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Plasmin in Milk: Activity Measurement, Effect of Environmental Factors, and Correlation with Milk Coagulation

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Plasmin in Milk: Activity Measurement, Effect of Environmental Factors, and Correlation with Milk Coagulation

by

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My family has supported me in every phase of my education, never allowing me to give up or be discouraged for very long. I'm especially grateful for my parents who gave me a desire to be successful and taught me to work. I am forever in their debt.

This work is dedicated to Julie and Kyle. They were beside me all the way.

Eric Bastian

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ABSTRACT

Plasmin in Milk: Activity Measurement, Effect of Environmental Factors, and Correlation with Milk Coagulation

by

Eric D. Bastian, Doctor of Philosophy

Utah State University, 1989

Major Professor: Dr. C. Anthon Ernstrom

Department: Nutrition and Food Sciences

Bovine plasmin activity was measured on H-D-valyl-L-leucyl-L-lysyl-4-nitroanilide by following absorbance changes at 405 nm. Steady-state kinetic parameters V_{max}, K_m, K_I, and K_I' were estimated. Bovine plasmin is competitively inhibited by casein and has a K_{cat} of .0158 ΔA_{405}/min/n*M*, K_m of .107 m*M* substrate, and K_I of .86 mg/ml casein. Bovine plasmin can be measured directly in bovine milk without interference from casein.

A total of 380 milk samples from nineteen Holstein (one herd) and nineteen Jersey (one herd) cows was collected monthly during one lactation period. Samples from each cow were analyzed for fat, protein, plasmin activity, plasminogen, pH, SCC, clotting time, curd firming rate, and final curd firmness. Three age groups from each breed/herd were chosen; first, third, and fourth and later lactations.

Plasmin activity in milk was most affected by lactation number, with milk from fourth- and later-lactation cows having higher activity than milk from first- or third-

lactation cows. Plasmin activity in milk increased during lactation but was not affected by breed/herd, pH, protein, or fat. Plasminogen averaged 5.4 times the plasmin activity in milk and increased during the first five months of lactation. Plasmin activity was higher in milk collected during summer and fall but plasminogen was higher in milk collected during fall and winter. Percentage of the total (plasmin + plasminogen) enzyme activated to plasmin increased in late-lactation milk and in milk from fourth- and later-lactation cows.

Plasmin activity did not affect any milk clotting parameters in this study. Increased protein in milk resulted in shorter clotting times. When statistically adjusted for protein content, clotting time was longer in milk from the Holstein herd compared to the Jersey herd. Curd firming rate was increased in milk with higher protein and fat. Milk samples collected in the fall had faster firming rates than milk from other seasons. Firming rates remained constant during lactation but increased with higher protein and fat content. Jersey herd milk produced firmer curd than Holstein herd milk, and milk collected in the fall had firmer curd than during the other seasons. (100 pages)

PART 1. PLASMIN IN DAIRY PRODUCTS: A REVIEW

ABSTRACT

Plasmin is secreted as plasminogen, which is activated in blood and milk. Its role in blood is to proteolytically break down blood clots. The enzyme has affinity for lysine residues and preferentially cleaves lys–x bonds. Plasmin purified from bovine milk and blood are identical when compared kinetically and immunologically. Plasmin purified from milk has optimum activity at pH 7.5–9.0 and 37°C. Methods for determining plasmin activity in milk have been developed. Synthetic chromogenic subtrates are often used because of their sensitivity and specificity. Plasmin is associated with casein micelles in milk and degrades β-casein and α_S-casein to γ-casein, proteose-peptones, and possibly λ-casein. κ-casein is resistant to proteolysis by plasmin. Plasmin activity in milk increases proteose-peptone fragments and affects the functional properties of casein by reducing milk viscosity, decreasing micelle size, and increasing the surface activity of casein. Plasmin contributes to proteolysis during cheese ripening depending on pH, NaCl application method, and cheese variety. The enzyme is heat resistant and survives many UHT treatments, but its role in gelation of UHT-treated milk is not fully understood. Enzyme activity increases in milk with stage of lactation, level of mastitis, and lactation number.

INTRODUCTION

Babcock and Russel (9) first reported proteolytic activity in fresh raw milk, which some researchers attributed to bacterial enzymes (52) or a native enzyme called galactase (97). However, more recent work has shown it to be caused by a nonbacterial, native milk proteinase (34, 46, 99). It is an alkaline serine proteinase analogous to the bovine blood serum enzyme, plasmin (EC 3.4.21.7) (19, 53, 59, 75).

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Properties and importance of bovine alkaline milk proteinase or bovine plasmin have been reviewed by Humbert and Alais (52), Fox (33), Visser (107), and Grufferty and Fox (41). Plasmin activity also has been observed in milk of other species (41).

STRUCTURE AND FUNCTION OF PLASMIN

Structure of Bovine Plasminogen

Bovine plasminogen has 786 amino acid residues. Based on its amino acid sequence, it has a molecular weight of 88,092 (94). Experimental reports of molecular weight vary from 48,000 (53) to 100,000 (26) as determined by gel filtration. Bovine plasminogen is different than human plasminogen because the amino acid composition and sequence of the proteins are not the same. However, distribution of cysteine residues is identical, suggesting that the five characteristic triple-loop structures, called kringles, which are stabilized by three disulfide bonds, are conserved between the two species (94). There are five lysine binding sites in human and bovine plasminogen, one associated with each kringle. One binding site has high affinity for lysine ($K_d = 9\ \mu M$), and the other four have lower affinity ($K_d = 5$ mM) (61).

Physiological Importance of Plasmin

Clotting of blood is an important defense mechanism against loss of blood and bacterial invasion. During healing, blood clots are removed by fibrinolysis (36). Plasmin is important in the fibrinolytic system of mammals because it cleaves fibrin, the blood clotting protein. It is secreted as a proenzyme, plasminogen, and activated by hydrolysis of Arg^{560}-Val^{561} in human plasminogen (32) and Arg^{557}-Ile^{558} in bovine plasminogen (94). The amino acid substitution around the activation site in bovine

plasminogen, with other substitutions, makes it resistant to activation by streptokinase, though streptokinase readily activates human plasminogen (94).

Human plasminogen has an N-terminal glutamic acid. Traces of plasmin cleave the first 76 residues, leaving an N-terminal lysine. Bovine plasminogen has an N-terminal aspartic acid, and cleavage of the first 77 residues by plasmin gives an N-terminal arginine (94). This N-terminal fragment of plasminogen has been called the prepeptide, but its release does not activate plasminogen; rather it causes a conformational change in plasminogen that exposes Arg^{557}–Ile^{558} allowing increased rates of activation (16). During bovine plasminogen activation, the prepeptide is cleaved by plasmin, leaving a structurally modified inactive protein. Then Arg^{557}–Ile^{558} is cleaved by urokinase or tissue-specific plasminogen activators (t-PA) to give an active two-chain molecule consisting of a heavy chain joined to a light chain by a single disulfide bond (86). The active site of plasmin is in the light chain and, like trypsin, consists of His^{598}, Asp^{641}, and Ser^{736} (16, 94).

