ABSTRACT

Plasmin is by far the predominant and most completely studied endogenous protease in bovine milk. Plasmin-induced proteolysis can have either beneficial or detrimental effects on the texture and flavor of dairy products, depending on the extent of hydrolysis and type of dairy product. In cheese, the breakdown of protein can help develop desirable flavors and texture during ripening, whereas in pasteurized milk and ultra-high-temperature milk, proteolysis causes undesirable gelation. Plasmin is part of a complex protease–protease inhibitor system in milk that consists of active and inactive forms of the enzyme, activators, and inhibitors. Considerable research has been done to isolate and characterize components of the plasmin system, determine how they interact, develop and compare quantitation methods, and determine how they are affected by cow characteristics, processing conditions, other milk components, storage conditions, and bacterial proteases. Considerable research has focused on enhancing or minimizing the activity of plasmin system components. The intent has been to control protease activity in casein and whey fractions, depending on the final food or ingredient application. Controlling the activity of plasmin has a great potential to improve dairy product quality and reduce their processing costs.

Key words: plasmin, milk quality, proteolysis, dairy product quality

INTRODUCTION

Proteolysis in milk was shown to be caused by native proteases and proteases produced by psychrotrophic microorganisms during refrigerated storage of milk (Fairbairn and Law, 1986; Grufferty and Fox, 1988a). Proteolysis induced by the native proteinase plasmin (PL; EC 3.4.21.7) is one of the most important contributors to the quality of microbiologically wholesome milk and its products. Plasmin-induced proteolysis has gained much interest from researchers because of its complexity and versatile effects on the quality of milk and dairy products. Plasmin activity can be essential and desirable for flavor development and texture changes during ripening of cheese, thus enhancing the product quality. The loss of PL from the casein micelle may slow the cheese ripening process and consequently increase the processing expense. Conversely, uncontrolled proteolysis can have detrimental effects on the quality of pasteurized milk, UHT milk, and nonfat dry milk (NFDM), causing undesirable precipitation or gelation. Many researchers have linked gelation and decreased stability of stored pasteurized milk to PL activity (Kohlmann et al., 1991b; Enright et al., 1999; Newstead et al., 2006). Additionally, uncontrolled proteolysis can result in poor curd formation (Srinivasan and Lucey, 2002) and degradation in stored casein products intended to be used as functional ingredients (Nielsen, 2002).

Plasmin is part of a complex system including its inactive form plasminogen, plasminogen activators, and inhibitors. The PL system components interact with each other and with other components of milk to promote or inhibit proteolysis in milk and milk products. Many factors, such as thermal processing, pH, mineral content, whey proteins, and storage temperature can influence the kinetics of the PL-induced hydrolysis. This review presents the current state of knowledge and future trends of the PL system activity and significance in various dairy applications.

THE PLASMIN SYSTEM

Plasmin is part of a complex protease–protease inhibitor system in milk, commonly referred to as the PL system. Plasmin exists in milk primarily in its zymogen form, plasminogen (PG), which can be converted into active PL by plasminogen activators (PA; Grufferty and Fox, 1988a). The conversion of PG to PL is mediated by at least 2 types of PA, tissue-type (t-PA) and urokinase-type (u-PA) (Bastian and Brown, 1996). The PL system also includes plasminogen activator
inhibitors (PAI) and plasmin inhibitors (PI), whose effects on PA and PL, respectively, are greatly dependent on the processing conditions (Richardson, 1983a; Precetti et al., 1997). The PL system components (Figure 1) interact together and with other components of milk, such as whey and casein proteins, and promote or inhibit proteolysis depending on the processing and storage conditions of milk.

