catalytic properties of the enzyme indicate that sulfhydryl oxidase is a distinct enzyme from xanthine oxidase and thiol oxidase (EC 1.8.3.2).

SO is capable of oxidizing reduced ribonuclease and restoring enzymatic activity, suggesting that its physiological role may be the nonrandom formation of protein disulfide bonds, e.g., during protein biosynthesis.

SO immobilized on glass beads has the potential to ameliorate the cooked flavor arising from sulfhydryl groups exposed by protein denaturation on UHT processing of milk, but the commercial viability of this system is not known. In any case, sulfhydryl groups are effective antioxidants and may serve a beneficial role in UHT milk.

The production of sulfur compounds is believed to be very important in flavor development in Cheddar and other varieties of cheese. Residual sulfhydryl oxidase activity may play a role in reoxidizing sulfhydryl groups exposed upon heating cheesemilk; the sulfhydryl groups thus protected may be reformed during the ripening process.

XI. SUPEROXIDE DISMUTASE (EC 1.15.1.1)

Superoxide dismutase (SOD) scavenges superoxide radicals, \( O_2^- \), according to the reaction:

\[
2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2
\]  
(6)

The \( H_2O_2 \) formed may be reduced to \( H_2O + O_2 \) by catalase, peroxidase or suitable reducing agents. SOD has been identified in many animals and bacterial cells. Its biological function is to protect tissue against oxygen free radicals in anaerobic systems (8, 9, 70).

SOD, isolated from bovine erythrocytes, is a blue-green protein due to the presence of copper, removal of which by treatment with EDTA results in loss of activity which is restored by adding \( Cu^{2+} \); it also contains \( Zn^{2+} \), which does not appear to be at the active site. The enzyme, which is very stable in 9 M urea at neutral pH, consists of two identical subunits of molecular weight 16 kDa held together by one or more disulfide bonds.

Milk contains trace amounts of SOD which is present exclusively in the skim milk fraction. This enzyme has been isolated and characterized. It appears to be identical to the bovine erythrocyte enzyme. Assay methods for SOD are described by Stauffer (86).

SOD inhibits lipid oxidation in model systems. The level of SOD in milk parallels that of XO (but at a lower level), suggesting that SOD may be excreted in milk in an attempt to offset the pro-oxidant effect of XO. Attempts have been made to correlate the stability of milk to oxidative rancidity with the SOD activity in the milk, but these results have been equivocal. Milk contains several pro- and antioxidants, including exogenous factors such as light, the precise balance of which, rather than any single factor, determines overall oxidative stability. The possibility of using exogenous SOD to retard or inhibit lipid oxidation in dairy products has been considered. A marked improvement in the oxidative stability of milk with a high level of linoleic acid was achieved by adding low levels of SOD.

SOD is more heat stable in milk than in purified preparations. In milk it is stable at 71°C for 30 min (i.e., it is not affected by HTST pasteurization) but loses activity rapidly at even slightly higher temperatures. Slight variations in pasteurization temperature are therefore critical to the survival of SOD in heated milk products and may contribute to variations in the stability of milk to oxidative rancidity.

XII. CATALASE (EC 1.11.1.6)

An indigenous catalase in milk was first recognized in 1907. The catalase activity of whole milk is associated either with membranes in the skim milk phase or the MFGM. The pellet obtained from buttermilk on centrifugation at 10,000 g is a particularly rich source, from which catalase has been highly purified and crystallized (8, 9, 70).

Milk catalase is a heme protein with a molecular weight of 200 kDa and an isoelectric pH of 5.5. It is stable between pH 5 and 10 but rapidly loses activity outside the range. Heating at 70°C for 1 h at pH 7.0 causes complete inactivation. Like other catalases, it is strongly inhibited by \( Hg^{2+} \), \( Fe^{2+} \), \( Cu^{2+} \), \( Sn^{2+} \), \( CN^- \), and \( NO_3^- \). Analytical methods for catalase are described by Stauffer (86).

Catalase activity in milk varies with feed, stage of lactation, and especially with mastitic infection, for which it may be used as an index. However, it is not usually used for this purpose, since somatic cell count and N-acetylglucosaminidase activity are superior indices of mastitis. Catalase may act as a lipid pro-
oxidant via its heme iron (i.e., nonenzymatically), but it is probably not very significant.

There is general agreement that cheese made from raw milk ripens more quickly and develops a more intense (although not always a more desirable) flavor than cheese made from pasteurized milk (87). However, for public health reasons and in the interest of producing a consistent product, pasteurized milk is now generally used for cheese making. Many cheeses are still made from raw milk, especially in southern Europe. Many countries require that if raw milk is used for cheese making, the cheese must be ripened for at least 60 days during which it was presumed that pathogens die off, a presumption that is now considered to be invalid. Subpasteurized or thermized milk (e.g., heated at 63–65°C for 16 sec) has been considered as a compromise between raw and pasteurized milk for cheese making. The acceptance of such a practice would require an appropriate validation test, but no suitable test is available at present for identifying thermized milk owing to the production of catalase. The thermal inactivation of catalase was also studied in the cheese during ripening, especially by yeasts. The occurrence of a peroxidase, lactoperoxidase (LPO), in milk was recognized as early as 1881. It is one of the most heat-stable enzymes in milk. Its inactivation was used as an index of thermized milk was investigated by Hirvi and Griffiths (88). Although catalase was a useful index of thermization of milk (it was almost completely inactivated by heating at 65°C for 16 sec), it was unsuitable as an index of cheese made from thermized milk owing to the production of catalase in the cheese during ripening, especially by yeasts. The thermal inactivation of catalase was also studied by Hirvi et al. (89).

XIII. LACTOPEROXIDASE (EC 1.11.1.7)

The occurrence of a peroxidase, lactoperoxidase (LPO), in milk was recognized as early as 1881. It is one of the most heat-stable enzymes in milk. Its inactivation was used as an index of flash pasteurization (now very rarely used) and is now used as an index of super-HTST pasteurization, e.g., temperatures > 75°C for 15 sec. LPO was first isolated in 1943; several isolation procedures have since been published (8, 90).

LPO is a heme protein containing ~ 0.07% Fe, with an absorbance peak (Soret band) at 412 nm (A_{412}/A_{280} ~ 0.9). The pH optimum is ~ 8.0, its molecular weight is 77.5 kDa, and it consists of two identical subunits. Two principal forms (A and B) occur, each of which exhibits microheterogeneity with regard to amide groups (glutamine and/or asparagine) and carbohydrate content, giving a total of 10 variants.

A. Assay of Lactoperoxidase

The principle generally used in assays for peroxidases is the use of a chromogenic or fluorogenic reducing agent, AH2, a number of which have been used (86). A highly recommended substrate for lactoperoxidase is 2, 2'-azinobis(3-ethylbenzythiazoline-6-sulfonic acid) [ABTS] (91).

B. Significance

1. As for many other indigenous enzymes, the level of LPO in milk increases on mastitic infection and is therefore a possible index of mastitis; however, it is not well correlated with somatic cell count, and superior methods, including enzyme-based methods (Sec. VII) are available to monitor mastitis.

2. LPO causes nonenzymic oxidation of unsaturated lipids, probably due to its heme group. The heat-denatured enzyme is more active than the native enzyme. Compared with other pro-oxidants, LPO is probably not significant in milk and dairy products.

3. LPO has been used in the Storch test for flash pasteurized milk (67), but this process is not used in modern milk processing. However, the pasteurization of milk at a temperature higher than the HTST minimum, i.e., > 72°C for 15 sec, has recently become quite common. The assay based on the inactivation of alkaline phosphatase is not applicable under such circumstances. Griffiths (67), who evaluated the suitability of several indigenous enzymes as indices of the super-pasteurization of milk, concluded that assay of LPO activity was the most promising method for detecting heat treatments in the order of 76°C for 15 sec. Andrews et al. (68) reported the results of a generally similar study, in which LPO was not included. They conclude that N-acetylglucosaminidase, γ-glutamyl transpeptidase (γ-GGTP), and α-mannosidase or xanthine oxidase may be the most suitable indicators of heat treatment of 65–75, 70–80, and 80–90°C, respectively. The relative suitability of GGTP and LPO were compared by McKellar et al. (57). The kinetics of the thermal denaturation of LPO was reported by Martin-Hernandez et al. (92).

4. Milk contains bacteriostatic or bacteriocidal substances, referred to as lactenins. One of these is LPO, which requires H2O2 and thiocyanate (SCN−) to cause inhibition. The nature, mode of action, and specificity of the LPO-SCN−-H2O2 system have been widely studied. LPO and thiocyanate, which is produced in the rumen by enzymatic hydrolysis of thio-
glycosides from Brassicae plants, occur naturally in milk although at variable and probably suboptimal levels. Milk does not contain indigenous H₂O₂, which can be generated metabolically by catalase-negative bacteria, or produced in situ through the action of exogenous glucose oxidase on glucose which may be added to milk or produced in situ from lactose by exogenous β-galactosidase or the action of indigenous XO on added xanthine or hypoxanthine, or from added sodium percarbonate or it may be added directly.

Immobilized glucose oxidase has been used to generate H₂O₂ in situ in thiocyanate- and glucose-enriched milk or whey. A self-contained LPO-H₂O₂-SCN⁻ system using coupled β-galactosidase and glucose oxidase, immobilized on porous glass beads, to generate H₂O₂ in situ from lactose in milk containing 0.25 mM thiocyanate has been developed.

The bacteriocidal effect of the LPO-H₂O₂-SCN⁻ system has several applications which have been reviewed extensively (90, 91, 93, 94):

1. Sanitization of immobilized enzyme columns in which LPO is coimmobilized with the enzyme of interest. The requisite H₂O₂ could be generated by immobilized enzymes—e.g., xanthine oxidase, glucose oxidase, or β-galactosidase/glucose oxidase.
2. As a bacteriocidal agent in toothpaste.
3. In the therapy of mastitis during the non-lactating period.
4. For the preservation of milk in regions lacking refrigeration or pasteurization facilities.
5. To reduce the incidence of enteritis in calves or piglets fed milk replacers. The indigenous LPO is inactivated during the manufacture of these products and LPO isolated from milk or whey is added.

LPO (and lactoferrin) is cationic at the natural pH of milk at which all the principal proteins are anionic. LPO and lactoferrin can therefore be readily isolated from milk or whey by using cation exchange resins. The ion exchangers may be used as columns (95), batchwise (96), or as membranes (97). At least some of these methods are applicable on an industrial scale, making it possible to isolate LPO for use as a food ingredient.

C. Biochemistry of Lactoperoxidase System

LPO catalyzes the peroxidation of −SCN to products which are nontoxic to mammalian cells but which kill or inhibit the growth of many species of microorganisms. The net reaction is:

\[
\text{H}_2\text{O}_2 + ^{-}\text{SCN} \xrightarrow{\text{LPO}} ^{-}\text{OSCN} + \text{H}_2\text{O} 
\]  (thiocyanate anion) (hypothiocyanite anion) (7)

Microbial membranes have low permeability for −OSCN but are quite permeable to HOSCN (pKₐ = 5.3). HOSCN or −OSCN oxidizes sulfhydryl groups:

\[
\text{RSH} + ^{-}\text{OSCN} \rightarrow \text{R-S-SCN} + ^{-}\text{OH} 
\]  (sulfenyl thiocyanate) (8)

\[
\text{R-S-SCN} + \text{H}_2\text{O} \rightarrow \text{R-S-OH} + \text{H}^+ + ^{-}\text{SCN} 
\]  (sulfonic acid) (9)

Any reaction involving a sulfhydryl group, e.g., thiol enzymes, will be inhibited by this oxidation. The effect may be reversed by thiol compounds such as glutathione or cysteine.

XIV. OTHER ENZYMES

In addition to the enzymes described above, several other indigenous enzymes have been isolated and partially characterized (Table 2) (70). Although a fairly high level of some of these enzymes occurs in milk, they have no apparent function or significance in milk which contains no substrate for many of them. It is possible that some of these enzymes will assume importance in the future as indices of animal health or of product quality. These enzymes will not be discussed further.