Plasmin's action is prevented in fluid blood by antiproteolytic enzymes: α_2-antiplasmin and α_2-macroglobulin (16). Tissue-plasminogen activators do not easily convert plasminogen to plasmin in blood serum but have high binding affinity to fibrin clots and become effective in converting plasminogen to plasmin when bound. Plasminogen interacts only weakly with fibrin (through lysine binding sites), but it has high affinity for t-PA bound to fibrin. Bound t-PA activates plasminogen and increases plasmin concentration at the fibrin matrix. Plasmin formed on fibrin surfaces already has its active site and lysine binding sites occupied and is, therefore, slowly inactivated by α_2-antiplasmin. A tissue-specific plasminogen activator has been isolated from human mammary epithelial cells (32) and tissue-specific activators probably exist in bovine mammary epithelial cells. Plasminogen activators are found in animal tissues and in milk (59). Blood trypsin inhibitors occur in milk and may affect plasmin activity (51) .

Purification of Plasmin

A number of workers have isolated and purified plasmin from bovine milk (26, 45, 54, 79). Dulley (26) purified alkaline milk proteinase 629–fold from acid casein with ammonium sulfate (40% w/v) precipitation, CM cellulose chromatography, and Sephadex G–200 gel filtration. Kaminogawa et al. (54) purified plasmin from acid casein by a sulfuric acid and dimethylformamide method, 20% w/v ammonium sulfate precipitation, DEAE–cellulose ion exchange chromatography, and Sephadex C–50 followed by Sephadex G–100 gel filtration. They increased the specific activity of native milk proteinase 3900-fold compared to that of unfractionated acid casein. Halpaap et al. (45) and Reimerdes and Petersen (79) used affinity chromatography to purify milk proteinase and in a single step purified the enzyme 90-fold and 142-fold (giving total purification of ~17,000–fold (41)). They used lysine-Sepharose columns since plasmin has a specificity for lysine residues. Affinity chromatography coupled with ammonium sulfate precipitation and gel filtration yielded .3 mg of milk proteinase from one liter of milk. Halpaap et al. (45) purified plasmin from bovine blood using the same procedure and reported a yield of 200 mg/L of blood.

Characterization of Plasmin

Diisopropyl-fluorophosphate (DFP) is a serine proteinase inhibitor. Tomich et al. (101) and Kaminogawa et al. (55) reported total inhibition of plasmin by DFP combined with iodoacetic acid. Plasmin has at least one serine residue in its active site. Chen and Ledford (13) found total inhibition of plasmin by soybean trypsin inhibitor, but Kaminogawa et al. (55) observed only partial inhibition. These conclusions led to acceptance of native milk proteinase as a trypsin-like serine proteinase.

Optimum temperature and pH ranges for alkaline milk proteinase or bovine milk plasmin have been reviewed by Humbert and Alais (Table 1) (52).

Table 1. Properties of purified native proteinases from bovine milk

	Optimum Conditions	
Authors	pH	Temperature (°C)
Warner & Polis (1945)	8.5	-
Kiermeier & Semper (1960)	6.8	37
Carini & Bozzolati (1970)	-	45
Kaminogawa et al. (1972)	8.0	37
Dulley (1972)	6.5-9.0	37
Reimerdes et al. (1975)	7.5-8.0	-
Richter et al. (1977)	6.5-7.5	37

Plasmin's optimum pH range is 6.8-9.0 although reports of its pH stability vary because of differences in purification and isolation methods. Impure preparations have often been used and may include enzymes other than plasmin. Recently it has been observed that two similar serine proteinases and an acid proteinase are extracted from milk by many purification procedures (53, 56, 73). Reimerdes (73) discovered that the two serine proteinases have different specificities for lysine and arginine peptide bonds. He separated the two by lysine-Sepharose affinity chromatography. One bound tightly to the column, but the other had no affinity. These two proteins were called proteinase I and proteinase II. In kinetic experiments, proteinase I was most like plasmin purified from bovine blood. There was a high degree of homology between the two enzymes. Eigel et al. (30) reported further evidence for the identity of alkaline milk proteinase as

plasmin by immunological crossreactivity between whole casein prepared from bovine milk and antisera raised against bovine blood plasmin.

PLASMIN IN MILK

Distribution of Plasmin in Milk

Purification of plasmin from the casein fraction of milk suggests its association with casein micelles. Milk proteinase-casein micelle interactions have been reported (30, 33, 78, 96). Grufferty and Fox (41) reviewed such associations with acid casein, rennet casein, or centrifuged casein micelles. Plasminogen association with milk fat globule membrane also has been reported (50). Korycka-Dahl et al. (59) concluded that most of the milk proteinase associates with casein micelles, but small amounts interact with milk fat globule membrane.

Plasminogen and plasminogen activators also are associated with the casein micelle but not with milk serum (20, 59,76, 81), although inhibitors of plasmin and plasminogen activators occur only in the serum phase of milk (59, 77). The postulated plasmin system in milk is shown in Figure 1. Without plasminogen activator inhibitor, plasminogen activator produces active plasmin. If plasmin inhibitors also are absent, then plasmin degrades casein.

Plasmin activity has been dissociated from casein micelles by ε-amino-n-caproic acid at levels up to 0.1 *M* (59, 81). Higher levels of this lysine derivative (K_I = 80 m*M*) will inhibit plasmin activity (59, 88). However, incubation of milk with 50 m*M* ε–amino–n–caproic acid enhances plasminogen activation (95) if the prepeptide has not been cleaved (41). Grufferty and Fox (43) studied release of plasmin activity from casein micelles using PAGE and free amino group measurement by

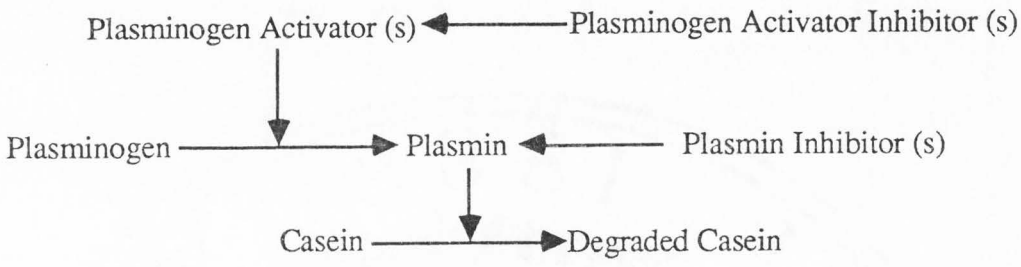

Figure 1. Schematic representation of plasmin enzyme system in milk

fluorescence to examine effects of low-temperature storage, addition of NaCl, and pH on plasmin association with centrifuged micelles. Centrifuged micelles from milks stored at 4°C and 20°C for 16 h had the same plasmin activity, indicating that temperature does not affect release of plasmin activity from casein micelles. Plasmin activity was lower in casein ultracentrifugates stored at 4°C than at 37°C, suggesting that the reaction is slower or activation of plasminogen does not occur at low temperatures. This conflicts with reports of increased plasmin activity in milk stored at 4°C by Reimerdes and Herlitz (74) but is in agreement with Donnelly and Barry (22), who showed extensive proteolysis of β-casein at 37°C (.06–.15 mg/h/ml) but little or no proteolysis in milk stored at 4°C (.003–.01 mg/h/ml). At low temperatures, proteolysis by plasmin is slow. Addition of 1 *M* NaCl caused complete loss of plasmin activity from micelles. Micelle-associated plasmin activity was not reduced in the pH range of 6.6–4.8; however, at pH 4.6 most of the activity was removed. This differs from observations of time-dependent dissociation of plasmin from casein starting below pH 5.7 with its rate increasing at pH 4.7 (83) . However, Richardson and Elston (83) isolated casein by acid precipitation at pH 4.7. This precipitation may have released some of the proteolytic activity that remained with casein micelles in Grufferty and Fox's (43) centrifugation studies.