**Plasmin and Plasminogen**

The PL and PG found in bovine milk are essentially identical to those found in bovine blood, as indicated by their heat and pH stabilities, pH optimum, specificity for casein hydrolysis, inhibition patterns (Reimerdes, 1983), and as indicated by their amino acid sequence (Benfeldt et al., 1995). The comparisons between human and bovine PL and PG, and the activation of human and bovine PG, have been reviewed (Schaller et al., 1985; Bastian and Brown, 1996). In blood, PL breaks down blood clots, and its activity is controlled by enzyme activators and inhibitors (Collen et al., 1985). Plasmin, mostly in its zymogen form, PG, enters milk from the blood via the mammary cell wall lining, and associates mainly with the casein fraction of the milk. In fresh milk, PG is the predominant form, where its concentration is 2 to 30 times that of PL (0.8–2.8 μg/mL PG compared with 0.1–0.7 μg/mL PL; Ozen et al., 2003). Therefore, any potential activation of PG could contribute significantly to PL activity in milk. The conversion of PG into PL by PA can occur while the milk is in the mammary lumen, before milking and during milk storage (Driessen and Van Der Waals, 1978; Schaar, 1985; Alichanidis et al., 1986). Urokinase-type PA and t-PA activate PG to PL by cleaving the Arg557–Ile558 bond in PG while the milk is in the mammary lumen before milking and during milk storage (Driessen and Van Der Waals, 1978; Schaar, 1985; Alichanidis et al., 1986). Plasmin autocatalytically cleaves a Lys77–Arg78 bond to produce Arg-PG. The cleavage of PG by PL at the Lys–Arg bond causes the release of the preactivation peptide and a marked conformational change in PG. The resultant PG form can be easily activated to PL by PA.

**Figure 1.** Plasmin system in bovine milk.
Compared with the other components of the PL system in bovine milk, little work has been done on PA. Plasminogen activators are thought to be even more heat stable than PL and PG (Lu and Nielsen, 1993a); thus, they survive pasteurization, whereas PAI does not (Richardson, 1983a; Prado et al., 2006). Therefore, PA in milk can have a significant effect on PL activity, which in turn can cause either beneficial or detrimental proteolysis in dairy foods.

The reported amounts of u-PA and t-PA, their activities, and molecular weight vary based on the nature of the milk, methods of separation, and assay methods (Ismail et al., 2006). Researchers have found that t-PA activity is significantly enhanced by fibrin (Rånby et al., 1982; Karlan et al., 1987), and both t-PA and u-PA activities are stimulated by casein (Markus et al., 1993; Politis et al., 1995a). Furthermore, it was observed that amiloride inhibits u-PA activity but has no effect on t-PA (Heegaard et al., 1994b). It is commonly believed that PA are associated with casein; however, conflicting results have been observed by several researchers as to which PA is predominantly associated with the casein micelle (Lu and Nielsen, 1993c; Heegaard et al., 1994b; White et al., 1995). Lu and Nielsen (1993c) have identified 5 proteins with u-PA–like activities associated with the casein fraction. Heegaard et al. (1994b) reported that t-PA was 100-fold more abundant than u-PA in the casein fraction. White et al. (1995) found that 50% of PA activity associated with casein is attributed to t-PA. Heegaard et al. (1994a) and White et al. (1995) confirmed the absence of PA from the whey fraction. Association of u-PA (Heegaard et al., 1994b; White et al., 1995) and t-PA (Zachos et al., 1992) with somatic cells (SC) has been observed. White et al. (1995) linked the association of t-PA with the SC to the presence of casein remnants in the cell extracts, and thus concluded that t-PA is the main PA associated with casein micelle and u-PA is associated with the SC. The controversy in results could be attributed to many factors. Urokinase-type PA can dissociate from the SC, and be picked up by casein and physically bind to it. Therefore, the SCC in milk, the time the SC stay in milk, and the heat treatment of milk could be factors affecting the dissociation of u-PA from SC. Presence of PL can lead to the conversion of single-chain u-PA and t-PA into 2-chain proteins with significantly enhanced activities (Ugwu et al., 1998); thus, the amount of PL in milk and in the isolated PA solution will be another source of variation affecting the results. Other factors leading to conflicting results could be differences in methods of PA isolation from the casein micelle, and the substrates used in the chromogenic assays (Ismail et al., 2006). In particular, less purified samples can give very misleading results because of the presence of PL in PA extracts. Ismail et al. (2006) indicated that a very small amount of u-PA can cause more PG activation than that caused by a larger amount of t-PA, especially in an extracted sample that has more PL. Prado et al. (2007) reported that u-PA is more thermally stable than t-PA. Therefore, based on the amount of PA present, their heat stability, and the fact that t-PA requires the presence of fibrin (or a fibrin-like compound), it seems that u-PA has the greater potential to affect PG activation in milk.