Nearly 40 other enzymatic activities have been detected in milk (Table 3) but have not been isolated, and only limited information on their molecular and biochemical properties in milk is available (70). Some of these enzymes have been evaluated as indices of the heat treatment of milk (67, 68). Perhaps further study will identify some important or useful attributes of some of these enzymes.

XV. SUMMARY

Although milk contains ~60 indigenous enzymes, only two, lipoprotein lipase (LPL) and plasmin, are really technologically significant. LPL has the potential to cause serious problems but these can be avoided through good milking practices on the farm. The enzyme is relatively heat labile and hence does not cause problems in heat-treated products. Although milk contains considerable potential plasmin, relatively
little is expressed owing to the presence of inhibitors. The significance of plasmin is mainly negative but its action is probably positive in cheese during ripening. Acid phosphatase may be significant in the dephosphorylation of phosphopeptides in cheese. Several oxidoreductases—xanthine oxidase, lactoperoxidase, catalase, superoxide dimutase, and sulfhydryl oxidase—are, at least potentially, significant in milk stability. Lysozyme and lactoperoxidase may have antimicrobial effects in the intestine of the consumer.

Perhaps the most significant and most useful aspect of the indigenous enzymes in milk is as indicators of mastitis, especially N-acetylglucosaminidase, and of thermal treatments. The thermal stabilities of the indigenous enzymes cover quite a wide range of temperatures which make it possible to determine at what temperature milk has been heat-treated in the range 65–90°C for 15 sec. The principal advantage of enzymes compared with other heat-induced changes in analytical applications is the relative ease with which their activity can be quantified.

Most dairy products should undergo no change during storage, and hence the indigenous enzymes capable of causing undesirable changes are inactivated by heating. Cheese is an exception; a very complex series of microbiological, biochemical, and perhaps chemical reactions occur which lead to desirable characteristic taste, aroma, and texture of the finished cheese (98, 99). At least four indigenous enzymes contribute to cheese ripening—plasmin, lipoprotein lipase, acid phosphatase, and xanthine oxidase. Perhaps others contribute, but information is lacking.

Most of the indigenous enzymes in milk remain to be isolated and characterized. As for the principal enzymes, these minor enzymes probably originate from the mammary tissue. Most of these enzymes are unlikely to be technologically significant as milk contains no substrates. However, they may be significant as indicators of animal health or thermal history; if so, they will be isolated and characterized.

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Exogenous Enzymes in Dairy Technology

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I. INTRODUCTION

Milk from domesticated animals and products formed therefrom have been components of the human diet for \(\sim 10,000\) years. Some dairy products are consumed in most or all regions of the world and they are major dietary items in many regions, e.g., Europe, North America, and South America. Total milk production is \(\sim 530 \times 10^6\) metric tons per year, of which \(\sim 85\%\) is bovine milk; sheep, goats, water buffalo, camels, and mares are important dairy animals in some regions. Milk is a very perishable commodity and hence there has been a strong incentive to convert it to more stable products, which is facilitated by certain properties of milk. Classically, milk has been preserved by fermentation, usually with salt addition (cheese, fermented milks, butter). Newer preservation methods include drying, pasteurization/sterilization, and freezing. Some characteristics of milk also render it very amenable to modification by enzymes; that milk is a liquid facilitates enzyme addition. Cheese production is probably the oldest, and is still the largest, application of exogenous enzymes in food processing.

Since the principal components of milk are proteins (\(\sim 3.5\%\)), lipids (\(\sim 3.6\%\)), and lactose (\(\sim 4.8\%\); a disaccharide containing galactose and glucose), the principal enzymes used in dairy technology are proteinases and peptidases, lipases, and \(\beta\)-galactosidase (lactase). However, several oxidoreductases have significant applications.

The principal applications of enzymes in dairy technology will be considered in this review. Earlier reviews include Fox and Grufferty (1), Fox (2), Fox and Stepaniak (3), Brown (4), and Desmazeaud and Spinnler (5).

II. PROTEINASES

Bovine milk contains \(\sim 3.5\%\) proteins which can be resolved into two groups based on solubility at pH 4.6 and 20°C: the caseins, which are insoluble under these conditions and which represent \(\sim 80\%\) of the total protein; and the whey (serum) proteins, which are soluble. The casein fraction of bovine milk and that of the other main dairying species comprises four proteins, \(\alpha_s1\), \(\alpha_s2\), \(\beta\), and \(\kappa\)-caseins, which, although they have certain features in common, e.g., insolubility at pH 4.6, are distinctly different proteins. The whey protein fraction also comprises four main proteins—\(\beta\)-lactoglobulin, \(\alpha\)-lactalbumin, blood serum albumin, and immunoglobulins—and several minor proteins, including \(\sim 60\) indigenous enzymes. Readers are referred to Fox (6) for a detailed description of the milk protein system.

As discussed in Section II.A.1, the colloidal stability of the caseins is extensively changed by limited proteolysis, leading to gelation of the milk system, which is the first step in the production of many cheese varieties. The milk proteins can be easily separated from the other milk constituents and the caseins and whey...
proteins readily separated from each other on an industrial scale. Both the caseins and whey proteins have good and diverse functional properties; consequently, milk proteins are the preferred functional food proteins for a wide range of applications (7). Some functional properties can be improved and/or modified by limited proteolysis.

Proteolytic enzymes are the most widely used enzymes in dairy technology and will be discussed below under three headings: cheese manufacture, modification of functional properties, and production of protein hydrolyzates for nutritional and other applications.

A. Cheese Manufacture

The manufacture of all cheese varieties essentially involves concentrating the protein and fat of milk sixfold to 12-fold, depending on the variety. Concentration is achieved by (a) coagulating the principal milk proteins, i.e., the caseins (if present, the milk fat is occluded in the coagulum); (b) cutting or breaking the coagulum and inducing it to synerese under the influence of heat and acid; (c) separation of curds and whey; and (d) acidification, pressing, and salting of wheyed curd.

Coagulation of the casein is induced by one of three methods:

1. Limited proteolysis by a crude proteinase (rennet), which is exploited in the manufacture of all ripened and some fresh cheeses (~75% of total production).

2. Isoelectric precipitation at ~pH 4.6, used for fresh cheeses, usually by in situ production of lactic acid by a culture (starter) of lactic acid bacteria and less frequently by direct acidification with preformed acid, usually HCl, or acidogen, usually gluconic acid–δ-lactone.

3. Acid plus heat, i.e., acidification to ~pH 5.2 with acid whey, acid milk, citrus juice, vinegar, or acetic acid at 80–90°C; this method is used to produce a small number of relatively minor varieties, e.g., Ricotta.

Concentration of the total colloidal phase of milk (i.e., fat and total protein) to the level present in cheese, i.e., to a “pre-cheese,” by ultrafiltration is now used commercially for the manufacture of several cheese varieties. Additions of rennet and starter to the concentrate are still necessary for texture and flavor development.

1. Enzymatic Coagulation of Milk

The principal gastric enzyme of neonatal ruminants is chymosin rather than pepsin. Chymosin has low general proteolytic activity but high milk-clotting activity. Presumably, it evolved to coagulate milk in the stomach and thus delay its discharge into the intestine and increase the efficiency of digestion. Shortly after the domestication of dairy animals (~8000 B.C.), humans learned to exploit the ability of chymosin and some other proteinases, collectively referred to as rennets, to coagulate milk for the production of cheese, which was probably the first application of enzymes in food processing.

The rennet coagulation of milk is a two-stage process. The first (primary) phase involves the enzymatic production of “para-casein” and TCA-soluble peptides (glycomacropeptides), while the secondary phase involves the Ca-induced gelation of para-casein at a temperature in the range of 30–35°C. Proteolysis is essentially complete before the onset of coagulation.

The enzymatic coagulation of milk exploits certain properties of the caseins. As discussed above, bovine casein consists of four proteins—αs1-, αs2-, β-, and κ-caseins—in the approximate ratio of 40:10:35:12. These contain 8–9, 10–13, 4–5, and 1–2 mol of P per mol, respectively. Owing to their high phosphate content, αs1-, αs2-, and β-caseins bind Ca2+ strongly and precipitate at Ca2+ > 6 mM. However, κ-casein binds Ca2+ weakly and is soluble at high Ca2+. It also reacts hydrophobically with αs1-, αs2-, and β-caseins and can stabilize up to 10 times its weight of these Ca2+-sensitive caseins against precipitation by forming colloidal aggregate, called micelles.

In milk, >95% of the casein exists as micelles, which consist, on a dry weight basis, of ~94% protein and 6% of other species, mainly Ca2+ and PO43− with some Mg2+ and citrate, collectively called colloidal calcium phosphate (CCP). The micelles are spherical, 50–600 nm (mean, ~120 nm) in diameter, with particle weights of ~109 daltons; i.e., a typical micelle contains ~5000 monomers (Mw = 20–24 kDa). The micelles typically bind ~2 g H2O/g protein.

Within the micelles, the caseins are held together by CCP bridges, hydrophobic interactions, and hydrogen bonds. There is a widely held view that the casein monomers are organized as submicelles (spherical particles, Mw ~ 5 × 106 daltons). The micelles dissociate when CCP is removed (e.g., by Ca2+ chelators or acidification/dialysis), or when the pH is increased above ~9, or on addition of detergents (e.g., sodium dodecyl
sulfate) or urea. In most models of the casein micelle, it is envisaged that the Ca\(^{2+}\)-sensitive \(\alpha_{s1}\), \(\alpha_{s2}\), and \(\beta\)-caseins interact hydrophobically to form the core of the micelles, with \(\kappa\)-casein located predominantly on the surface. The N-terminal two-thirds of \(\kappa\)-casein is hydrophobic and reacts hydrophobically with the core proteins, leaving the hydrophilic C-terminal region projecting into the surrounding environment. It has been proposed that submicelles contain variable amounts of surface \(\kappa\)-casein and aggregate such that \(\kappa\)-casein-rich submicelles predominate at the surface of the micelles with the \(\kappa\)-casein-deficient submicelles buried within. The micelles are stabilized by a zeta potential of \(\sim -20\) mV and by steric factors caused by the protruding C-terminal segments of \(\kappa\)-casein which form a “hairy” layer, 7–10 nm thick, on the surface of the micelles, preventing close approach.

\textit{a. Primary Phase of Rennet Action.} During the primary phase of rennet action, \(\kappa\)-casein is the only protein hydrolyzed to a significant extent. It is cleaved by chymosin, and for most of the other proteinases used as rennets, at the bond Phe105-Met106, which is cleaved by chymosin, and for most of the other proteinases protein hydrolyzed to a significant extent. It is cleaved primarily as intact \(\kappa\)-casein and aggregate such that the \(\kappa\)-casein-rich submicelles predominate at the surface of the micelles with the \(\kappa\)-casein-deficient submicelles buried within. The micelles are stabilized by a zeta potential of \(\sim -20\) mV and by steric factors caused by the protruding C-terminal segments of \(\kappa\)-casein which form a “hairy” layer, 7–10 nm thick, on the surface of the micelles, preventing close approach.

A number of attempts have been made to explain the unique sensitivity of the Phe-Met bond. Di-, tri-, or tetrapeptides containing a Phe-Met bond are not hydrolyzed. However, the Phe-Met bond is hydrolyzed in the pentapeptide, H.Leu-Ser-Phe-Met-Ala-OMe, i.e., a derivative of \(\kappa\)-CN f103–107. The length of the peptide and the sequence around the cleavage site are important determinants of enzyme-substrate interaction. Ser\(_{104}\) is particularly important and its replacement by Gly or Ala in the above pentapeptide renders the Phe-Met bond very resistant to hydrolysis by chymosin but not by pepsins. Even substituting D-Ser for L-Ser markedly reduces the sensitivity of the adjacent Phe-Met bond. Extension of the above pentapeptide from the N- and/or C-terminal to reproduce the sequence of \(\kappa\)-casein increases the efficiency of hydrolysis of the Phe-Met bond by chymosin; the tetradecapeptide, \(\kappa\)-CN f98–111, is hydrolyzed as efficiently as intact \(\kappa\)-casein and \(\sim 66,000\) faster than the parent pentapeptide, \(\kappa\)-CN f103–107, with a k\(_{\text{cat}}\)/K\(_M\) of \(\sim 2\) M\(^{-1}\) sec\(^{-1}\).