Determination of Plasmin Activity in Milk

Several methods for measuring alkaline milk proteinase activity in milk have been described. Driessen and van der Waals (24) used PAGE and densitometry to determine rates of formation of γ-caseins in sterilized milk. Dulley (26) and Yamauchi et al. (110) used casein hydrolysis assays. They precipitated casein with acid before and after incubation and measured free tyrosine. Other workers have used labelled proteins such as azocasein (111) and ^{14}C-methylated β-casein (23).

Andrews (5) immobilized casein on Sepharose and linked it to a reporter enzyme that was resistant to proteolysis. When casein was proteolyzed by plasmin, the reporter enzyme was released from Sepharose. During subsequent incubation, reporter enzyme then reacted with many of its own substrate molecules in proportion to the initial cleavage. The above methods suffer from being nonspecific, laborious, or requiring much sample preparation.

Richardson and Pearce (84) developed a fluorometric assay using a coumarin peptide with lysine in the position next to coumarin. Plasmin cleaves this bond releasing 7-amido-4-methyl-coumarin, a fluorescent product. Rate of increase in fluorescence is proportional to plasmin activity. They reported plasmin and plasminogen contents of pasteurized skim milk to be .14–.73 g/ml and .55–2.75 g/ml. A similar method was reported by Rollema et al. (89) using a chromogenic substrate, H-D-valyl-L-leucyl-L-lysyl-4-nitroanilide. Plasmin cleaves the lysine–nitroanilide bond and releases 4-nitroaniline, a compound which absorbs light at 405 nm. Change in absorbance per unit time is a measure of proteolytic activity. Plasminogen is assayed after activation with urokinase using the nitroaniline substrate. These fluorogenic and chromogenic substrates are sensitive and specific for plasmin in milk and require little sample preparation to measure plasmin activity.

Effects of Plasmin on Casein

Plasmin is primarily secreted in normal milk as plasminogen. It is activated during storage (2, 24) or even while milk is held in the mammary lumen before milking (22, 92). Because plasmin is associated with casein micelles, degradation of micellar casein is expected. Yamauchi and Kaminogawa (109) identified decomposition products of β-casein caused by alkaline milk proteinase or plasmin. These peptides had mobilities equal to γ-caseins in electrophoretic gels. They later showed that amino acid

composition, molecular weight, sedimentation coefficients, and terminal amino acids of these β-casein breakdown products were equivalent to γ-caseins (57), suggesting that γ-caseins are proteolytic products of β-casein. Gordon and Groves (37) sequenced γ_1-, γ_2-, and γ_3- caseins, compared them to β-casein, and confirmed that γ-caseins are pieces of β-casein. Eigel (27), and Snoeren and Van Riel (96) showed that proteolysis of β-casein by plasmin from blood and milk resulted in carboxyl-terminal fragments corresponding to γ-caseins. Later, Andrews (3) and Eigel (29) showed that proteose-peptone component 5 has two plasmin derived N-terminal pieces of β-casein corresponding to residues 1–105 and 1–107. Remaining peptides, comprised of residues 106–209 and 108–209, correspond to γ_2-, and γ_3-casein. After incubating β-casein with milk and blood plasmin, Andrews (4) showed that proteose-peptone component 8-fast is produced from the N-terminal end of β-casein. Cleavage of β-casein at 28–29 gives γ_1-casein and component 8–fast. Although Andrews and Alichanidis (8) reported proteose-peptone component 8–slow to be a heterogeneous mixture not derived from β-casein, Eigel and Keenan (31) showed that it is two pieces of β-casein corresponding to residues 29–105 and 29–107. The postulated degradation scheme of β-casein by plasmin is shown in Figure 2.

Snoeren and Van Riel (96) reported that after 4 h incubation, proteolysis of α_{S2}-casein by bovine blood and milk plasmin results in degradation of 80% of α_{S2}-casein. They also found that the degradation rate of α_{S2}-casein by plasmin is equivalent to that of β-casein.

When purified α_{S1}-casein is incubated with bovine plasmin at 37°C, the electrophoretic band corresponding to α_{S1}-casein B disappears in 30 min with formation of several bands having higher electrophoretic mobilities (6, 8, 19, 28). A lower susceptibility of α_{S1}-casein to plasmin compared to β-casein was observed. Aimutis

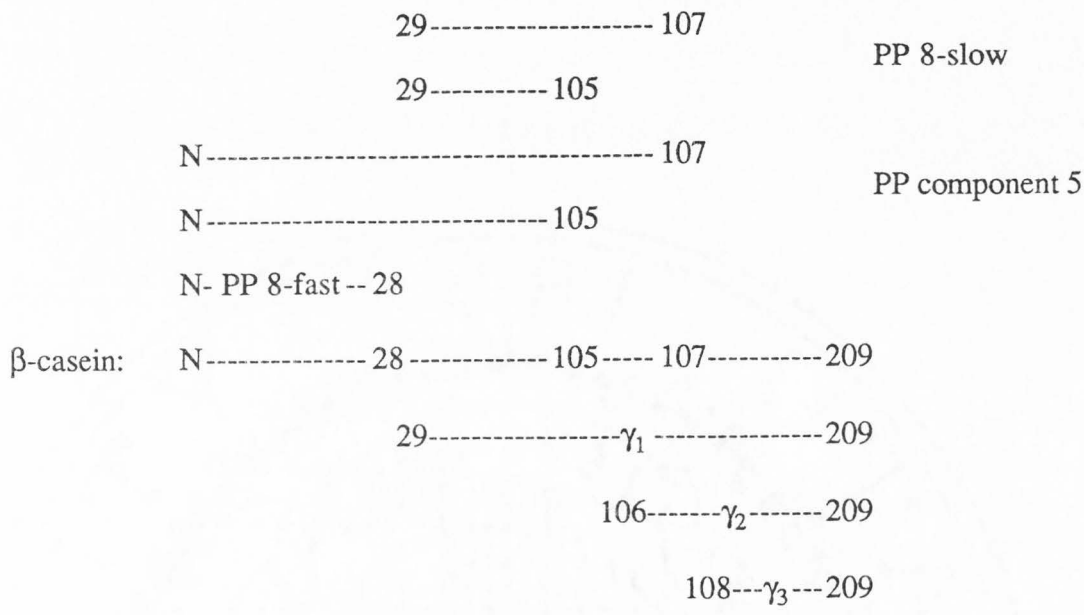

Figure 2. γ-casein and proteose-peptone (PP) fragments of β-casein after plasmin hydrolysis (100)

and Eigel (1) have indicated that the electrophoretic patterns of crude λ-caseins are identical to patterns of peptides derived from α_{S1}-casein incubated with plasmin.