**Plasmin System Inhibitors**

The conversion of PG to PL can be slowed by the action of PAI. Similarly, the proteolysis of casein induced by PL can be slowed by the action of PI. Plasmin inhibitor and PAI are present mainly in milk serum (whey), and their activity is affected by pH fluctuation and heat treatment. Inhibitors of PL and PA have been studied as a potential means of controlling PL-related activities (Precetti et al., 1997). Unlike PL, PG, and PA, PL system inhibitors are relatively heat labile (Richardson, 1983a). Little is known about the heat stability of PAI and PI, and it has been generally proposed that the inhibitors are thermally unstable. Richardson (1983a) suggested that PAI is inactivated by mild thermal treatments. An increase in activity of PL and a subsequent decrease in concentration of PG were observed in pasteurized milk compared with raw milk after incubation at 37°C for up to 80 h (Richardson, 1983a). However, the decrease in PG concentration was greater than the increase of PL activity, suggesting that PAI might have been inactivated by pasteurization while some PI remained active. The thermal stability of the PL system components will be discussed further in a subsequent section.

**Association of Plasmin-Related Activities with Casein Micelle**

Lysine binding and, to a lesser extent, electrostatic forces are involved in the binding of PL, PG, and PA with the casein micelle (Baer et al., 1994). Occurrence of PL in the whey fraction is the result of its dissociation from the casein micelle due to several factors. Milk storage temperature, pH, ionic strength, hydrolysis of casein by PL, and action of microbial proteases have been studied as possible factors that influence PL dissociation from casein micelles. Evidence on the effect of temperature is conflicting (Reimerdes and Herlitz, 1979; Donnelly and Barry, 1983; Grufferty and Fox, 1988b). However, pH clearly influences PL dissociation from casein micelles of fresh milk, with most or all of the PL activity dissociated at pH 4.6 to 4.7 (Richard-
son and Elston, 1984; Grufferty and Fox, 1988b). This effect of pH explains, at least in part, the higher levels of PL in acid whey (44 μg/g of protein) than in sweet whey (4 μg/g of protein). Any shift of PL from the casein to the whey fraction, by various mechanisms, may negatively affect the quality of food products containing whey protein as a functional ingredient.

Compared with our understanding of PL and PG association with and dissociation from casein micelles, we know very little about this for PA. Although no empirical data exist, information on the nature of the binding interaction of u-PA with the specific binding protein in blood (i.e., u-PA receptor) suggests the distinct possibility that, like PL and PG that associate with the casein micelle by lysine binding sites, u-PA may have binding sites on the casein micelle (Politis et al., 1995a; Ellis et al., 1999). The literature offers some insight in this area. Mangel et al. (1990) concluded that PG bound to a lysine-binding site underwent a ligand-induced conformational change that results in PG being more accessible to u-PA for activation. Stephens et al. (1992) suggested that the enhanced u-PA activity caused by hirudin is caused by a simultaneous binding of u-PA (through polyanionic interactions) and PG (through lysine binding) to heparin. The milk literature reports association with and dissociation from casein micelles. Kinetics in this case is related to the enzyme (PL or PA) properties, namely the efficiency and speed of their activity during the chemical reaction (hydrolysis of caseins by action of PL or conversion of PG to PL by action of PA). Interactions between the PL system components and with other milk components as affected by the aforementioned factors are discussed below.

**Processing Conditions**

**Heat Treatment of Milk.** The heat treatment of milk varies depending on the end usage of milk. In general, milk can be pasteurized at various temperatures (65–75°C) and times (15–30 s), or commercially sterilized following UHT processing (135–150°C for few seconds). Also, before processing into products such as NFDM, a preheating treatment is applied, which includes low heat (75°C for 15 s), medium heat (75°C for 1–3 min), high heat (80°C for 30 min), or ultra-high heat (120°C for 1 min). Having such a wide range of heat treatments applied, various effects on PL system components are expected. Reported results show that the PL system components vary considerably in their thermal stability, highlighting the complexity of the PL system activity (Dulley, 1972; Lu and Nielsen, 1993a; Prado et al., 2006, 2007).