The two residues, Phe-Met, are not intrinsically essential for chymosin action. Replacement of Phe by Pheno or cyclohexylalanine decreases k\(_{\text{cat}}\)/K\(_M\) about threefold and \(\sim 50\)-fold, respectively. Oxidation of Met\(_{106}\) decreases k\(_{\text{cat}}\)/K\(_M\) \(\sim 10\)-fold but substitution of Ile for Met increases this ratio about threefold. In fact, the chymosin-susceptible bond in porcine or human \(\kappa\)-caseins is Phe-Ile, which is readily hydrolyzed by calf chymosin. Thus, the sequence around the Phe-Met bond, rather than the bond itself, contains the important determinants of hydrolysis. The sequence Leu\(_{103}\)-Ser-Phe-Met-Ala-Ile\(_{108}\) of \(\kappa\)-casein, which may exist as a \(\beta\)-structure, fits into the active site cleft of acid proteinases. The hydrophobic residues, Leu\(_{103}\), Phe\(_{105}\), Met\(_{106}\), and Ile\(_{108}\), probably interact with hydrophobic residues along the active site cleft while the hydroxyl group of Ser\(_{104}\) forms a hydrogen bridge with a counterpart on the enzyme. Residues 98–102 and 109–111 probably form \(\beta\)-turns around the edges of the active site cleft in the enzyme-substrate complex; this conformation is stabilized by Pro residues at positions 99, 101, 109, and 110. One or more of the three His residues, 98, 100, 102, and Lys\(_{111}\), are probably involved in electrostatic bonding.

Pepsins and most other acid proteinases used as rennets hydrolyze \(\kappa\)-casein at Phe\(_{105}\)-Met\(_{106}\) but the acid proteinase of Cryphonectria parasitica hydrolyzes Ser\(_{104}\)-Phe\(_{105}\). Although the specificity of cathepsin D on \(\alpha_{s1}\) and \(\beta\)-caseins is generally similar to that of chymosin, it has very poor milk-clotting activity. The aggregation characteristics of micelles vary with the rennet used, suggesting differences in the extent and/or specificity of the hydrolysis of \(\kappa\)-casein or perhaps of the other caseins. Furthermore, the commonly used rennets have markedly different specific activities on synthetic \(\kappa\)-casein-related peptides.

Reviews on the enzymatic coagulation of milk include Dalgleish (8, 9), Fox (10–12), Fox and Mulvihill (13), and Fox and McSweeney (14).

\textit{b. Rennets.} The rennets used to coagulate milk are crude preparations of selected proteinases. Many proteinases can coagulate milk but most are too proteolytic relative to their milk clotting activity and hydrolyze the coagulum too quickly, causing reduced cheese yield and/or defective, e.g., bitter cheese. Traditionally, rennets were prepared from calves’, kids’, or lambs’ stomachs; the principal proteinase in such rennets is chymosin. The molecular and enzy-
matic properties of chymosins have been studied extensively (15–17).

Owing to increasing world production of cheese (~3% per year over the past 30 years), concomitant with a reduced supply of calf vells, the supply of veal rennet has been inadequate for many years, which has led to a search for rennet substitutes. Although many proteinases can coagulate milk, only six have been found to be more or less acceptable as rennets: bovine, porcine, and chicken pepsins and the acid proteinases from Rhizomucor miehei, R. pusillus, and C. parasitica. Chicken pepsin is the least suitable of these and is used only in special circumstances. Bovine pepsin gives generally satisfactory results with respect to cheese yield and quality; many commercial “calf rennets” contain a substantial proportion of bovine pepsin. Although the proteolytic specificity of the three commonly used fungal rennets on α_{s1}- and β-caseins is considerably different from that of calf chymosin, they generally yield acceptable cheese and were widely used in the United States before the introduction of microbial recombinant chymosin. Acid proteinases from flowers of the genus Cynara are used to coagulate sheep’s milk for some artisanal cheeses in Portugal and Spain, especially Serra d’Estrala cheese; these proteinases are not generally suitable as rennets. The extensive literature on rennet substitutes has been reviewed (18–21).

The gene for calf chymosin has been cloned in selected bacteria, yeasts, and molds. Chymosin from genetically engineered Kluyveromyces marxianus var. lactis (Gist-brocades), Escherichia coli (Pfizer), and Aspergillus nidulans (Hansens) is commercially available and used extensively, with excellent results; however, these products are not yet permitted in all countries. Reviews on microbial recombinant chymosin include Teuber (22) and IDF (23).

Microbial recombinant chymosin preparations contain no pepsin whereas 5–50% of the milk-clotting activity of calf rennets may be due to pepsin; hence, some minor differences in the pattern of proteolysis in cheese made with microbial recombinant chymosin or calf rennet are observed, most notably the formation of the peptide Asp^244^-Phe^246^-CN f110–199 in cheese made using calf rennet (owing to the action of pepsin). For those wishing to simulate the action of calf rennet more closely, blends of microbial recombinant chymosin and bovine pepsin are commercially available.

Calf chymosin contains three isoenzymes—A, B, and C. A and B are gene products that differ from each other by one amino acid; A has Asp at position 243 while B has Gly at this position. Chymosin C appears to be a degradation product of chymosin A which lacks three residues, Asp^244^-Phe^246^-CN. Commercially available microbial recombinant chymosins contain only chymosin A or B; it is not known if the different forms of chymosin differ in specificity, but it is claimed by rennet manufacturers that the cheesemaking properties of chymosin A and B are not equivalent.

The primary, and probably higher, structures of commercially available microbial recombinant chymosins are identical to that of calf chymosin. However, several modified chymosins have been produced from genetically engineered microorganisms. At present, the objective of these investigations is to study the mechanism of chymosin action at the molecular level, but it is probable that chymosin with improved cheese-making properties will emerge from such studies, e.g., enzymes with increased activity on certain bonds shown to produce cheese with improved quality or reduced activity on other bonds, cleavage of which results in flavor or textural defects. It should be remembered that chymosin evolved to coagulate milk in the neonatal stomach (to improve the efficiency of digestion) and not to produce cheese. It is fortuitous that chymosin is the best protease for cheese production, not just for milk coagulation, but it is highly probable that it can be improved. For reference on genetically engineered chymosins, see (14, 17).

Most (70–90%) of the rennet added to cheese milk is lost in the whey. Therefore, the possibility of immobilizing rennet has been investigated as a means of extending its working life. Several rennets have been immobilized but their efficacy as milk coagulants has been questioned. There is widespread support for the view that properly immobilized enzymes can not coagulate milk owing to inaccessibility of the Phe-Met bond of κ-casein and that the apparent coagulating activity of immobilized rennets is due to leaching of enzyme from the support. Even if immobilized rennets could hydrolyze micellar κ-casein, operational difficulties would exist at the cheese factory level. Furthermore, as discussed in Section II.A.2.d, the residual rennet in cheese curd plays an essential role in cheese ripening and it would be necessary to add some chymosin or similar enzyme to the curd after coagulation, which would be difficult or impossible (see 8, 9, 24 for reviews).

### c. Factors Affecting the Hydrolysis of κ-Casein

The pH optimum for chymosin and bovine pepsin is ~ 4.7 on small Phe-Met-containing peptides and 5.3–5.5 on κ-CN f98–111 or on whole κ-casein. The pH optimum for the first stage of rennet action
in milk is ~6.0. Milk for most cheese varieties is renneted at about pH 6.5.

Increasing ionic strength (0.01–0.11) decreases the rate of hydrolysis of κ-CN f98–112, especially if the pH is also increased. At 1 mM, NaCl, CaCl₂, and MgCl₂ stimulate the hydrolysis of κ-casein in isolated form or in sodium caseinate.

The optimum temperature for the coagulation of milk by calf rennet at pH 6.6 is ~45°C. The temperature coefficient (Δ rate/10°C) for the hydrolysis of κ-casein in solutions of Na-caseinate is ~1.8, the Ɛₒ is ~10,000 cal mol⁻¹ and the activation entropy is ~−39 cal deg⁻¹ mol⁻¹; generally similar values have been reported for the hydrolysis of isolated κ-casein.

The efficacy of κ-casein as a substrate for chymosin decreases as its level of glycosylation increases. At pH 6.6, κ-casein hydrolysis decreases from ~43 sec⁻¹ for carbohydrate-free κ-casein to ~25 sec⁻¹ for κ-casein containing 6 moles N-acetyl neuraminic acid (NANA) per mol. However, Ɛₒ is lowest for the κ-casein component containing 3 moles NANA per mol. Polymerization (aggregation) markedly increases Ɛₒ with little effect on κ_cat.

d. Secondary (Nonenzymatic) Phase of Rennet Coagulation. Hydrolysis of κ-casein removes its highly charged, hydrophilic C-terminal segment from the surface of the casein micelles, thereby reducing their zeta potential from ~−20 mV to ~−7 mV and removing the steric stabilizing layer. When ~85% of the total κ-casein has been hydrolyzed, the casein micelles begin to aggregate and eventually form a gel. Reducing the pH or increasing the temperature from the normal (~6.6 and ~31°C, respectively) induces coagulation at a lower degree of κ-casein hydrolysis.

The mechanism involved in the coagulation of rennet-altered micelles is not known precisely. Coagulation is dependent on Ca²⁺ and on colloidal calcium phosphate, which are exchangeable to a certain extent. Ca²⁺ may function by neutralizing negative charges on the caseins. Coagulation is highly temperature dependent; rennet-altered micelles do not coagulate below ~20°C, above which the Q₁₀°C for coagulation is ~16. The high temperature dependence of coagulation suggests that hydrophobic bonds may be involved or that multiple bonds are formed.

The rennet coagulability of milk is adversely affected by heat treatments at temperatures >65°C and is prevented by very severe heat treatments (>90°C for 10 min). Although changes in the equilibria of milk salts, especially calcium phosphate, are contributory factors, complexation of κ-casein with β-lactoglobulin and/or α-lactalbumin is primarily responsible for the increased rennet coagulation time of heated milk; both the primary phase and especially the secondary phase are adversely affected. The adverse effects of heating can be reversed by acidification before or after heating or by addition of CaCl₂.

Rennet-coagulated milk gels are relatively stable if left undisturbed but syneresis strongly if cut or broken. The rate and extent of syneresis are promoted by reducing the pH, increasing the temperature, and applying pressure, e.g., agitation. By controlling the extent of syneresis, the cheese maker can control the moisture content of cheese which is a major factor affecting the rate and pattern of ripening and the stability of cheese. Differences in moisture content are, in fact, a major factor responsible for the diversity of cheese flavor and texture.

2. Proteolysis During Cheese Ripening

Acid-coagulated cheeses are usually consumed fresh, but the vast majority of rennet-coagulated cheeses are ripened (matured) for a period ranging from ~3 weeks to >2 years; the rate of ripening is directly related to the moisture content of the cheese. During ripening, numerous microbiological, biochemical, and chemical events occur, as a result of which the principal constituents of the cheese—the proteins, lipids, and lactose—are transformed to primary, and later to secondary, products. Among the principal flavor compounds present in most cheese varieties are: peptides, amino acids, amines, acids, thiols, and thioesters (derived from proteins); fatty acids, methyl ketones, lactones, esters, and thioesters (derived from lipids); organic acids (lactic, acetic, and propionic); carbon dioxide; esters; and alcohols (derived from lactose). At appropriate concentrations and combinations, these compounds are responsible for the characteristic flavor of the various cheese varieties.