Eigel (28) detected no change in electrophoretic patterns before and after incubation of κ-casein A with bovine plasmin at 37°C for 1 h. Doi et al. (21) purified five κ-casein fractions with different amounts of carbohydrate. These fractions contained identical polypeptide chains but had carbohydrate moieties of increasing size. They incubated human plasmin with components which had no carbohydrate (P-2) or which contained a high amount of carbohydrate (P-5) and measured proteolysis using SDS PAGE. Although component P-5 was not hydrolyzed, component P-2 was hydrolyzed. Andrews and Alichanidis (8) showed κ-casein proteolysis only when plasmin was added at 20 times the amount required to achieve proteolysis of the other caseins. κ-casein is resistant to proteolysis by plasmin, and the degree of susceptibility of casein to plasmin is $\beta = \alpha_{S2} > \alpha_{S1} >> \kappa$ (8, 28, 96).

Andrews and Alichanidis (8) compared 38 components of the proteose-peptone fraction with proteolytic fragments derived from plasmin degradation of individual caseins. They found that 90% of these correspond to plasmin–casein fragments and that changes in proteose-peptone components occurred, depending on time and temperature of storage. They concluded that milk history is important in defining the proteose-peptone fraction. Schaar (92) found that variation in plasmin activity explained only 38% of the variability of proteose-peptones during lactation. He estimated that only 23% of proteose-peptones formed during cold storage was a result of plasmin activity (41). When the regression of plasmin activity and proteose-peptones was extrapolated to zero plasmin activity, proteose-peptones were not zero. This suggests that plasmin activity alone cannot explain the proteose-peptone content of fresh, high quality milk.

Grufferty and Fox (42) observed more hydrolysis by plasmin in redispersed micellar and non–micellar casein solutions than in the milk from which these caseins were prepared. Reimerdes et al. (77) found plasmin inhibitors only in the serum phase of milk. Centrifugation followed by redispersion would remove inhibitors from the system. Grufferty and Fox (42) determined that ethanol stability is not affected by plasmin activity. They also found smaller micelle size, reduced viscosity, increased solubility in pH 3.5–5.0 solutions, and increased surface activity (reduced interfacial tension) at the air–water interface after casein hydrolysis by plasmin.

EFFECTS OF PLASMIN ON CHEESE MAKING

Plasmin Influence on Cheese Curd Formation

Poor clotting properties are often observed in late-lactation milk (42, 66, 108) (longer clotting times and reduced curd firmness). Increased incidence of proteose-peptones (65), γ-caseins, and other casein breakdown products (10, 66) corresponds to observations of decreased β-casein and α_S-casein in such milk. This has led to theories of increased plasmin activity being responsible for poor clotting of late lactation milk. When Pearse et al. (69) incubated artificial micelle milk with porcine plasmin (.13 units/ml milk for 3 h) and 50% of the β-casein was degraded, a normal coagulum was formed. After 8 h incubation, a rennet clotting time was observed, but the curd formed was fragile (clotting time was not delayed until 20 h incubation). Artificial micelle milk incubated with plasmin for longer than 8 h did not form a coagulum. In comparison, Grufferty and Fox (42) found no change in rennet clotting time of casein micelle dispersions before and after incubation for 72 h at 37°C (no plasmin was added). After 96 h, the rennet clotting time doubled.

McMahon and Brown (62) have reviewed factors affecting clotting time in milk. These include pH, temperature, calcium ion activity, and enzyme used. Increased casein concentration has little effect on clotting time. When milk protein is concentrated by ultrafiltration, almost no change in clotting time occurs. However, curd firmness is significantly affected by increased protein. Pearse et al. (69) and Grufferty and Fox (42) did not measure curd firming, which would be more affected by plasmin activity than is clotting time. Okigbo et al. (67) reported that milk from some individual cows clots quickly but has poor curd firming properties. When pH of such milk was adjusted to 6.3, the clotting time decreased but curd firmness showed little improvement. Even pooling poor and good coagulating milk samples did not improve the clotting properties of the poor coagulating milk.

Role of Plasmin in Cheese Ripening

Traditional Cheese. The importance of plasmin in cheese ripening is still being debated. Stadhouders (98) followed amino acid nitrogen during ripening of two Dutch cheeses made from aseptically drawn milk. The milk for one of these cheeses was heated at 100°C for 5 min to destroy plasmin. He found no difference between the two and concluded that plasmin is not important in cheese ripening. Reiter et al. (80) found only low levels of amino acids liberated in aseptic starter-free Edam. In contrast, Visser (103) manufactured aseptic rennet-free, starter-free, and rennet- and starter-free Gouda. Any ripening in the rennet- and starter-free cheese could be attributed to plasmin. Plasmin produced soluble and amino acid nitrogen during ripening (105) but did not contribute to bitter flavor development (104).

Visser and deGroot-Mostert (106) reported formation of minor caseins from β-casein by plasmin during ripening of Gouda. However, this proteolysis was low compared to proteolysis by rennet and bacterial enzymes. Creamer (14) reported major

proteolysis of α_{s1}- and β-casein in Gouda and Cheddar by plasmin. Contrary to Visser and deGroot-Mostert (106), he attributed β-casein degradation in these cheeses to plasmin. In Meshanger–type cheese, plasmin activity was considered to be unimportant for normal ripening; but at high pH (6.2), β-casein was degraded more quickly than α_s-casein (64). Proteinase activity was stimulated by low concentrations of NaCl (2%) and higher cheese moisture.

Trieu–Cuot and Gripon (102) studied proteolysis in Camembert during ripening and found that plasmin activity was important after 21 d of ripening at the cheese surface and after 35 d in the center of the cheese. This increased activity coincided with increased pH at the surface and center. They concluded that plasmin is important in the ripening of Camembert.

Grappin et al. (38) have reviewed proteolysis of cheese proteins during ripening and concluded that plasmin is important, especially if rennet has been inactivated and the pH is favorable. Fox (35) also reported that plasmin activity is influenced by pH and NaCl and suggested that differences in plasmin activity between cheese varieties are based on salting procedures. When cheese is salted and pressed, the salt dissociates plasmin from casein, and plasmin is lost in the whey. Brine cheeses, however, lose little whey during brining; and plasmin remains in the cheese. Fox (35) considered the pH effect only as it pertained to release of plasmin from casein micelles and did not recognize that some plasmin, already associated with micelles, may be inactivated at lower pH. For example, as pH in Camembert increases, plasmin activity also increases, suggesting reactivation of plasmin already associated with casein. Fox (35) also suggested that plasmin is possibly important in high cook-temperature cheeses in which rennet is inactivated. So depending on the pH, NaCl application method, cook temperature, and cheese variety, proteolytic breakdown of casein by plasmin does play a role in peptide and amino acid release during cheese ripening (14, 35, 38, 106).