The thermal treatment associated with pasteurization of milk has been shown to increase PL levels and PG activity. Further heat treatment has been shown to reduce PL levels. This initial increase in PL activity has long been attributed to inactivation of PI and PAI, but could also be related to the thermal stability of t-PA and u-PA or the enhanced activation of denatured PG. Possible explanations for the decrease in PL levels with further heat treatment have included interactions of PL, PG, or PA with denatured whey proteins. Recent studies were undertaken to investigate the thermal stability of the PL system and the consequent effect on activities of its components and their interactions with other milk constituents.

Plasmin and PG can fully survive pasteurization conditions (72°C for 15 s) (Dulley, 1972), and are somewhat resistant (20–40% remaining activity) to certain high-temperature, short-time (HTST), and UHT heat treatments. Reported PL D-values are 35.7 (Driessen and Van der Waals, 1978) and 12.4 min (Alichanidis et al., 1986) at 72.5°C, and 7 (Driessen and Van der Waals, 1978) and 10 s (Alichanidis et al., 1986) at 142.5°C. Plasmin levels actually increase upon pasteurization, an observation attributed to the enhanced PG activation because of PAI inactivation (Richardson, 1983a; Prado et al., 2006). However, there is more to that than just the direct effect of heat on PL and PG. Like any protein, PL and PG are prone to denaturation and
structural modification upon heating. The temperature range for denaturation of PG is between 50.1°C and 61.6°C (Burbrink and Hayes, 2006). In this temperature range, PG loses its naturally occurring tertiary structure but is not yet inhibited. On the contrary, PG becomes more accessible to the action of PA by unfolding of its kringle domains. Burbrink and Hayes (2006) showed that activation of PG is enhanced upon heating, where the overall catalytic efficiency of activation, as measured by $k_{cat}/K_m$ (where $k_{cat}$ = reaction rate; $K_m$ = Michaelis-Menten constant; $k_{cat}/K_m$ = catalytic efficiency of the enzyme), increased when PG was heated to temperatures above the onset of denaturation (60°C and above).

Almost complete inactivation of PAI occurs upon pasteurization (75°C for 15 s); however, PI retains two-thirds of its activity (Prado et al., 2006). The inhibition of PAI in milk enhances PG activation by PA, and the remaining PI may inhibit part of the PL generated. However, PA is even more heat stable than PL and PG, being quite stable during pasteurization and UHT processing, with a D-value of 109 min at 70°C and 32 s at 140°C in a buffer system (Lu and Nielsen, 1993a). In a milk system, PA survived UHT treatment (Kelly and Foley, 1997; Kennedy and Kelly, 1997). The heat stability of PA was inferred from the marked increase of PG activation in UHT milk with high SCC as compared with that of UHT milk with low SCC (Kelly and Foley, 1997). The PA that mostly associates with somatic cells is u-PA (White et al., 1995). Thus, the thermal stability of PA referred to by Kelly and Foley (1997) is most likely that of u-PA. A recent study differentiated the thermal stability of t-PA and u-PA in milk containing β-LG (Prado et al., 2007). Half the t-PA activity was lost in milk heated at 85°C for 15 or 30 s, compared with only 30% loss of u-PA activity heated at 85°C for 30 s. The u-PA activity was even quite stable at 90°C for 30 s. Therefore, in spite of the remaining active PI, PL activity may continue to increase during storage of processed milk because of the enhanced activation of the unfolded PG by the heat-stable PA, especially u-PA.

Having previously mentioned the complexity of the PL system, it is important here to draw attention to the complexity of the milk system as a whole. Upon severe heat treatment, such as UHT processing, PL activity is significantly affected due to interactions with β-LG, which contains free SH groups that cause irreversible denaturation of PL by S-S/S-H interaction (Enright and Kelly, 1999). Unfolding of PG also can promote interaction with β-LG, more so under elevated temperatures, and thus hinder PG activation. However, the PL system is not completely shut down upon heating, even at elevated temperatures. Although intense heat treatments can irreversibly inactivate most of the PL in the presence of β-LG (Enright et al., 1999), any residual PG and PA in the system, coupled with the inactivation of the system’s inhibitors, will result in active PL during storage of milk. The effect of whey proteins on PA activation of PG to PL was not reported in previous studies that have examined the effect of whey proteins on PL activity (Lo and Bastian, 1997, 1998; Benfeldt et al., 1998). When milk was stored after heat treatment (up to 75°C), there was an increase in PL activity and a decrease in PG activity (Lu et al., 2009).