The biochemistry of cheese ripening has been reviewed by Fox et al. (25, 26) and Fox and Wallace (27); only proteolysis is discussed here.

a. Significance of Proteolysis. Proteolysis is essential in all rennet-coagulated cheese varieties, especially internal- and surface-bacterially ripened cheeses in which it is probably the principal biochemical event during ripening. Proteolysis contributes to cheese ripening in at least four ways: (a) makes a direct contribution to flavor, or off-flavor, e.g., bitterness, or indirectly since free amino acids are catabolized to amines, acids, thiols, thioesters, etc.; (b) facilitates the release of sapid compounds during mastication; (c) the
production of NH$_3$ from amino acids released by proteolysis affects flavor and texture; (d) changes in texture due to breakdown of the protein network, increase in pH, and greater water binding by the newly formed amino and carboxyl groups. There is a good correlation between the intensity of Cheddar cheese flavor and the extent and depth of proteolysis.

Considerable information is available on the level and type of proteolysis in the principal cheese groups (14, 25, 26, 28–33).

b. Proteolytic Agents in Cheese. Four, and in some varieties five, agents contribute to proteolysis in cheese during ripening: rennet or rennet substitute; indigenous milk enzymes, especially plasmin; starter bacteria and their enzymes, released on cell lysis; non-starter bacteria, which either survive pasteurization of the cheese milk or gain access to the pasteurized milk; and secondary inocula, the cheese milk or gain access to the pasteurized milk starter bacteria, which either survive pasteurization of bacteria and their enzymes, released on cell lysis; non-indigenous milk enzymes, especially plasmin; starter cheese during ripening: rennet or rennet substitute; some varieties five, agents contribute to proteolysis in (14, 25, 26, 28–33).

The proteolytic specificity of calf chymosin on $\alpha_{s1}$-, $\alpha_{s2}$-, and $\beta$-caseins in solution has been established and these findings can, largely, be extended to cheese (14, 33).

The principal chymosin cleavage sites on $\alpha_{s1}$-casein in cheese are: Phe$_{23}$-Phe$_{24}$, which is hydrolyzed rapidly and completely in cheese (e.g., within ~ 3 months in Cheddar), and Leu$_{101}$-Lys$_{102}$, which is cleaved fairly extensively; Phe$_{33}$-Gly$_{34}$ and Leu$_{98}$-Leu$_{99}$ are also cleaved to some extent in cheese. Surprisingly, the Trp$_{164}$-Tyr$_{165}$ bond, which is the second most susceptible bond in $\alpha_{s1}$ casein in solution, does not appear to be hydrolyzed in cheese. The small peptide, $\alpha_{s1}$-CN f1–23, does not accumulate in cheese but is hydrolyzed rapidly by the cell envelope-associated lactococcal proteinase, with a specificity dependent on the strain (34).

Some peptide bonds in $\beta$-casein in solution are hydrolyzed quite rapidly by chymosin in the order: Leu$_{192}$-Tyr$_{193}$, Ala$_{189}$-Phe$_{190}$, Leu$_{163}$-Ser$_{164}$, and Leu$_{139}$-Leu$_{140}$. In cheese, these bonds are hydrolyzed to a very limited extent or not at all, probably because the C-terminal region of $\beta$-casein is very hydrophobic and undergoes hydrophobically driven interactions in cheese. These interactions appear to be accentuated by NaCl; even in solution, the hydrolysis of $\beta$-casein is strongly inhibited by 5% NaCl, which is the typical NaCl concentration in the aqueous phase of many cheese varieties. Inhibition of the hydrolysis of $\beta$-casein in cheese is desirable since the peptide $\beta$-CN f193–209 and fragments thereof are very bitter.

Although $\alpha_{s2}$-casein in solution is fairly readily hydrolyzed by chymosin, its fate in cheese is not clear, and para-$\kappa$-casein ($\kappa$-CN f1–105) is very resistant to chymosin (and to other proteases in cheese).

The specificity of pepsins is generally similar to that of chymosin but has not been established precisely. Bovine pepsin cleaves the Leu$_{109}$-Glu$_{110}$ bond of $\alpha_{s1}$-casein quite rapidly, a bond which is cleaved very slowly by chymosin. The specificity of the fungal rennet substitutes is quite different from that of chymosin (35). The principal cleavage sites of R. miehei proteinase in $\alpha_{s1}$-casein in solution are Phe$_{23}$-Phe$_{24}$, Met$_{123}$-Lys$_{124}$, and Tyr$_{165}$-Tyr$_{166}$, while those in $\beta$-casein are Glu$_{31}$-Lys$_{32}$, Val$_{59}$-Val$_{59}$, Met$_{93}$-Gly$_{94}$, and Met$_{93}$-Gly$_{94}$.
of sapid compounds—amines, acids, NH₃, thiols—alterations via chemical mechanisms leads to a range of amino acids by microbial enzymes and perhaps if the activity of such enzymes is excessive. Catabolism least to background flavor and perhaps to bitterness small peptides and amino acids. These contribute at microbial proteinases and peptidases which produce particularly bitter.

The proteolytic activity of the coagulant in cheese influences quality in four ways:

1. Some rennet-produced peptides may have a positive influence on flavor but excessive or unbalanced proteolysis, e.g., too much or excessively proteolytic rennet or unsuitable environmental conditions, e.g., too much moisture or too little NaCl, leads to bitterness. The peptide β-CN f193–209 derived from the C-terminal of β-casein and fragments thereof are particularly bitter.

2. Rennet-produced peptides serve as substrates for microbial proteinases and peptidases which produce small peptides and amino acids. These contribute at least to background flavor and perhaps to bitterness if the activity of such enzymes is excessive. Catabolism of amino acids by microbial enzymes and perhaps alterations via chemical mechanisms leads to a range of sapid compounds—amines, acids, NH₃, thiols—which are major contributors to characteristic cheese flavors.

3. Alterations in cheese texture appear to influence the release of flavorful and aromatic compounds, arising from proteolysis, lipolysis, glycolysis, and secondary metabolic changes, from cheese during mastication, and this may be the most significant contribution of proteolysis to cheese flavor.

4. Texture is an important attribute of all cheeses and is critical in some varieties. Protein forms a continuous solid matrix in cheese, and its hydrolysis leads to a softening of the texture. Chymosin is primarily responsible for textural changes during the early stages of ripening. The functionality (stretchability and meltability) of mozzarella is strongly influenced by proteolysis; a low level of proteolysis improves functionality, but quality deteriorates on further proteolysis.

3. Acceleration of Cheese Ripening

The original objective of cheese manufacture was conservation of the principal nutrients in milk (i.e., lipids and proteins) by a combination of acidification, dehydration, low E₅₀, and salting. Chemical and biochemical changes do occur during storage but stability was the prime objective. While still important, stability is no longer the primary objective of cheese manufacture, and since ripening is expensive, its acceleration, especially in low-moisture, slow-ripening varieties, is desirable, at least under certain circumstances, provided the whole process can be maintained in balance.

Some high-moisture cheeses develop an intense flavor through a very active secondary flora, e.g., internal blue mold, external white mold, or a bacterial surface smear; these cheeses ripen quickly, e.g., 4–16 weeks. High-moisture internal bacterially ripened cheeses also mature rapidly but develop a low flavor intensity; if the ripening of such cheeses is extended, they will probably develop off-flavors. It is possible to develop an intense flavor in internal bacterially ripened cheeses only if the moisture content is low and they are ripened for a long period, e.g., Parmesan, extramature Cheddar, or extramature Gouda, which are ripened for 2–3 years. Owing to the high cost of ripening facilities and stocks, the ripening of extramature cheeses is expensive. Consequently, there is commercial interest in accelerating the ripening of these cheeses, provided quality can be maintained. It might also be possible to apply similar techniques to medium-moisture, medium-flavor cheeses, e.g., regular Cheddar and Gouda, with the objective of accentuating their flavor. Most of the work on accelerating cheese ripening has in fact been on Cheddar. Techniques for accelerating ripening may also be applicable to reduced-fat cheeses, which tend to ripen slowly. A substantial literature on attempts to accelerate cheese ripening has accumulated and has been reviewed regularly (36).

Glycolysis is rapid in all cheeses and does not require acceleration. Lipolysis is limited in most cheeses and excessive lipolysis is undesirable. Consequently, most studies on accelerated ripening of cheese have focused on proteolysis, which contributes to flavor and is mainly responsible for changes in texture.

Methods for accelerating cheese ripening fall into six categories: elevated ripening temperature, exogenous enzymes, chemically or physically modified cells, genetically modified starters, adjunct starters, and enzyme-modified cheeses. These methods either seek to make the conditions under which indigenous enzymes function more favorable (i.e., elevated temperature) or to increase the level of certain key enzymes which are considered to be particularly important in cheese ripening.
A complex cascade of enzymes is involved in cheese ripening, and most key enzymes have not been identified. Not surprisingly, the use of single enzymes, e.g., additional coagulant (which is mainly responsible for primary proteolysis), plasmin (for which some benefits are claimed), or Neutrase (from \textit{B. subtilis}), while accelerating proteolysis, does not accelerate flavor development and may cause off-flavors. It has been claimed that a combination of exogenous proteinases and lactococcal cell-free extracts (rich in peptidases) accelerate ripening, but the results are equivocal. Uniform incorporation of the enzyme preparation into cheese curd poses problems. For Cheddar, the enzyme preparation, diluted with salt, may be added to milled curd, but this method is not applicable to most varieties. Addition of microencapsulated enzyme(s) to cheese milk is technically feasible, but microencapsulation techniques currently available are not very efficient. At present, exogenous enzymes are not being used commercially to accelerate the ripening of natural cheese.

Exogenous lipase, traditionally pregastriac esterase, is added to certain hard Italian cheeses, e.g., Romano and provolone. It has been claimed that inclusion of selected lipases in the blend of exogenous enzymes accelerates the ripening of other cheeses, e.g., Cheddar and Ras.

Most of the enzymes involved in cheese ripening are produced by microorganisms that grow in or on the cheese. By increasing the number of these organisms it should be possible to accelerate ripening. The use of whole cells should have two advantages over isolated enzymes: they contain the “natural” cocktail of enzymes found in cheese and they are cheaper to produce. Three approaches have been considered in the use of bacterial cells to accelerate cheese ripening: attenuated starter cells, genetically modified cultures, and adjunct cultures.

The starter, in addition to its essential role in curd acidification, is also essential for secondary proteolysis and flavor development. Therefore, it might be expected that increasing the number of starter cells in cheese would accelerate ripening. However, increasing the level of active starter added to the cheese milk causes an excessively rapid rate of acid production which has undesirable effects, e.g., a crumbly texture and overacid flavor. The acid-producing ability of starter cells may be attenuated or destroyed by heat-shocking, freeze-shocking, or solvent treatment with very little effect on their enzyme activities. These attenuated cells are in effect packages of enzymes and their use has been reported to accelerate the ripening of a number of cheese varieties.

A simpler and probably more effective approach is the use of lactose-negative strains of \textit{Lactococcus}, which cannot grow in milk or cheese curd but serve as a balanced “natural” source of enzymes important in cheese ripening. Such cultures are available commercially and are claimed to give satisfactory results.

Cells of selected non-lactic-acid bacteria, which do not grow in a particular cheese, either because they are aerobic, e.g., \textit{Pseudomonas} spp. or \textit{Brevibacterium} spp., or because they have a high growth temperature, e.g., \textit{Propionibacterium}, might also serve as suitable “packages of enzymes” for addition to cheese curd; however, such cultures do not appear to be used commercially at present.

It is probable that certain enzymes are rate limiting in cheese ripening. At present, the key limiting enzymes are unknown, but studies are in hand with the objective of identifying these enzymes using deficient or overproducing mutants. It is possible to genetically modify \textit{Lactococcus} to overproduce certain desirable enzymes, to delete undesirable genes, or to introduce foreign genes for putatively important enzymes. It is highly probable that genetically modified bacteria with the ability to accelerate ripening and improve cheese quality will become available in due course for use as primary or secondary cultures.