UF Cheese. Some cheeses are made from ultrafiltered milk (UF cheese) and contain higher amounts of whey protein than traditional cheese. This affects plasmin activity because 50% inhibition of plasmin by β-lactoglobulin has been observed (13, 53). Specifically, it is the heat denatured form of β-lactoglobulin that inhibits plasmin activity (40).

Casein breakdown in UF cheese is slower than in traditional cheese (17, 48, 72). Qvist et al. (72) found retarded proteolysis and flavor development in UF Havarti cheese. Although α_S-casein was degraded at a rate similar to traditional Havarti cheese, degradation of β-casein was much slower. They recorded some evidence that inhibition of plasmin by β-lactoglobulin has a retarding influence on UF cheese ripening. Creamer et al. (15) observed that the proteolytic action of rennet is also inhibited by whey protein.

There are inhibitors of plasmin in milk, other than β-lactoglobulin, that would be concentrated during UF and included in some cheese varieties. Casein concentration is higher in cheese than milk and probably has binding sites for plasmin which are not cleaved and act in an inhibitory fashion. All these factors would be included in observations of reduced proteolysis in UF cheese. To say that β-lactoglobulin is the only protein that reduces proteolysis in UF cheese is too simplistic.

HEAT STABILITY OF PLASMIN

Heat Inactivation

Plasmin is a heat resistant enzyme, and many heat treatments have been used in an effort to reduce proteolysis by plasmin in dairy products (2, 24, 26, 59, 63). Noomen (63) reported a 30-40% increase in proteolytic activity during storage (37°C) of HTST pasteurized milk (15 s at 72°C) and attributed it to destruction of a plasmin inhibitor.

Heat stabilities of plasmin, plasminogen, and trypsin inhibitor in milk are similar (81), and reports of increased plasminogen activation (19), after pasteurization, led Grufferty and Fox (41) to suggest that increased plasmin activity in pasteurized milk is because an inhibitor of plasminogen activator is destroyed by pasteurization. After storing samples for 3 d, Richardson (81) found activity in pasteurized milk to be three times greater than in raw milk. De Rham and Andrews (19) and Andrews (6) observed a similar phenomenon. Plasmin activity decreases 10–17% following HTST pasteurization of milk but increased plasminogen activation after pasteurization leads to more proteolysis during subsequent storage of pasteurized compared to raw milk (41).

Driessen and van der Waals (24) reported that heat inactivation of plasmin in aseptically drawn milk follows a first order reaction. The D–values were 55.6 min at 67.5°C and 7 s at 142°C. To limit proteolysis in sterilized milk, they recommend heating at 142°C for 16 s. Alichanidis et al. (2) also reported that heat inactivation of porcine plasmin added to skim milk, 25 mg/ml casein, 3 mg/ml α-lactalbumin, or 6 mg/ml β-lactoglobulin follows first order kinetics. Their D–values were 25 s and 10 s at 142°C for casein solution and skim milk. Increased levels of β-lactoglobulin accelerate heat inactivation but α-lactalbumin has no effect (2). Carboxymethylated β-lacto-globulin also has no effect. This suggests that an interaction between free sulfhydryl groups of β-lactoglobulin and plasmin increases the heat inactivation rate. Plasmin's lower heat stability in milk than in whey-protein-free micelle dispersions is attributed to this disulfide interchange (44, 87). Rollema and Poll (87) also found first order heat inactivation of plasmin and plasminogen in skim milk. Plasmin inactivation lagged behind that of plasminogen at all temperatures, and the rate constant was proportional to β-lactoglobulin concentration. They obtained D–values of 1.5 min at 85°C and 6 s at 140°C in skim milk. Plasmin's heat stability is lower in non–micellar than in micellar casein solutions, suggesting protection of the enzyme by casein micelles (44). Heat

stability is increased by adding .05 *M* lysine or ε-amino-n-caproic acid, showing that lysine binding increases heat stability.

Plasmin in UHT milk

Age gelation often happens during storage of UHT-milk (30, 41). Proteolytic action of plasmin has been suggested as a causative factor in this reaction (18, 58). There has been conflicting evidence for this, and many researchers have been unable to correlate proteolysis with age gelation (47, 60, 91). Plasmin and plasminogen survive most UHT heat treatments (e.g. 140°C for 4 s), and proteolysis by this enzyme system does occur in UHT-treated dairy products (12, 18, 25, 85). When UHT-milk is stored at room temperature, β-casein concentration decreases and γ-casein concentrations increase (12). Milk stored at 4°C does not exhibit this proteolysis.

Payens (68) reported that the aggregation kinetics of age gelation are similar to rennet clotting. However, other aspects of rennet coagulation and age gelation are different. The extent of κ-casein hydrolysis in gelled UHT-milk is only 5–10%; in rennet coagulated milk, it is 80–90% (49). Plasmin does not hydrolyze κ-casein, and the microstructure of gelled UHT-milks and rennet-coagulated milks is different (49).

Manji et al. (60) stored UHT-milk at 4°C, 23°C, and 37°C. These milks had received direct (steam injection) and indirect (plate heat exchange) heat treatments. They measured plasmin and plasminogen activity in these samples but found no correlation between plasmin/plasminogen activity and age gelation. Gelation was observed only in samples that had received direct UHT treatment.

Plasmin, plasminogen, urokinase, trypsin and trypsin inhibitor have been aseptically added to UHT-treated (14 s at 135°C) milk (58). Samples with added plasminogen and low levels of plasmin gelled faster than milk with no added enzyme. Addition of these proteins to UHT milk may not simulate properties of indigenous

proteins. de Koning et al. (18) added di-isopropyl-fluorophosphate and aprotinin to UHT-treated milk samples and observed no proteolysis or gelation, even after 9 mo storage at 20°C; but the control without inhibitor exhibited proteolysis and gelled in 60 d.

A thinning of UHT custard (heated for 2, 4, or 8 s at 142°C) occurs during storage at 20°C, accompanied by plasmin proteolysis (25). Custards treated for 18 or 36 s at 142°C are resistant to thinning. This suggests that plasmin actually causes thinning rather than gelation. The physiological role of plasmin is to break down blood clots. It also generates fragile curd in clotted milk after excessive proteolysis (see above). It is unlikely that it would contribute to gelation; rather it would contribute to thinning. However, the actual role of plasmin will remain speculative until the mechanisms of age thinning and age gelation are resolved.

FACTORS AFFECTING PLASMIN ACTIVITY IN MILK

Mastitis

Plasmin activity is higher in mastitic than in normal milk (39, 70, 71, 90). Mastitic milk has less β- and α_S-casein and correspondingly more γ- and κ-casein compared to milk from healthy quarters of the same cow (11). These differences were more evident after the milk was incubated for 8 h at 37°C unless soybean trypsin inhibitor was added to the milk before incubation. From this, Barry and Donnelly (11) concluded that plasmin activity accounted for the differences in casein composition between mastitic and normal milk. However, three different proteolytic enzymes that degrade casein have been found in mastitic milk (20). Two of these are inhibited by soybean trypsin inhibitor. Some of these enzymes may originate in somatic cells. When somatic cells in milk are disrupted, there is increased proteolysis (7, 20). This is

attributed to plasminogen activators (41) and proteolytic enzymes (11) being released from the somatic cells. However, when Grieve and Kitchen (39) added disrupted somatic cells to good quality milk and stored it at 5°C, no effect on milk proteins was observed. Incubating intact somatic cells in milk at 37°C for 24 h also gives limited activation of plasminogen, suggesting that intracellular activators and enzymes remain with the cells in milk and do not affect milk plasmin concentrations until released (71).