However, when milk was heated to higher temperatures of 85°C or 90°C, we observed a larger decrease in PG activity and no further increase in PL activity. To test the hypothesis that this was due to PG interaction with β-LG, PG activity was tested in the presence of β-LG or cysteine, which caused a marked decrease in PG activity. Two-dimensional electrophoresis confirmed that heating of PG in the presence of β-LG causes polymerization of PG into dimers, trimers, and polymers through disulfide interchange. Formation of polymers hindered the activation of PG into PL.

**Cheesemaking Conditions.** Many factors in cheesemaking systems can affect the activity of the PL system. For instance, the cook temperature of the curd was shown to affect the PL activity. Higher levels of PL activity were observed for Swiss cheese compared with Cheddar cheese (Richardson and Pearce, 1981; Farkye and Fox, 1990). The higher cook temperature during the production of Swiss cheese (50°C) compared with that of Cheddar cheese (37°C) was thought to be the contributing factor, because the higher temperature inactivates rennet, thus the cheese relies primarily on PL for ripening (Farkye and Fox, 1990). Somers and Kelly (2002) indicated that increased cooking temperature causes higher PL activity due to PG activation.

The effect of salting of the curd has also been investigated. Salt concentration has been found to have little to no effect on PL activity (Farkye and Fox, 1990). However, Fox et al. (2000) found that low concentrations of salt (up to 2% NaCl) slightly stimulated PL, whereas higher concentrations (8% NaCl) decreased PL activity. Aggeler et al. (1981) investigated the effect of salt on PA under physiological condition. They observed that physiologic salt concentration (0.15 M NaCl) inhibited PA of cells from several mammalian species. Choi et al. (2006) determined the effects of curd cook temperatures and salt concentrations on the activities of PL, PG, and PA. Plasmin, PG, and u-PA activities were lower in curd cooked at 21°C compared with that cooked at 37°C or 55°C. Activity assays showed that only PG was affected by salt concentration (i.e., higher
PG-derived activity at 0% salt than at 5% salt). However, electrophoresis results showed that PL and t-PA activities increased with salt concentration.

**The pH of Milk**

Plasmin is most active at pH 7.5 to 8.0 and at 37°C (Fox, 1981), but is stable and active over a broad pH range as indicated in part by its activity in various cheeses (Bastian and Brown, 1996). Lowering the pH of milk, such as in the case of cheese and yogurt production, will cause the precipitation of casein proteins, thus forming a curd. Low pH causes dissociation of the casein micelle and with it dissociation of PL and PG from the micelles into the whey fraction of milk. The effect of pH on PG activation has not been fully researched.

**Storage Conditions**

**Cold Storage of Milk.** In the United States, extended refrigerated storage of milk on the farm, in transport, at the dairy plant, and in supermarkets (following thermal processing) have led to a total age of the milk before consumption of 20 to 21 d. Refrigerated storage (typically at 2 to 5°C) of raw and pasteurized milk for long periods has resulted in quality problems for the dairy industry. Refrigerated storage can affect milk quality either by promoting the growth of psychrotrophs or by affecting the PL system. Although the optimal temperature for PL activity is 37°C, PL can still be active during cold storage. Similarly, PA can be active during refrigeration and thus mediate the conversion reaction of PG to PL. On the other hand, changes occur in milk protein micelles during refrigerated storage; β-casein becomes more soluble at lower temperatures. This change in location of β-casein may allow PL more access, resulting in more PL-mediated proteolysis during refrigerated storage of milk. Crudden et al. (2005) noted significantly more autolysis of PL during storage at 5°C compared with storage at 20°C and 37°C. In their study, the authors also observed significant dissociation of β-casein into the serum fraction during storage at 5°C, supporting previous observation by Davies and Law (1983) and Ali et al. (1980).