All cheese acquires an adventitious nonstarter microflora, predominantly mesophilic lactobacilli, which grow from low numbers initially (e.g., \(10^3\) to \(10^7\) cfu/g) to \(10^7\)–\(10^8\) cfu/g and which dominate the microflora of cheese after \(~ 3\) months owing to the death of the primary starter. These nonstarter lactic acid bacteria (NSLAB) probably affect cheese ripening, and there is considerable interest in inoculating cheese milk with selected strains of NSLAB to accelerate or modify cheese flavor development (37).

An extreme form of accelerated ripening is practiced in the production of enzyme-modified cheese (EMC); the subject has been reviewed by Kilecawley et al. (38). EMCs are produced by adding a cocktail of enzymes (proteinases, peptidases, lipases) and perhaps bacterial cultures to homogenized, pasteurized fresh curd or young cheese. The mixture is incubated for a requisite period and repasteurized to terminate the microbiological and enzymatic reactions. The preparation may be spray-dried or commercialized as a paste.

Although the flavor of EMCs does not approximate that of natural cheese, they have the ability to potentiate cheeselike flavor in various food products, e.g., processed cheese, cheese analogs, cheese sauces, and dips, and products containing cheese, e.g., crackers, crisps, etc. For such applications, EMCs may replace...
20–50 times their weight of natural cheese and are cheaper. Cheddar EMCs are the most important commercially, but EMCs that stimulate several varieties have been developed, e.g., blue, Swiss, and Romano.

B. Other Applications of Proteinases

In comparison with their use in cheese making, the other applications of proteinases in dairy technology are quite small but some have considerable growth potential. The more important of these are discussed below.

1. Dietary Products

Protein hydrolyzates for use in soups, gravies, flavorings, and dietetic foods are generally prepared from soy proteins, gluten, milk proteins, meat, or fish protein by acid hydrolysis. Neutralization results in a high salt content which is acceptable for certain applications but may be unsuitable for dietetic foods and food supplements. Furthermore, acid hydrolysis causes total or partial destruction of some amino acids. Enzymatic hydrolysis is a viable alternative (39), but bitterness due to hydrophobic peptides is frequently encountered. Caseins are strongly hydrophobic and yield very bitter hydrolyzates, but bitterness may be eliminated, or at least reduced, by one of several treatments (39, 40).

There is increasing interest in the production of casein-derived peptides with special nutritional or physiological properties; some of the possibilities have been reviewed (41, 42). Apart from the interest in casein hydrolyzates for the nutrition of patients with digestive problems, interest has been focused recently on phosphopeptides derived from casein which it is claimed stimulate the absorption of calcium and iron, but views on this are not unanimous (43). Methods for the production of caseinphosphopeptides for nutritional and/or medical applications have been developed (44–46).

The casein(glyco)macropeptide (CMP), \( \kappa-CN f106–169 \), produced from \( \kappa \)-casein during the enzymatic coagulation of milk for cheese or rennet casein, is lost into the whey. CMP is devoid of aromatic amino acids and hence is a suitable nutrient for patients suffering from phenylketonuria. Several biological activities have been attributed to the CMP, including inhibition of adhesion of oral actinomyces and streptococci to erythrocytes, effects on gastrointestinal motility, growth factors for \textit{Bifidobacterium} spp., inhibition of the binding of cholera toxin, inhibition of influenza virus hemagglutinin, stimulation of cholecystokinin release from intestinal cells, and inhibition of acid secretion in the stomach (47, 48). Methods have been developed for the industrial production of CMP from whey (48, 49).

There is considerable interest in the fortification of performance-enhancing drinks with protein hydrolysates; bitterness is a problem here, and whey protein hydrolysates are preferred to casein hydrolysates.

2. Physiologically Active Peptides from Milk Proteins

Peptides with various physiological activities have been isolated from milk protein hydrolysates; at least some of these peptides are produced in vivo and may play a physiological role (50). The best-studied of these are the \( \beta \)-caseinomorphins, a family of peptides containing 4–7 amino acids (representing \( \beta-CN f60–63/7 \) with opioid activity. These peptides are produced in the intestine in vivo but it is still unclear whether or not they reach the brain. Peptides with opiate properties have also been isolated from hydrolysates of \( \alpha_4 \)- and \( \kappa \)-caseins, lactotransferrin, \( \alpha \)-lactalbumin, and \( \beta \)-lactoglobulin.

Other biologically active peptides that have been isolated from hydrolysates of milk proteins include: immunomodulating peptides, an inhibitor of angiotensin-converting enzyme, blood platelet modifiers, and stimulators of DNA synthesis (50). Whether any of these peptides are active in vivo remain to be established, but their formation has led to casein being referred to as a pro-hormone (51).

The release of biologically active peptides requires precise hydrolysis of the parent molecules at specific bonds. If the application of these peptides develops as predicted, very interesting applications for proteinases in the dairy industry will emerge.

3. Modification of Protein Functionality

Milk proteins are among the principal functional proteins used in food products (52). In general, milk proteins possess very good functional properties but suffer some limitations, notably the insolubility of casein in the pH range 3.0–5.5. The functional properties of milk proteins may be improved by limited proteolysis (1, 40, 53, 54). An acid-soluble casein, free from off-flavor and suitable for incorporation into beverages and other acid foods, has been prepared by limited proteolysis. The antigenicity of casein is destroyed by proteolysis, and the hydrolysate is suitable for use in milk protein-based foods for infants allergic to cow’s milk. Controlled proteolysis improves the meltability of directly acidified cheese but excessive proteolysis
causes bitterness. Casein solutions are very viscous at concentrations > 20%, w/v, which increases the cost of drying caseinates. Viscosity may be reduced by limited proteolysis, but only a small increase in solids is possible owing to bitterness after even moderate levels of proteolysis.

The surface activity of sodium caseinate can be increased considerably by the treatment with plasmin, apparently owing to the formation of γ2- and γ3-caseins (β-CN f106–209, β-CN f108–209) which are very surface active. Generally, the emulsifying and foaming properties of small peptides are poor, since they form very thin interfacial layers.

Limited proteolysis of lactalbumin (heat-denatured whey protein), which is insoluble and has very poor functional properties, yields a product with greatly improved solubility and functionality. Limited proteolysis of whey protein concentrate (WPC) reduces its emulsifying capacity and increases its specific foam volume but reduces foam stability. The heat stability of WPC may be improved considerably by limited hydrolysis without concomitant impairment of other functional properties or off-flavor development.

The plastein reaction has been proposed as a mechanism by which the functional and nutritional properties of proteins may be improved. The reaction is of little relevance to milk proteins because they already have good functional and nutritional properties and yields of plastein are low.

One of the principal food applications of whey protein (WP) and whey protein isolates (WPI) is in the production of thermo-set gels. The gelation of WPCs at low temperatures has been achieved by limited proteolysis by certain enzymes (55); perhaps gelation occurs through the plastein reaction.

III. LIPASES

Lipases have a number of relatively low-volume applications in the dairy industry. Some of these applications are traditional and essential for the manufacture of particular products: others are emerging but hold considerable potential.

A. Lipases in Cheese Production

The principal application of lipases in dairy technology is in cheese manufacture, particularly, some Italian varieties, e.g., Romano and provolone. The characteristic “piquant” flavor of these cheeses is due primarily to short-chain fatty acids resulting from the action of lipase(s) in the rennet paste traditionally used in their manufacture.

Rennet pastes are prepared from the stomachs of calves, lambs, or kids slaughtered after suckling; the stomachs and contents are held for a considerable period prior to maceration. Because of possible risks to public health, the use of rennet pastes, which have proteolytic and lipolytic activities, is prohibited in some countries. The lipase in rennet paste is of oral origin and its secretion is stimulated by suckling: the secreted lipase is washed into the stomach with the ingested milk. Oral (lingual) lipase, commonly referred to as a pregastric esterase (PGE), is secreted by several species and probably makes a significant contribution to the digestion of lipids by the neonate in which the activity of pancreatic lipase is limited. The considerable literature on PGE has been comprehensively reviewed (3, 56). PGE shows a high specificity for short chain fatty acids, especially butyric, esterified on the Sn-3 position of glycerol, although some interspecies differences in specificity have been reported. They are maximally active at 32–42°C, pH 4.8–5.5, and in the presence of 0.5 M NaCl. Calf, kid, and lamb PGEs have been partially purified from commercial preparations (57). Calf PGE has been isolated from oral tissue and characterized with respect to pI (7.0), molecular weight (∼49 kDa), and amino acid composition (58, 59). The secondary structures of rat lingual lipase and pancreatic lipase were studied and compared (60). The gene for rat lingual lipase has been cloned and sequenced, and the amino acid sequence of the enzyme has been deduced (61).

PGE-containing extracts from calf, kid, or lamb tissue are commercially available as alternatives for rennet pastes. Slight interspecies differences in specificity render one or another more suitable for particular applications. Particularly large differences in the ability of lipases to release 4-methylcetanoic acid, which exhibits a goat-muttony aroma, have been found (62). Such differences in specificity permit the generation of a range of flavors in cheese products. The propensity of PGE to synthesize triglycerides is increased in the αw range 0.75–0.90 by the addition of ethanol to the reaction mixture (63); this property may be exploited to modify cheese flavors.

Although PGE extract is now widely used in the manufacture of hard Italian cheese varieties, connoisseurs of Italian cheese claim that rennet paste gives superior results. Perhaps rennet paste contains enzymes in addition to chymosin and PGE. A second lipase, termed gastric lipase, was identified in an extract of cleaned gastric tissue and partially charac-
terized. A combination of calf gastric lipase and goat PGE gave Cheddar and provolone of superior quality to cheese made with PGE alone (64). However, Nelson et al. (56) expressed reservations on the occurrence of a gastric lipase distinct from PGE. Traditional rennet paste would be expected to contain both PGE and gastric lipase.

*R. miehei* secretes a lipase that is reported to give satisfactory results in Italian cheese manufacture (65). The enzyme has been characterized (66) and is commercially available as Piccantase. The lipases secreted by selected strains of *Penicillium roqueforti*, *P. candidum*, or *A. niger* are considered to be potentially useful for the manufacture of Romano, provolone, and other cheese varieties (67, 68). Unlike PGE, the fungal lipases do not preferentially release short-chain fatty acids. The use of different lipase preparations or blends of lipases opens possibilities for producing Italian cheeses with different degrees of sharpness.

*A. oryzae* secretes a lipase which has an exceptionally high specificity for C₆–C₈ acids (69). Another interesting characteristic of this enzyme is that it forms micelles, ~0.2 μm in diameter, in aqueous media, as a result of which ~94% of the enzyme added to milk is recovered in cheese curds. The formation of short-chain fatty acids was reported to parallel flavor intensity in Cheddar cheese containing this enzyme. In contrast to the FFA profile caused by calf PGE, which liberated high concentrations of C₄₀, the FFA profile in the cheese containing *A. oryzae* lipase was similar to that in the control cheese, but the level of FFA was much higher.

Extensive lipolysis also occurs in blue cheese varieties, and in addition to making a direct contribution to flavor, the free fatty acids serve as substrates for fungal enzyme systems in the biosynthesis of methyl ketones, which are the principal contributors to the typical flavor of blue cheese (70). *P. roqueforti* lipase predominates the ripening of blue cheeses. However, blue cheese ripening may be accelerated and quality improved by the addition of exogenous lipases (71, 72).

Blue cheese is a popular ingredient for salad dressings and cheese dip. High-quality natural cheese is not normally required for these applications and there is considerable interest in the production of cheaper substitutes. Various methods have been developed for the production of blue cheese flavor concentrates; most of these methods involved the use of fungal lipases and usually *P. roqueforti* spores (1, 2, 73).