An 82% increase in plasmin activity in mastitic milk compared to normal milk has been observed (93). Plasminogen (measured after activation) increases only 21%. A close relationship between sodium and plasmin in milk also suggests that plasmin increases sodium leakage into milk by degrading membrane proteins (93).

Saeman et al. (90) induced mastitis in six Holstein cows and measured SCC, total proteolytic activity, plasmin activity, and nonplasmin activity during preinfection, infection, and postinfection. All parameters increased significantly during infection. Although nonplasmin activity and SCC decreased to preinfection levels during the postinfection period, total activity remained significantly higher than preinfection levels. Most of the higher activity was attributed to increased plasmin. They concluded that even after curing mastitis, milk quality does not return to its preinfection status.

Other Factors Affecting Plasmin Activity

Stage of Lactation. Plasmin and plasminogen (measured after activation) activities increase at the end of lactation (22, 70, 71, 92, 93). There is more γ-casein and less β- and α_S-casein in late-lactation milk (10, 11). Politis et al. (70) showed increased plasmin activity in late-lactation milk, even after adjusting for decreased milk production. Korycka-Dahl et al. (59) found no increase in plasmin in late-lactation milk, but plasminogen increased 2-fold. Richardson (82) suggested that increased plasmin activity occurs because more plasmin enters the mammary gland rather than increased

plasminogen activation (because of increased plasminogen activators). However, Politis et al. (71) reported plasminogen to plasmin ratios of 6.55 during early lactation and 3.29 at the end of lactation, suggesting increased activation of plasminogen to plasmin in milk during late-lactation.

Lactation Number. Milk from older cows has higher plasmin activity although plasminogen levels are constant (92, 93). Plasmin activity is constant in milk during the first three lactations of cows and increases for the fourth and later lactations even after statistically adjusting for SCC, season, stage of lactation and milk yield.

Breed of Cow. Higher plasmin activity has been observed in milk from American Holstein Friesian cows compared to Jersey cows, .15–.37 mg/L and .27–.53 mg/L (82). A similar trend has been observed in Swedish Friesian and Jersey cattle (92). However, when plasmin activity is adjusted for differences in casein content between these breeds, the difference was removed. From this, Schaar (92) concluded that the negative correlation between plasmin activity and casein content is probably caused by competition between casein and the synthetic substrate used for measuring plasmin activity.

CONCLUSIONS

It is generally accepted that alkaline milk proteinase is bovine blood plasmin. However, the homology of amino acid sequences of plasmin purified from blood and milk has not been verified. There is possibly an additional serine proteinase and an acid proteinase in milk that can be included in this trypsin-like system. More research identifying the properties of these enzymes is needed.

Milk plasmin has been purified and shown to have optimum proteolytic activity at pH 7.4 and 37°C. It exists in milk in inactive and active forms. Both forms are

associated with casein micelles. Plasminogen activators also are associated with casein micelles in milk and activate the proenzyme. Inhibitors of plasmin and plasminogen activators are found only in milk serum and can be separated from casein micelles by ultracentrifugation.

Plasmin activity in milk can be determined by PAGE of degraded casein, degradation of labelled casein, and by chromogenic/fluorogenic substrates. These methods differ in specificity, sensitivity, and sample preparation. Synthetic substrates are often used in milk-plasmin research.

Hydrolysis of β-casein to yield γ-caseins and proteose-peptones depends on plasmin activity in milk. Degradation of α_{s2}-casein occurs at the same rate as that of β-casein. Plasmin hydrolyzes α_{s1}-casein at a slower rate, and κ-casein is resistant to hydrolysis by plasmin. Excessive hydrolysis of susceptible caseins results in smaller micelles, reduced curd forming properties of milk, and increased surface activity of casein at air–water interfaces.

Activity of plasmin in cheese is affected by pH and cheese moisture. It is more active and plays a more important role in ripening of high moisture, high pH cheeses, such as Camembert; rather than in low moisture, low pH cheeses, like Cheddar. Cheese from ultrafiltered milk probably has reduced activity because of plasmin inhibition by β-lactoglobulin and other inhibitors in milk.

Plasmin in milk is heat resistant and survives most UHT treatments. Proteolysis occurs but does not always correlate with age gelation of UHT-milk products, rather it may be more causative in age thinning of UHT-treated dairy products.

Increased plasmin occurs in milk at the end of lactation, in milk from older cows, and in mastitic milk. Its highest levels have been observed in mastitic milk; futhermore, after the mastitic infection is cured, it does not return to preinfection levels. This probably explains why older cows have high plasmin activity levels. Somatic cells do

not affect plasmin activity if they remain intact, and even lysed cells have little effect on milk protein degradation when the milk is stored at cold temperatures.

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PART 2. INHIBITION OF BOVINE PLASMIN BY CASEIN IN ASSAYS USING SYNTHETIC SUBSTRATES

ABSTRACT

Bovine plasmin (EC 3.4.21.7) activity was measured on H-D-valyl-L-leucyl-L-lysyl-4-nitroanilide by following absorbance change at 405 nm. Initial rates of reactions with all combinations of .4, 4, and 40 × K_m substrate concentrations and .068, .68, and 6.8 × K_I casein concentrations were measured. Steady-state kinetic parameters V_{max}, K_m, K_I, and K_I' were determined by nonlinear least squares fitting of the data to the equation:

$$V_O = \frac{V_{max}}{1 + \frac{K_m}{S} + \frac{I}{K_I'} + \frac{K_m \times I}{K_I \times S}}$$

Bovine plasmin is competitively inhibited by casein ($K_I' \longrightarrow \infty$) and has a K_{cat} of .0158 ΔA_{405}/min/n*M*, K_m of .107 m*M* substrate, and K_I of .86 mg/ml casein. Bovine plasmin can be measured directly in bovine milk without interference from casein.

INTRODUCTION

Plasmin (MW 89,000) is a blood enzyme that enters the mammary gland and causes breakdown of α_S- and β-caseins in milk (4). Its concentration and activity increase in mastitic milk (12) and late-lactation milk (7, 11, 12). One way to measure plasmin activity in dairy products is to use synthetic chromogenic or fluorogenic substrates. Because plasmin has high affinity for lysine residues and cleaves lys-x bonds (2), lysine peptides are linked to chromogenic or fluorogenic tags. N-succinyl-L-alanyl-L-phenylalanyl-L-lysyl-7-amido-4-methyl coumarin does not fluoresce, but after plasmin hydrolysis, 7-amido-4-methyl coumarin, a fluorescent compound, is released (9). Rate of fluorescence increase is proportional to plasmin activity. Plasmin

concentration in skim milk was reported to be .14–.73 mg/L using this method. It is sensitive, specific, requires little sample preparation and only 30 min analysis time.