Guinot-Thomas et al. (1995) observed a decrease in caseinated calcium in curd over a period of 6 d of storage at 4°C, which could be due to the transfer of the calcium into the soluble phase. The researchers attributed the decrease in casein-fraction calcium composition to casein hydrolysis by microbial proteases. In milk, calcium can be present as free ions or as calcium phosphate. Both the free calcium and the calcium phosphate can be bound to casein to form caseinated calcium or micellar calcium phosphate, respectively. Total levels of calcium in cheese have been shown to influence cheese texture (Solorza and Bell, 1995). Manufacturing parameters, such as preacidification, were found to decrease the water-insoluble calcium level in cheese (Metzger et al., 2001), which was associated with decreased post-melt chewiness. Upon acidification, some of the calcium associated with the caseins was lost in the serum fraction. The dissociation of calcium from the casein micelle and its solubilization in the serum might influence PL activity and PG activation during cold storage. Schroeder et al. (2008) investigated the effect of calcium ion on the PL system under refrigerated storage of milk. The researchers kinetically characterized the effect of calcium on PL activity and PG activation. The researchers showed that storage at 2.2°C resulted in lower PG activation and higher serum calcium compared with storage at 4.4°C. Further testing showed that calcium actually reduced the catalytic efficiency of PG activation, as measured by kcat/Km. Results suggest that although storage of milk at 2.2°C would be desirable for fluid milk, it might be advantageous to store cheese milk at a higher temperature. Further research is needed to investigate closely the effects that different refrigeration temperatures and time have on PG activation, taking into account subsequent changes in the levels of calcium as well as the level, structure, and solubility of other milk components.

**Room Temperature Storage of Milk.** Ultra-high-temperature milk and NFDM are normally stored at room temperature. Plasmin can undergo autolysis with prolonged storage, thus losing some of its activity. However, the chances for PG activation are high, because the temperature (22–25°C) is close to optimum (37°C) and PAI are already inhibited by the heat treatment applied to the UHT milk and NFDM. Therefore, more casein hydrolysis is prone to take place, leading ultimately to the gelation of UHT milk and poorer protein quality of NFDM, which is used in many food products as a functional ingredient.

**Bacterial Proteases**

Heat-stable metalloproteinases produced by psychrotrophic microorganisms during refrigerated storage (Cousin, 1982) can contribute to proteolysis in milk. The current trend in the dairy industry is to reduce the frequency of milk collection, thus the refrigerated storage of milk has been lengthened, allowing the psychrotrophic bacteria to dominate the microflora. The heat-stable proteases produced by the psychrotrophic bacteria can destabilize the casein micelles by hydrolyzing κ-CN (Ewings et al., 1984; Mitchell and Marshall, 1989; Cromie, 1992), resulting in reduced cheese quality,
production of small peptides that contribute to bitter flavor, UHT gelation, and fouling of heat exchangers (Grufferty and Fox, 1988a; Champagne et al., 1994). In refrigerated raw milk, gram-negative psychrotrophs are responsible for spoilage; of these, Pseudomonas spp. are predominant. Pasteurized milk, on the other hand, is spoiled primarily by gram-negative psychrotrophs that recontaminate the milk after pasteurization, or by gram-positive psychrotrophs that survive pasteurization. Among microorganisms that survive pasteurization, spore-forming Bacillus spp. dominate (Cousin, 1982; Sorhaug and Stepaniak, 1997). An extracellular protease from Pseudomonas fluorescens M3/6, produced after incubation in reconstituted NFDM stored at 7°C, was characterized and shown to have activity on α-, β-, and κ-CN (Kohlmann et al., 1991a,b). Matta and Punj (1998) isolated and partially characterized a protease from Bacillus polymyxa B-17. The B. polymyxa protease, referred to as “Milcozyme” in the literature, has been used as a microbial rennet substitute (Jarmul et al., 1983). However, Milcozyme was found to be an unacceptable substitute because of a lower yield and softer curd than that produced with rennet (Reps et al., 1974a).