The low level of lipolysis that occurs in most other varieties is catalyzed by lipases/esterases derived from the starter or nonstarter lactic acid bacteria. Considerably more lipolysis occurs in cheeses made from raw milk than in pasteurized milk cheeses, possibly owing to the indigenous milk lipoprotein lipase and/or a more diverse nonstarter microflora in the former. It has been claimed that the flavor of many cheeses can be intensified or their ripening accelerated by incorporating PGE or fungal lipases, although their use appears to be very limited (1–3). Relatively extensive lipolysis occurs in Parmigiano-Reggiano, which can probably be attributed to the use of raw milk (which contains an indigenous lipase) and the long ripening time (2 years).

Lipases, probably mainly of fungal origin, are used in the production of some enzyme-modified cheeses (38).

**B. Other Applications of Lipases**

Lipases are used to hydrolyze milk fat for a variety of uses in the confectionery, candy, chocolate, sauce, and snack food industries. The partially hydrolyzed fat imparts a greater intensity of butterlike flavor to the products and delays staling, presumably as a result of the emulsifying effect of di- and monoglycerides (56, 71, 74).

An important new application of lipases is in the trans/interesterification of fatty acids on triglycerides (75). This approach can be used to modify the melting point of triglycerides, and hence their rheological properties. An important application of this technology is in the production of cocoa butter substitutes for chocolate manufacture. Mono- or polysaturated fatty acids may also be introduced to relatively saturated milk lipids to improve their nutritional qualities. Immobilized lipase systems have been developed for these applications (74).

**IV. β-GALACTOSIDASE**

Lactose is a reducing disaccharide containing galactose and glucose linked by a β-1-4 O-glycosidic bond (O-β-D-galactopyranosyl-(1-4)-α- or β-D-glucopyranose, α- and β-lactose, respectively). Lactose is by far the dominant carbohydrate in milks which are, in turn, the only significant natural sources of lactose. The concentration of lactose in milk ranges from 0% in the milk of marine mammals to ~10% in milk from some species of monkey; bovine and human milk contain ~4.8% and 7% lactose, respectively. Lactose is an important source of energy for the newborn mammal, but when a very energy-dense milk is required (mammals in aquatic or polar environments), the lipid rather than the lactose content is increased. In fact, there is a fairly
good inverse relationship between the level of fat and that of lactose.

Among sugars, lactose possesses many fairly unique properties: low solubility (~18 g/100 mL H₂O at 20°C); a marked tendency to form supersaturated solutions which are difficult to crystallize; when crystallization does occur, the crystals are hard and sharp and, unless kept to dimensions < 20 μm, cause a sandy texture in foods; crystallization is complicated by its mutarotation characteristics since α- and β-lactose differ considerably in solubility and degree of hydration; it has low sweetness (16% as sweet as sucrose at 1%, w/w); owing to its crystallization and mutarotation characteristics, it is hygroscopic and may cause “caking” of dairy powders; it has a strong tendency to absorb flavors and odors.

At 20°C, α-lactose is considerably less soluble (~7 g/100 g H₂O) than β-lactose (~50 g/100 g H₂O). However, the solubility of the α-anomer increases more sharply with increasing temperature than that of the β-anomer and the solubility curves intersect at ~93.5°C; therefore, when lactose is crystallized at a temperature < 93.5°C, α-lactose is obtained. α-Lactose crystallizes as a monohydrate while β-lactose crystals are anhydrous.

When milk is spray-dried, there is insufficient time for lactose to crystallize and an amorphous lactose glass is formed. If the moisture content of the powder is low, the glass is stable, but if the moisture content increases, the glass becomes hygroscopic, and lactose crystallizes as α-lactose monohydrate, leading to caking. In practice, the problem is solved by precrystallizing the lactose, usually induced by seeding with powdered lactose crystals.

When milk is frozen, the lactose usually does not have time to crystallize and the concentration of inorganic solutes in the liquid aqueous phase increases. On holding in the frozen state, calcium phosphate crystallizes as Ca₃(PO₄)₂, releasing H⁺ and reducing the pH to ~5.8, and lactose crystallizes as α-lactose monohydrate, reducing the amount of solvent water, further increasing the concentration of inorganic solutes. The combination of low pH and high Ca²⁺ destabilizes the casein micelles which aggregate when the milk is thawed. Unless properly controlled, these characteristics of lactose may cause defects in concentrated, dehydrated, and frozen dairy products. However, some of the same characteristics may be exploited to make lactose an interesting and useful food additive—e.g., as a free-flowing agent, an agglomerating (instantizing) agent, an additive to stabilize color, flavor, and texture, especially when concomitant sweetness is undesirable, and as a reducing sugar in products in which Maillard browning is desirable as a source of color and flavor. The chemistry, properties, problems, modifications, and applications of lactose and lactose derivatives have been reviewed (76–85).

It is claimed that lactose promotes the intestinal absorption of calcium and phosphorus and hence should be nutritionally beneficial, especially in infant nutrition. However, lactose is involved in two enzyme deficiency diseases: lactose intolerance and galactosemia. There are in fact two forms of galactosemia, both arising from the congenital deficiency of an enzyme in their Leloir pathway for galactose metabolism (86). Classical galactosemia is due to a deficiency of galactose-1-phosphate:uridyl transferase. Ingested galactose (from lactose or other source) is phosphorylated to galactose-1-phosphate which is not metabolized further, leading to the accumulation of galactose and galactose-1-phosphate. Galactosemic infants appear normal at birth but develop various symptoms, including mental retardation, unless put on a galactose-free diet within 2–3 months. The second form, galactokinase-deficient galactosemia, results in the failure to phosphorylate galactose, some of which is metabolized to galactitol which accumulates in the eye, causing cataracts.

Disaccharides must be hydrolyzed to monosaccharides in the intestine prior to absorption, in the case of lactose by β-galactosidase. The vast majority of infants secrete adequate levels of β-galactosidase in the brush border of the small intestine to hydrolyze ingested lactose; however, a small minority of infants secrete an inadequate level of β-galactosidase. The level of intestinal β-galactosidase reaches a maximum shortly after birth and declines thereafter to a low level. With the exception of northwestern Europeans and a few African tribes, the level of intestinal β-galactosidase becomes so low within 6–8 years as to render the subject incapable of hydrolyzing ingested lactose at an adequate rate, leading to lactose intolerance. The unhydrolyzed lactose passes to the large intestine where it leads to flatulence, cramps, diarrhea, and possibly death. Thus, only a minority of the world’s population can consume large quantities of lactose-containing foods with impunity. The subject of lactose intolerance has been reviewed extensively (87, 88).

The third feature of the lactose problem is the development of economic outlets for the large quantities (~5 x 10⁴ kg/year) available from cheese and casein wheys. The technology for lactose production is well developed but < 10% of the potentially available lactose is recovered as such; although lactose has a num-
The dairy industry has developed methods for controlling the physicochemical problems posed by lactose, and lactose intolerance may not be too serious if lactose-containing products are introduced gradually to the diet. However, all the various problems posed by lactose (technological, nutritional, utilization) may be solved via hydrolysis by β-galactosidase (lactase) to the less problematic sugars, glucose and galactose.

β-Galactosidase (β-D-galactoside galactohydrolase; EC 3.2.1.23) catalyzes the hydrolysis of lactose to its component monosaccharides, glucose and galactose. The use of β-galactosidase in dairy technology has been considered as one of the most promising applications of exogenous enzymes in food processing. However, although many interesting applications have been demonstrated, the use of β-galactosidase has not yet become commercially successful for economic reasons. The voluminous literature on the preparation, properties, and uses of β-galactosidases has been the subject of several reviews (76, 81, 83, 89, 90).

Although β-galactosidase is widely distributed in plant, animal, and microbial sources, only the enzymes from Aspergillus niger, A. oryzae, Kluyveromyces marxianus var. lactis, K. fragilis, Bacillus stearothermophilus, and Escherichia coli are commercially available. β-Galactosidases from several sources have been isolated and characterized; the important properties have been summarized by Mahoney (89, 90). In general, β-galactosidases produced by molds have an acid pH optimum (2.5–4.5) and are therefore best suited for use in acid wheys, while yeast and bacterial β-galactosidases, with a pH optimum in the range 6–7.5, are more suitable for use in milk or rennet wheys. β-Galactosidases with high thermal stability have been isolated and are attractive because they can be used at high temperatures at which microbial growth is slow or absent. The heat stability of β-galactosidase from K. lactis and Streptococcus thermophilus is considerably greater in milk than in buffer systems owing to the combined effects of casein and lactose.

β-Galactosidases from several sources have been immobilized by encapsulation; entrapment in fibers, gels, or semipermeable membranes; adsorption or covalent attachment by a variety of techniques to various supports, e.g., porous glass, collagen, cellulose derivatives, and various resins (76, 83, 89, 90).

The principal applications of β-galactosidase in dairy technology are: (a) production of low-lactose milk and dairy products for β-galactosidase-deficient patients; (b) modification of dairy products for use in ice cream, baked goods, yogurt, etc.; (c) production of syrups and sweeteners for food applications; and (d) pretreatment of milk for freezing.

In spite of the widespread incidence of β-galactosidase deficiency among non-Caucasians, such subjects adjust to lactose-containing diets if lactose is introduced gradually and the response to lactose is moderated by other components in the diet. Researchers are divided as to the desirability of including milk in the diets of lactase-intolerant subjects (87, 88). However, it appears to be generally agreed that treatment with β-galactosidase would enhance the nutritional value of dairy products in such cases and render protein-rich dairy products suitable for supplementation of nutritionally deficient diets. Lactose-hydrolyzed milk is commercially available but is more expensive than normal milk. β-Galactosidase is also available in powder or liquid form for home use (89, 90). Direct addition of β-galactosidase to milk at mealtime ("enzyme replacement therapy") has also given satisfactory results. A promising method for reducing treatment cost is the addition of a very low level of soluble β-galactosidase to UHT milk which, during prolonged storage, induces extensive hydrolysis. This approach has been commercialized by the Tetra-Pak Company (Lund, Sweden).

The increased sweetness of lactose-hydrolyzed milk does not appear to be a problem and may actually be preferred by some individuals.

β-Galactosidase can act as a transferase resulting in the synthesis of several oligosaccharides (galacto-oligosaccharides), the range and concentration of which appear to vary with the source of the β-galactosidase and the duration of treatment. Many of the oligosaccharides are β-(1 → 6) galactosides which are not hydrolyzed in the small intestine and pass into the large intestine where they are acted on by bacteria leading to intestinal disturbances, mainly flatulence. However, galacto-oligosaccharides stimulate the growth of Bifidobacterium spp. in the lower intestine, which is believed to be beneficial. A product (oliginate, 6'-galactosyl lactose) is produced commercially by the Yokult Company in Japan for addition to infant formulae. Some galacto-oligosaccharides have interesting functional properties and may find commercial applications (91, 92).

Hydrolysis of lactose in milk for yogurt has been suggested as a means of increasing the sweetness of yogurt without a concomitant increase in calories; it
is reported to reduce fermentation time (89, 90). Some authors have reported that lactose-hydrolyzed yogurt has superior texture and consistency, with less wheying off, than controls. Off-flavors have been reported in some lactose-hydrolyzed yogurts, perhaps owing to the action of contaminating proteinases. Yogurt and other cultured milks are well tolerated by lactose-intolerant subjects, apparently owing to the secretion of \( \beta \)-galactosidase by the thermophilic culture used or to slower gastric emptying (88).

It is also claimed that the manufacturing time for Cheddar and other cheeses is reduced by pretreatment of cheese milk with \( \beta \)-galactosidase, and, possibly more significantly, the quality of the cheese is improved and ripening accelerated. However, a proteinase present in the commercial \( \beta \)-galactosidase preparations used, rather than to the action of \( \beta \)-galactosidase, was probably responsible for the accelerated ripening.

Hydrolysis of lactose improves the functionality of milk powder in bakery products. The glucose moiety is fermentable by baker’s yeast (Saccharomyces cerevisiae), leading to increased loaf volume, while the non-fermentable galactose contributes to flavor and crust color through Maillard browning. Prehydrolysis of lactose using \( \beta \)-galactosidase renders milk stable to freezing (89, 90, 93).