The chromogenic substrate H-D-valyl-L-leucyl-L-lysyl-4-nitroanilide also can be used to determine plasmin activity (5, 10, 12, 13). Plasmin hydrolyzes this peptide to release 4–nitroaniline, which absorbs light at 405 nm. This method may be more convenient because a spectrophotometer can be used rather than a fluorometer (which is not always standard laboratory equipment).

Using the fluorogenic substrate mentioned above, Richardson (7) found that plasmin concentration in early and late-lactation milks was .15 and .37 mg/L in Jersey milk and .27 and .53 mg/L in Friesian milk and suggested that plasmin activity was dependent on breed of cow. Schaar (11) measured plasmin activity in milks from four breeds of dairy cows. He reported .112 7-amido-4-methyl coumarin (AMC) units/ml for Friesian milk and .084 AMC units/ml for Jersey milk. However, when plasmin activity was adjusted for casein content, no difference between Holstein and Jersey milk was found. Schaar (11, p. 375) concluded that, " The negative correlation between casein content and plasmin activity...is probably due to competition between casein and the synthetic substrate for the enzyme." Such competition is often the mode of action of many enzyme inhibitors.

Although casein is a natural substrate for plasmin in milk, it probably competes for plasmin's active site during analysis with synthetic substrates. This type of interference can be considered reversible competitive inhibition, though casein is cleaved and then released by plasmin. This is because casein has many different sites that can bind plasmin, and a 30 min assay time would not be sufficient to cleave all possible sites. Digestion of casein by plasmin at optimal pH and temperature takes several hours even when extra plasmin is added to milk (6).

There are three types of enzyme inhibition; competitive, uncompetitive, and mixed. Only competitive inhibition can be overcome by increasing concentration of substrate to a level high enough that the probability of the inhibitor binding to the enzyme's active site is minimal. If casein is competitively inhibiting plasmin in assays using synthetic substrates, the inhibition could be removed by increasing substrate concentration.

The aim of this research was to determine whether casein inhibits plasmin from reacting with synthetic substrates and to determine the type of inhibition; competitive, uncompetitive or mixed.

MATERIALS AND METHODS

Reagents

H-D-valyl-L-leucyl-L-lysyl-p-nitroanilide (catalog # V 7127), plasmin purified from bovine blood (.25 Sigma units/mg, catalog # P 7911)), and ε-amino-n-caproic acid (catalog # A 2504) were purchased from Sigma Chemical Company (St. Louis, MO). Purified casein was purchased from Fisher Scientific (Pittsburgh, PA).

Kinetic Assays

An assay buffer was prepared containing 50 m*M* Tris, 110 m*M* NaCl, and 3 m*M* ε-amino-n-caproic acid and its pH adjusted to 7.4 with HCl. The enzyme solution contained 6.3 mg of plasmin per 25 ml assay buffer. H-D-valyl-L-leucyl-L-lysyl-p-nitroanilide was also dissolved in the buffer (5 mg/ml). Casein solution was prepared by dissolving 4.0 g in 100 ml buffer, mixing 3 ml of this solution with 1 ml .4 *M* sodium citrate, and centrifuging at 27,000 *g* for 10 min. Casein concentration of the supernatant was estimated by Kjeldahl nitrogen determination using a Kjeldahl factor of 6.36 (14).

Preliminary estimates of K_m and K_I (competitive inhibition constant) were obtained from Hanes (3) linear transformations of initial rate data using reaction mixtures with several combinations of substrate and casein. Using these estimates, three levels of substrate corresponding to .1, 1 and $10 \times K_m$ were combined with three levels of casein corresponding to .1 1 and $10 \times K_I$ to give nine different reaction mixtures. Plasmin solution (150 µl) was added to each reaction mixture at time = 0 to give a final concentration of 26.8 n*M* (total volume was 850 µl). Final substrate concentrations were .04, .40, and 4.00 m*M*, and final casein concentrations were .0585, .585, and 5.85 mg/ml. Rate of release of 4-nitroaniline by plasmin was followed by measuring increase in absorbance at 405 nm using a Beckman DU–8B spectrophotometer with a kinetics compuset module. Absorbance measurements were made over 30 min and reaction rates were measured in duplicate.

Initial Rate Calculation

It was assumed that the early part of the absorbance by time curve is a rectangular hyperbola, and initial reaction rates of each mixture were determined by fitting absorbance–time data to equation 1 (equation for a rectangular hyperbola) using the NLIN procedure of SAS (Marquardt method).

$$A = \frac{A_{max} \times t}{K + t} \qquad (1)$$

Where: A = absorbance

A_{max} = the maximum absorbance approached by the curve

t = time

K = time at 1/2 A_{max}

Both A_{max} and K were estimated from the fitted data and initial rates determined from the derivative of equation 1 (shown in equation 2) at zero time.

$$\frac{dA}{dt} = \frac{A_{max}}{K} \quad \text{at } t=0 \tag{2}$$

Determination of Kinetic Constants

Kinetic constants were determined by fitting experimental data to equation 3, the general equation for describing enzyme inhibition kinetics.

$$V_o = \frac{V_{max}}{1 + \frac{K_m}{S} + \frac{I}{K_I'} + \frac{K_m \times I}{K_I \times S}} \tag{3}$$

Where: V_o = initial velocity

V_{max} = maximum velocity

K_m = the substrate concentration at 1/2 Vmax

K_I = inhibitor constant for competitive inhibition

K_I' = inhibitor constant for uncompetitive inhibition

S = substrate concentration

I = casein concentration (inhibitor)

The four kinetic parameters in this equation, V_{max}, K_m, K_I, and K_I', were estimated using SAS (Marquardt method of NLIN procedure).

RESULTS AND DISCUSSION

A typical reaction curve is shown in Figure 3. The initial rate, estimated by SAS, has been added to the graph. This method of determining initial rates is superior to simply taking the slope of the line between the first two points or trying to subjectively decide the initial rate because the slope at t = 0 is obtained rather than at t = some short time. All the data collected were fitted to the equation. This reduces the error caused by omitting the data in later parts of reaction curves.

Richardson and Elston (8) reported plasmin activity in some commercially prepared caseins. The casein solution used in this experiment was assayed for residual plasmin activity. Absorbance increased .0027 units over 30 min for the highest casein concentration used in the inhibition study. This was negligible compared to increases of .3 absorbance units for the slowest reaction mixtures and did not affect the reaction rates.

Brown (1) proposed that enzyme inhibition patterns are a function of the inhibition constants (Table 2), and that type of enzyme inhibition can be determined from estimates of K_I and K_I'. If either of these parameters becomes large, the terms in which they are found in the denominator of equation 3 approaches zero. If both terms approach zero, there is no inhibition and equation 3 becomes the simple Michaelis–Menten equation for enzyme kinetics. Estimates of the kinetic parameters for this experiment are given in Table 3. The term I/K_I' is negligible so casein is reacting in a competitive manner.