Several studies have shown that bacterial proteases affect the PL system, in which turn will affect the quality of dairy products. Plasmin activity has been reported to decrease with microbial growth and storage time. Decreased PL activity was observed in fresh raw milk after 4 d of storage at 4°C, with the psychrotrophic bacterial count reaching 10^6 to 10^7 cfu/mL (Guinot-Thomas et al., 1995). The PL decrease was attributed to psychrotrophic bacterial protease activity and PL autolysis (Guinot-Thomas et al., 1995). Studies with reconstituted NFDM (Fajardo-Lira and Nielsen, 1998) and fresh milk (Fajardo-Lira et al., 2000) stored under refrigerated conditions indicated that proteases produced by P. fluorescens M3/6 affected PL location by disrupting the casein micelle to release enzymes of the PL system into the whey. A reduced PL activity in the casein fraction and an increased activity in the whey fraction were observed with the growth of psychrotrophic microorganisms and the presence of proteases they produced (Fajardo-Lira and Nielsen, 1998; Fajardo-Lira et al., 2000). The 2 studies demonstrated clearly the effect of the bacterial protease on PL activity in the casein and whey fractions, when casein was separated from whey by acid treatment (Fajardo-Lira and Nielsen, 1998) and by centrifugation (Fajardo-Lira et al., 2000). Further studies have shown that some bacterial proteases can enhance the activity of PA, or actually act as a PA, to increase plasmin activity. Pseudomonas fluorescens M3/6 protease was shown to enhance PA activity by enhancing its catalytic activity (kcat/Km). The M3/6 protease caused the conversion of u-PA into the more active 2-chain protein of lower molecular weight (Frohbieter et al., 2005). Bacillus polymyxa protease, on the other hand, acted as a PA, hydrolyzing PG into lower molecular weight proteins with activity similar to PL (Larson et al., 2006). These findings suggest that the PL system and the metalloprotease system may be functionally interactive and cooperate in extracellular proteolysis. Therefore, when the intent is to study PL system activity in milk, it is crucial to minimize or eliminate the presence of bacterial proteases, ideally by starting with very fresh milk. Also, bacterial proteases can be shut down by the addition of a metalloproteinase inhibitor, such as EDTA.

**IMPORTANCE OF PLASMIN ACTIVITY IN VARIOUS DAIRY PRODUCTS**

The reported advantages and disadvantages of PL activity in various dairy products indicate the need to control the activation of PG and therefore the activity of PL. Cheeses and UHT milk are the principal dairy products for which the advantages and disadvantages of PL activity have been studied. Properties and importance of PL in bovine milk and dairy products have been reviewed (Grufferty and Fox, 1988a; Fox, 1992; Bastian and Brown, 1996). However, PL activity may influence the quality of other dairy products such as casein and whey protein products, and NFDM. Understanding the function of the PL system components and their interactions with other milk constituents is crucial for efficient control of PL activity. Currently the dairy industry is trying to find the best conditions for the processing of the aforementioned products to enhance the quality. Although attempts are being made, complete control or shut down of the PL system has not yet been achieved.

**Cheese**

Research on milk that contains high levels of PL suggests that hydrolysis of casein by PL does not greatly influence the time it takes for rennet to clot milk during cheesemaking, but this hydrolysis may reduce the strength of renneted milk gels (Bastian and Brown, 1996). The more commonly studied question about PL and cheesemaking is its importance for cheese ripening. Proteolysis in cheese during ripening results in texture modifications, an increase in pH through NH₃ formation, and the production of flavor compounds (Fox et al., 1993). Results of numerous studies with various cheeses indicate that the importance of PL in cheese ripening differs by variety, depending on the cooking temperature during cheesemaking and the pH during
ripening (Fox, 1989; Farkye and Fox, 1990; Bastian and Brown, 1996).

Increased PL activity, because of either PG activation (Bastian et al., 1991c,1997) or addition of PL (Farkye and Fox, 1992; Farkye and Landkammer, 1992), has been shown to improve the flavor and overall quality of certain cheeses. Even in Cheddar cheese, for which PL is considered less important than in Swiss cheese (Bastian and Brown, 1996), adding PL inhibitor during the cheesemaking process resulted in significantly less proteolysis of β-casein during ripening compared with the control cheese (Farkye and Fox, 1991).

Researchers have suggested that as cheese ripening progresses, PL activity decreases (Bastian and Brown, 1996). One way to overcome this PL loss would be to replenish the inactivated PL with newly activated PL from PG. In fact, ripening of Cheddar and Swiss cheeses has been accelerated through the addition of exogenous PA (Bastian et al., 1997; Barrett et al., 1999). The addition of exogenous PA, however, is likely to have limited practical applications because of cost and availability issues. Another possibility for increasing the amount of active PL in cheese may be activating endogenous PG with endogenous PA, through controlling the cheesemaking conditions to maximize PA activity.