Dulce de Leche, a sweetened concentrated dairy product, is popular in Latin America as a dessert or spread. Lactose crystallization is a major problem in this highly concentrated product. Growth of K. marxianus var. lactis in milk for the preparation of Dulce de Leche has been recommended for the control of lactose crystallization (94). About 50% of the lactose was hydrolyzed in 12 h (at 5.2 \times 10^7 cells/mL) or 100% in 24 h. When the delactosed milk was mixed in proportions of 1:2 with normal milk and concentrated, no lactose crystallization and no significant changes in flavor were noted. The use of permeabilized K. marxianus var. lactis cells for the same application was found to be very satisfactory (95). Presumably, isolated \( \beta \)-galactosidase could be used successfully in the preparation of Dulce de Leche.

Lactose crystallization may also be a problem in conventional sweetened condensed milk, although the problem can be controlled by appropriate manufacturing steps. Prehydrolysis with \( \beta \)-galactosidase offers an alternative solution.

Treatment with \( \beta \)-galactosidase prevented lactose crystallization in a whey retentate–buttermilk powder spread (96). Acid (mold) \( \beta \)-galactosidase was preferable to neutral (yeast) enzyme, and hydrolysis of 30% of the lactose was sufficient. Lactose-hydrolyzed whey appears to be suitable for incorporation into ice cream in which up to 25% of the skim milk solids may be replaced by hydrolyzed whey syrup without adverse effects on quality; 50% of the sucrose may also be replaced by whey syrup (89, 90, 97). Lactose-hydrolyzed whey may be fed in larger amounts than normal whey to animals, especially pigs.

Probably the principal commercial interest in \( \beta \)-galactosidase is for the production of syrups as a profitable outlet for lactose. Glucose-galactose syrups are \( \sim 70\% \) as sweet as sucrose and about four times sweeter than lactose. It has been known for a long time that lactose can be hydrolyzed to glucose and galactose by strong mineral acids, and there has been renewed commercial interest in the process, using free acid or ion exchange resins, as a means of producing glucose-galactose syrups. Production costs are reported to be lower than for enzymatic hydrolysis, but acid hydrolysis is applicable only to purified lactose solutions or perhaps ultrafiltration permeates.

Numerous applications have been reported for glucose-galactose syrups (in addition to the use of lactose-hydrolyzed whey) (83, 89, 90). Although effective systems for the production of glucose-galactose syrups and other lactose-hydrolyzed products are available and continued research will undoubtedly lead to improved systems, the cost of such syrups vis-à-vis alternatives, mainly glucose syrups from starch, remains a problem.

The sweetness of glucose-galactose syrups may be increased by isomerizing the glucose to fructose (which is about twice as sweet as glucose) using glucose isomerase, which is now widely used in the commercial production of high-fructose syrups from starch. Such a system has been patented and a glucose-fructose-galactose-lactose syrup with a sweetness equal to sucrose (both at 10% solution) has been prepared by treating a glucose-fortified lactose hydrolyzate with glucose isomerase. Conversion of lactose to lactulose offers another avenue for the production of novel sugar mixtures. Lactulose is a disaccharide consisting of galactose and fructose, which can be produced from lactose by mild alkaline treatment. At least some \( \beta \)-galactosidases can hydrolyze lactulose, although more slowly than lactose.

It will be apparent that \( \beta \)-galactosidase has numerous applications in the dairy industry. However, in spite of very considerable research and demonstrated technological feasibility in pilot-scale experiments, \( \beta \)-galactosidase is not yet exploited commercially on a significant scale, except for lactose-hydrolyzed pasteurized and UHT milks. Zadow (98) concluded that the
markets for lactose-hydrolyzed syrups are likely to remain limited for economic reasons.

V. LYSOZYME

Lysozyme (muramidase, EC 3.2.1.17), an enzyme which causes lysis of certain bacteria by hydrolyzing cell wall polysaccharides, is widely distributed in animal tissues and secretions. Some bacteria and bacteriophage secrete similar enzymes, lysins. Egg white is a particularly rich source of lysozyme, which is the best characterized of these enzymes. The molecular and biological properties of lysozyme and its use in food preservation and as a pharmaceutical have been comprehensively reviewed by Proctor and Cunningham (99) and Cunningham et al. (100).

The milks of most species contain an indigenous lysozyme; human and equine milks are particularly rich in this enzyme. Various aspects of indigenous milk lysozyme were reviewed by Farkye (101, 102).

In view of its antibacterial activity, the large difference in lysozyme content between human and bovine milks may have significance in infant nutrition. It is claimed that supplementation of baby food formulae based on cows’ milk with egg-white lysozyme gives beneficial results, especially with premature babies; however, results are equivocal (1).

Lysozyme has some other minor applications in dairy technology, but most current interest is focused on its use in Dutch, Swiss, Italian, and other cheese varieties to prevent late gas blowing and/or off-flavors caused by the growth of Clostridium tyrobutyricum. Contamination of cheese milk with Clostridium spp. can be reduced by good hygienic practices, and populations may be further reduced by bactofugation or microfiltration. However, in most countries it is normal practice to add sodium nitrate as a further precaution. The use of nitrate in foods is suspect because it leads to nitrosamine formation, and many countries have reduced permitted levels or prohibited its use.

Lysozyme is effective in killing Clostridium cells and preventing the outgrowth of their spores. It has been shown to be an effective alternative to nitrate in preventing the butyric acid fermentation and late gas blowing in several cheese varieties (1, 2, 99, 100, 103, 104). It was concluded (103, 104) that

the published information indicates that for some cheese types, lysozyme is a suitable substance for the control of late blowing, provided the number of clostridial spores is low. For these cheese types, which may be considered less sensitive to late blowing, the level of lysozyme already permitted by regulatory authorities in a number of countries appears to be satisfactory until further evidence is obtained. Lysozyme appears to be of value in the control of late blowing in countries which prohibit nitrate. Published information indicates that in some cheese types which are very sensitive to late blowing, such as Gouda, lysozyme used at the current normal addition under normal manufacturing and storage conditions is less effective than the usual amount of nitrate. In this case, lysozyme can not be considered a suitable alternative to nitrate at present. More information will become available for the various cheese types about the critical number of spores in the raw milk to cause defects when lysozyme is used. Combinations of lysozyme addition and other control measures can then be evaluated further.

Lysozyme appears to be quite effective against Listeria monocytogenes and other bacteria involved in foodborne diseases and food spoilage (105). Considering the widespread attention now focused on Listeria in dairy products, especially cheeses, it is likely that this application of lysozyme will be the subject of further research. The preservative effects of lysozyme in other foods were reviewed by Proctor and Cunningham (99) and Cunningham et al. (100). It is possible that some of these may be applicable to dairy products.

VI. GLUCOSE OXIDASE

Glucose oxidase (GO) catalyzes the oxidation of glucose to gluconic acid (via gluconic acid-δ-lactone) according to the following reactions (106):

\[
\text{glucose \text{ GO-FAD}} \rightarrow \text{gluconic acid-δ-lactone} + \text{GO-FADH}_2 \quad (1)
\]

\[
\begin{align*}
\text{GO-FAD} + \text{H}_2\text{O}_2 & \rightarrow \text{GO-FADH}_2 + \text{H}_2\text{O} \\
\text{GO-FADH}_2 + \text{H}_2\text{O} & \rightarrow \text{GO-FAD} + \text{H}_2\text{O}_2 + 1/2\text{O}_2
\end{align*}
\]

The hydrogen peroxide formed is normally reduced by catalase present as a contaminant in commercial preparations of GO (from P. notatum, P. glaucum, or A. niger) or added separately. GO, which has a pH optimum \( \sim 5.5 \), is highly specific for D-glucose and is used to assay specifically for D-glucose in the presence of other sugars, blood, urine, etc.
In the food industry, GO has four principal applications, which are not commercially very significant, especially in the dairy industry (1, 106, 107):

1. **Removal of residual, trace levels of glucose.** This application, which is particularly useful for the treatment of egg white prior to dehydration (although an alternative procedure using yeast fermentation is more commonly used), is of little if any significance in dairy technology.

2. **Removal of trace levels of oxygen.** Traces of oxygen in wines and fruit juices cause discoloration and/or oxidation of ascorbic acid. Chemical reducing agents may be used to scavenge oxygen, but enzymatic treatment with GO may be preferred. The GO system has been proposed as an antioxidant for high-fat products, such as mayonnaise, butter, and whole milk powder, but it does not appear to be used commercially for this purpose, probably because of cost vis-à-vis chemical antioxidants (if permitted) and the relative effectiveness of inert gas flushing of canned milk powder.

3. **Generation of hydrogen peroxide in situ.** The hydrogen peroxide generated by glucose oxidase has a direct bacteriocidal effect (which is a useful side effect of GO applied to egg products), but its bacteriocidal properties can be much more effectively exploited as a component of the lactoperoxidase/hydrogen peroxide/thiocyanate system (see Sec. X). Two components of this system occur naturally in milk: lactoperoxidase is present at ~30 mg/L, and thiocyanate, produced in the rumen by hydrolysis of thioglycosides from members of the Brassicae family, varies from 0.017 to 0.26 mM. Hydrogen peroxide does not occur naturally in bacteria-free milk, but it can be generated metabolically by catalase-negative bacteria, added directly (which is usually preferred) or produced in situ from sodium percarbonate or by the action of xanthine oxidase on added hypoxanthine or by the action of glucose oxidase on glucose, either added or produced in situ from lactose by β-galactosidase. Activation of the lactoperoxidase–hydrogen peroxide–thiocyanate system suppresses the growth of psychrotrophs in milk stored at 5°C and has given promising results as a milk preservative in tropical regions where refrigeration is lacking. It would appear that in such applications the use of exogenous hydrogen peroxide is the simplest and most appropriate.

4. **Production of acid in situ.** Direct acidification of dairy products, particularly cottage, feta-type, and mozzarella cheeses, is now fairly common. Acidification is normally performed by addition of acid or acidogen (usually gluconic acid-δ-lactone) or by a combination of acid and acidogen. In situ production of gluconic acid from added glucose or from glucose produced in situ from lactose by β-galactosidase or from added sucrose by invertase has been proposed (108). It is possible to produce gluconic acid from glucose using immobilized glucose oxidase. However, it is doubtful whether immobilized glucose oxidase could be applied to the acidification of milk because of the high probability of fouling by precipitated protein even if low temperatures at which less extensive casein precipitation occurs were used.

### VII. SUPEROXIDE DIMUTASE

Superoxide dismutase (SOD) catalyzes the reduction of superoxide anions, O2-, according to the following:

\[ \text{2O}_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \]  

(2)

The hydrogen peroxide formed may be reduced by catalase, peroxidase, or a suitable reducing agent. SOD occurs widely in tissues where it plays a major antioxidant role in scavenging superoxide radicals which are produced through the action of several enzymes, e.g., XO and peroxidases.

Milk contains a low level of indigenous SOD which has been isolated and characterized (101, 102); the milk enzyme appears to be identical to SOD from bovine erythrocytes. The level of indigenous SOD in milk, which is entirely in the skim phase, varies considerably among individual cows, with stage of lactation and mastitic infection. It may play a role in the oxidative stability of milk, but attempts to correlate stability with SOD activity have been inconclusive, presumably owing to the interaction of various pro- and antioxidants. The tendency of fat in milk to oxidize is directly correlated with increases in XO and negatively with increases in SOD activity. A low level of exogenous SOD, together with catalase, is a very effective antioxidant in dairy products, and it has been suggested that treatment of milk with SOD may be effective in preserving the flavor of UHT milk which is prone to lipid oxidation (1). It has been reported that a combination of SOD and catalase is a more effective antioxidant than butylated hydroxyanisole. However, it appears to be ineffective as an antioxidant in the presence of 0.1 ppm Cu^{2+}, apparently because Cu^{2+} effectively competes with SOD for O_2 and converts it to lipid-reactive species such as *OH. The commercial feasibility of using SOD as an antioxidant depends on cost, particularly vis-à-vis chemical antioxidants, if permitted. As far as is known, SOD is not used commercially as an antioxidant in the dairy industry.
VIII. SULFHYDRYL OXIDASE

Milk contains an enzyme capable of oxidizing sulfhydryl groups to disulfides:

\[ 2RSH + O_2 \rightarrow RSSR + H_2O \]  

(3)

The enzyme, which has molecular and catalytic properties distinctly different from thioloxidase (EC 1.8.3.2), glutathione:protein disulfide oxidoreductase (EC 1.8.4.2), and protein disulfide isomerase (EC 5.3.4.1), has been isolated and well characterized (101, 102). Sulfhydryl oxidase (EC 1.8.3.–) also has been demonstrated in human milk.

It has been proposed that sulfhydryl oxidase has a physiological role in the formation of disulfide bonds in vivo to give proteins the correct three dimensional structure. Industrially, the potential of the enzyme lies in its ability to ameliorate the cooked flavor of UHT-treated milk (1, 109). The milk enzyme occurs exclusively in the serum (whey) from which it may be easily isolated by exploiting its marked tendency to aggregate, thus facilitating its isolation by chromatography on porous glass. The enzyme has been immobilized on porous glass and titanium oxide, and its effectiveness in ameliorating the cooked flavor of UHT-treated milk has been demonstrated on a pilot scale using immobilized enzyme columns. For industrial-scale use, an adequate supply of the enzyme from whey may be a limitation but production of the enzyme by genetically engineered microorganisms might be feasible.

IX. CATALASE

\[ 2H_2O_2 \rightarrow 2H_2O + O_2 \]  

(4)

Hydrogen peroxide is used for the cold-sterilization of milk in regions lacking refrigeration and perhaps in some developed countries also; e.g., it may be used to treat cheese milk in the United States (1, 107). In underdeveloped regions, treatment of milk with \( H_2O_2 \) is performed at ambient temperature and excess \( H_2O_2 \) is reduced by indigenous milk catalase or by chemical interaction with milk proteins in which it causes some physicochemical changes, principally oxidation of methionine, with adverse effects on cheese quality. Side effects can be decreased by short exposure to \( H_2O_2 \) at \( \sim 65^\circ C \), after which the residual \( H_2O_2 \) is removed by added catalase, usually from beef liver or \( Aspergillus niger \). Most of the recent interest in the use of \( H_2O_2 \) as a preservative for milk has focused on the lactoperoxidase-\( H_2O_2 \)-thiocyanate system in which low concentrations of \( H_2O_2 \) are required (see Sec. X). However, at 400 mg/kg, \( H_2O_2 \) alone is a more effective long-term bactericidal agent than 8.5 mg/kg \( H_2O_2 \) in the lactoperoxidase system. There was no significant difference in the quality of cultured milk made from either lactoperoxidase-activated or \( H_2O_2 \)-treated milk. The activity of lactic starter was significantly lower in the former, but the rennet coagulability of the latter was extended.

There is interest in using immobilized catalase reactors for milk pasteurization or for glucose oxidase-catalase reactors. Although catalase may be readily immobilized, it is inactivated rapidly on exposure to hydrogen peroxide. Hydrogen peroxide appears to be an effective agent for inactivation of aflatoxin \( M_1 \). As discussed above, catalase, usually as a contaminant, is necessary for many of the applications of glucose oxidase and also to optimize the antioxidative properties of SOD.

X. LACTOPEROXIDASE

\[ H_2O_2 + 2AH \rightarrow 2H_2O + 2A \]  

(5)

Bovine milk is rich in lactoperoxidase (~ 30 \( \mu g \)/mL), which is distinct from the myeloperoxidase of leucocytes and salivary peroxidase. Lactoperoxidase has been purified by several investigators and well characterized (109–112).

The most important physiological and technological feature of lactoperoxidase is its ability, in the presence of \( H_2O_2 \) and thiocyanate, to inhibit the growth of several bacteria. Since lactoperoxidase is relatively heat resistant, there is adequate lactoperoxidase activity even in milk that has been moderately to severely heat-treated. Thiocyanate occurs in many animal tissues and fluids. It is produced endogenously during the detoxification of thiosulfates and metabolic products of sulfur amino acids and cyanide and from foods containing thioglucosides. Cows on pastures containing clover (rich in RCN) and nongrasses (e.g., Cruciferae containing thioglucosides) yield milk containing higher concentrations of thiocyanate than cows on winter feed or lay pastures. Saliva contains high levels of thiocyanate which is also secreted by gastric mucosal cells. Milk does not contain indigenous \( H_2O_2 \) but it may be added or produced in situ (see Sec. VI). Thus, bovine milk possesses an effective antibacterial system which probably affects the intestinal microflora of calves (and presumably other species). Lactoperoxidase also appears to protect the mammary...
gland against mastitic infection, especially during the dry period. The antibacterial significance of lactoperoxidase in vivo has been reviewed (113, 114). Human milk appears to contain a low level of lactoperoxidase (< 0.1 μg/mL) as well as myeloperoxidase and eosinophil peroxidase. The lactoperoxidase system may be exploited in vitro to extend the shelf life of milk under conditions where refrigeration and pasteurization facilities are lacking (111–114).

While most interest in lactoperoxidase has focused on the indigenous enzyme, it may also acquire significance as an exogenous enzyme. Techniques have been developed for the commercial-scale purification of lactoperoxidase from milk (42). Large-scale trials have shown that addition of purified lactoperoxidase to calf milk replacers markedly reduces the incidence of diarrhea and increases weight gain. Although the antibacterial properties of human milk do not depend on lactoperoxidase, its addition to bovine milk-based infant formulae may have beneficial effects (111–114).

XI. TRANSGLUTAMINASE

Transglutaminase (TGase; protein-glutamine γ-glutamyltransferase; EC 2.3.2.13) catalyzes an acyl transferase reaction between the γ-carboxamide group of peptide-bound glutamine residues (acyl donors) and a variety of primary amines (acyl acceptors), including amino acids, and the ε-amine group of lysine residues in certain proteins. In the absence of an amine, TGase catalyzes the deamination of glutamine residues, with water molecules acting as acyl acceptors. Thus, TGase can modify proteins by incorporation of (a) an amine, (b) crosslinking, or (c) deamination:

\[
\begin{align*}
&\text{a} \quad \text{Gln} \quad \text{C} \quad \text{NH}_2 + \text{RNH}_2 \\
&\text{b} \quad \text{Gln} \quad \text{C} \quad \text{NH}_2 + \text{H}_2\text{N} \quad \text{Lys} \\
&\text{c} \quad \text{Gln} \quad \text{C} \quad \text{NH}_2 + \text{H}_2\text{O}
\end{align*}
\]

The amino group might be from a simple aliphatic or aromatic amine, an amino acid, an amino sugar, or a phospholipid (β-aminoethanol moiety).

TGase is present in several animal tissues and body fluids and is involved in several biological phenomena, including blood clotting, wound healing, keratinization of epidermal tissue, and stiffening of cell membranes. An enzyme with a similar activity has been found in the bacterium Streptovercillium mobaraense; this is referred to as MTGase (M = microbial). However, TGase and MTGase differ in a number of respects, most notably in molecular weight (~77 and ~38 kDa, respectively) and dependence on Ca\(^{2+}\); TGase requires Ca\(^{2+}\), MTGase does not.

The activity of (M)TGase suggests several possible applications in the food industry:

1. Gelation of proteins by crosslinking
2. Formation of restructured meat and fish products from smaller pieces or comminuted meats
3. Stabilization of protein-stabilized foams or emulsions (by crosslinking proteins in the stabilizing layer)
4. Modification of the functionality, e.g., solubility or water binding, of proteins by converting glutamine to glutamic acid residues or binding of amino sugars or phospholipids
5. Modification of the nutritional value of foods by enzymatically attaching essential amino acids to proteins
6. Conjugation of the same or different proteins
7. Conjugation of amino sugars with proteins
8. Conjugation of phospholipids with proteins
9. Immobilization of enzymes

The feasibility of these applications has been demonstrated in laboratory studies, but the limited availability and cost of the enzyme to date have restricted larger-scale trials (guinea pig liver is the usual source of TGase). However, the gene for TGase has been inserted into microorganisms (115) which would be expected to increase the availability and reduce the cost of the enzyme.

Various aspects of the chemistry of TGase and its applications in foods have been reviewed by Motoki and Seguro (116) and Dickinson (117).

XII. β-LACTAMASE

Residues of penicillin in milk, resulting from treatment of mastitis-infected cows with antibiotics, inhibit or
prevent the growth of lactic acid bacteria used in the production of cheese and fermented milks. They may also evoke an allergic response in susceptible consumers and create conditions for the selection of penicillin-resistant pathogens. The usual approach taken to prevent contamination of the milk supply with antibiotics is to withhold milk from treated cows for an adequate period after treatment; obviously, this results in economic losses to farmers.

Procedures developed for the removal of \( \beta \)-lactam-type antibiotics from milk include columns of activated charcoal, ultrafiltration or diafiltration (1). While these methods may be suitable under certain circumstances, they have obvious limitations. An alternative approach is the inactivation of penicillin by \( \beta \)-lactamase [Eq. (9)].

Korycka-Dahl et al. (118) demonstrated the ability of \( \beta \)-lactamase from Bacillus cereus to inactivate penicillin \( G \) such that Cheddar or Swiss cheeses of normal quality could be produced from the decontaminated milk. The enzyme was active at 4°C so that penicillin in milk could be inactivated during cold storage on the farm or at the factory. The enzyme lost only 50% of its activity following heating at 63°C for 30 min and hence would retain considerable activity in pasteurized milk, which may be illegal. These problems may be solved by using immobilized \( \beta \)-lactamase (119). This procedure appears promising and awaits further development.

An alternative approach to solving the problems caused by penicillin in fermented products is the selection of \( \beta \)-lactamase-producing mutants of lactic acid bacteria (120).

XIII. PERSPECTIVES

Owing to the physicochemical properties of its constituents, milk is very amenable to enzymatic modification and it will be apparent from the foregoing that many potential applications of enzymes in dairy technology exist. However, in spite of numerous studies, only a few of these applications are commercially significant. Many of the potential applications are unsuccessful commercially because the proposed application is not sufficiently significant economically or alternative solutions are available.

By far the most important application is the use of rennets in cheese making, for which no alternative exists. It is likely that rennets will remain the principal enzyme in dairy technology for the foreseeable future. An unlimited supply of chymosin from genetically engineered microorganisms is now available; it is possible that the cheese-making properties of these microbial recombinant chymosins will be improved through genetic engineering. The production of cheese is expanding worldwide, and the market for rennets will therefore continue to increase. Development of cheese-like products as food ingredients is a growth area, and it is likely that the application of proteinases and lipases in the production of such products will increase. The use of proteinases and peptidases to produce protein hydrolysates with specific functional, nutritional, or physiological properties appears to be very promising and has been attracting increasing attention.

Lipases have some traditional and novel applications in cheese technology, but the growth potential of these applications is probably limited. However, new and more effective lipases may be identified. Perhaps the greatest potential application of lipases is in the production of tailor-made lipids with superior nutritional or functional properties.

Although \( \beta \)-galactosidase has applications in dairy technology, most of these are not commercially viable at present, mainly for economic reasons. Niche markets exist for lactose-hydrolyzed milks and these may expand. Exploitation of the transferase activity of \( \beta \)-galactosidase in the production of new oligosaccharides with novel functional and/or nutritional properties appears to hold potential.

Transglutaminase should have several interesting applications in dairy technology. The availability of a cheaper enzyme should encourage work on the application of this enzyme.

With the possible exception of lactoperoxidase and lysozyme, the other enzymes discussed in this chapter appear to have very limited application in dairy technology, at least under present circumstances.

REFERENCES

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