Results of numerous studies suggest that PL could be a valuable enzyme for accelerated ripening and improving flavor development in natural cheese (Bastian and Brown, 1996). Because cheese ripening is a time-consuming and expensive process (Webster and Frye, 1987), considerable benefits are to be gained in accelerating it, and considerable costs are associated with slow ripening. In cheeses for which PL is important for ripening, reduced levels of PL in the casein micelle presumably would lead to reduced cheese quality and increased costs of production.

**UHT Milk Products**

The evidence on the role of PL in the age gelation of UHT milk is conflicting. Results obtained and conclusions reached seemingly depend on factors such as processing conditions, storage conditions, level of PL, concentration of milk, and other ingredients used in the product. However, it seems clear that when PG or a low level of PL is added to UHT milk, the milk gels faster than milk with no added enzyme (Bastian and Brown, 1996). Heat can cause the whey proteins to bind to κ-casein located on the surface of the casein micelles, causing aggregation. Increasing the heating time and temperature of milk results in increased whey protein association with casein micelles. Therefore, in the production of UHT milk, for instance, raw milk is preheated (80–95°C for 30–60 s) before high temperature heating (135–150°C for a few seconds) to avoid forming complexes between whey proteins and casein. This delays gelation but does not shut down the PL system completely.

**Milk Protein Products**

Although PL activity is probably important in various milk protein products, little is known about the amounts of PL present and its effect on the properties of such products. Caseinates and whey protein concentrates and isolates are produced in large quantities. These are important as functional ingredients for formulated foods, such as bakery, dairy, beverage, dessert, pasta, confectionery, and meat products. In addition, both casein and whey protein products are used in animal feeds, and caseinates have numerous industrial applications. Casein and whey protein products are used in foods and other products because of their gelation, coagulation, hydration, emulsifying, and foaming properties. These functional properties are largely controlled by the chemical composition and physicochemical properties of the protein products. These in turn are determined by the composition of the milk and the processing conditions used for their isolation. One factor that can affect the functional properties of milk protein products is the PL content. For example, the functional properties of β-LG are reportedly improved by a 4% degree of hydrolysis caused by PL (Cassens et al., 1999b). However, thinning has been reported in industrial products to which caseinates are added, possibly because of PL activity (Nielsen, 2002). For both casein and whey protein products, it may be desirable to have little or no PL present that would hydrolyze other proteins in a system to which they are added as functional ingredients.

**NFDM**

Nonfat dry milk is yet another common protein-derived ingredient used to enhance functional properties, such as viscosity, emulsion stability, and foam stability in many food products. Most yogurts, pasteurized processed cheeses, bread machine mixes, and ice cream products contain significant amounts of powdered milk ingredients. The PL, PG, and PA, which are associated with casein micelles, are retained in the NFDM. Plasmin activity in powdered milk can cause unwanted proteolysis, thus affecting the functional properties of milk powders and the quality of the food products thereafter. The processing of NFDM includes defatting, heat-treating, concentrating, and then spray drying.
Predrying heat treatment conditions typically employed during the production of NFDM have not been researched with regard to PL system activity.

**RESEARCH NEEDS AND CONCLUDING REMARKS**

Identifying and characterizing the components of the PL system have improved our understanding of this system and its effects on the quality of dairy products. However, further work is needed to better control PL system activity in various dairy products. Improved control of the PL system will be possible only by fully understanding the conversion of PG to PL, and thus the factors that influence the PA kinetics, which include calcium content, pH, storage temperature, and whey proteins. The onset of the interference, the amount of β-LG required, and the mechanism of the interaction between β-LG and PG need to be further studied. Additionally, determining the factors that enhance or inhibit PL activity and the factors that influence the shift of PL from the casein to the whey fraction can lead to better control of the PL system. Adjusting the heating and storage (refrigerated and room temperature) conditions might result in better control and possible shutdown of the PL system. Research in this area is a pressing need, with specific emphasis on UHT milk, NFDM, yogurt (viscosity), and cheese (ripening) applications. Utilizing this knowledge has the potential to improve the quality and reducing the production costs of these dairy products.

**REFERENCES**


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