Experimental data was fitted again to equation 3 with the K_I' term removed. Estimates of the remaining kinetic parameters were not different although the residual sum of squares was lower.

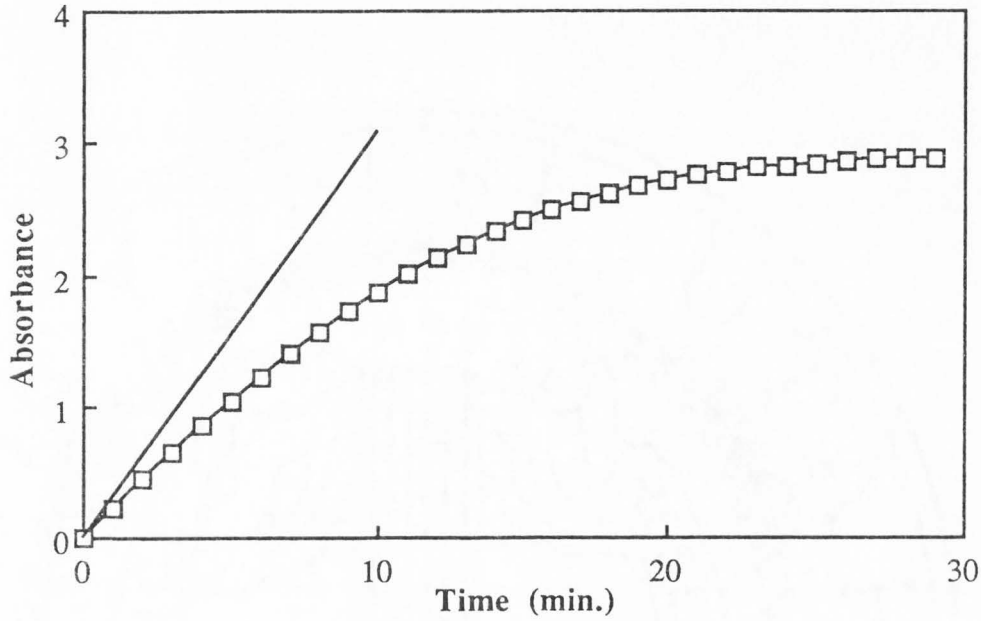

Figure 3. Increase in absorbance during 30 min with .4 m*M* substrate and .585 mg/ml casein. Initial rate or tangent line at zero time was determined using SAS estimates of maximum absorbance approached by the curve and the time at half the maximum absorbance

Table 2. Inhibition patterns as a function of inhibition constants

Type of inhibition	$\frac{I}{K_I}$	$\frac{I}{K_I'}$
None	0	0
Competitive	>0	0
Uncompetitive	0	>0
Mixed	>0	>0
Observed	.38	10^{-10}

Figure 4 is a plot of calculated reaction rates using estimates of K_m and K_I listed in Table 3. Equation 3 with the K_I' term removed was used for calculating these rates with different substrate and casein concentrations. If casein is increased at constant substrate concentration, the reaction rate is reduced. When substrate concentration is increased at constant casein concentration, inhibition is removed. Note the substrate concentration only increases to 1 m*M* rather than 4 m*M*. This is to expand the most interesting part of the graph that shows decreased rates at low substrate concentration and increasing casein concentration.

Table 3. Estimates of kinetic constants for casein inhibition of plasmin

Constant	Value	Standard error
K_{cat}	.0158 ΔA/min/n*M*	.01
K_m	.107 mM	.02
K_I	.860 mg/ml	.05
K_I'	$>10^9$	-

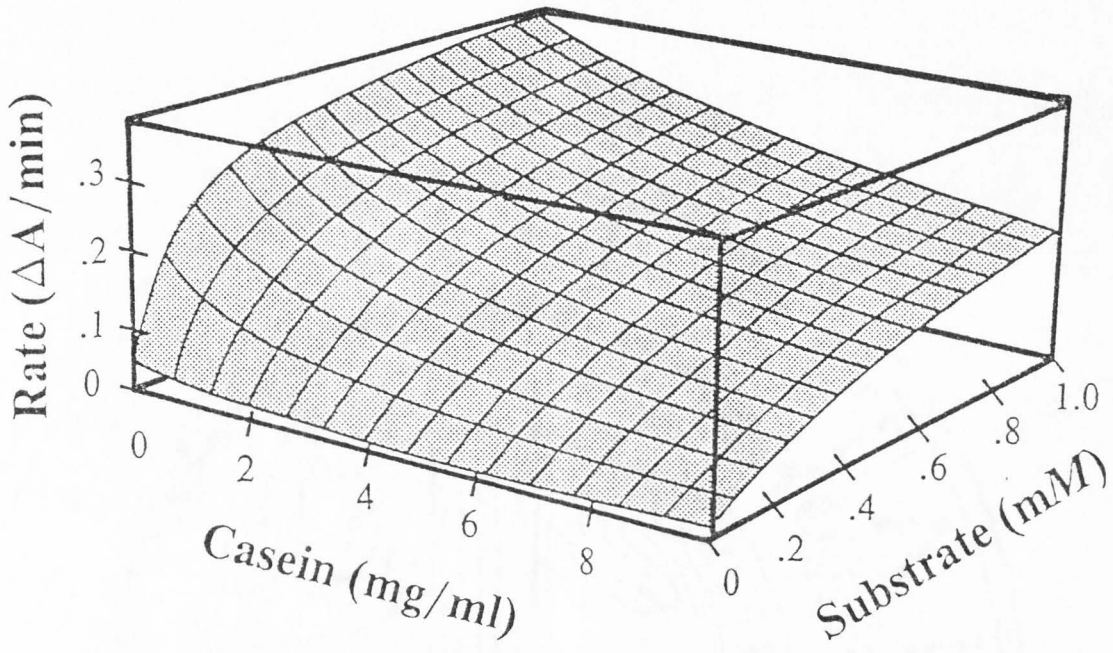

Figure 4. Effect of substrate and casein concentrations on initial rates of plasmin reactions. Rates were calculated using estimates of K_m and K_I determined for this study. The K_I' term was omitted for the calculation

CONCLUSIONS

Casein is an inhibitor of plasmin in activity assays using synthetic chromogenic or fluorogenic substrates. The type of inhibition is competitive and can be overcome by substrate manipulation. If sufficient substrate is added, the binding of enzyme to inhibitor will be negligible and no interference is observed.

Substrate concentration of .4 m*M* removed inhibition in 5 mg/ml casein. Sample preparation procedures for determining plasmin in the presence of casein would determine casein concentration, and substrate concentration would have to be adjusted.

Because casein inhibition can be removed, plasmin activity can be measured directly without interference from casein.

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PART 3. PLASMIN ACTIVITY AND MILK COAGULATION: EFFECT OF AGE, SEASON, AND STAGE OF LACTATION

ABSTRACT

A total of 380 milk samples from nineteen Holstein and nineteen Jersey cows (from one Holstein herd and one Jersey herd) were collected monthly during ten-month lactations. Milk sampling followed Dairy Herd Improvement Association procedures for collecting individual cow milk samples. Samples were analyzed for plasmin activity, plasminogen (activity after activation), protein, fat, pH, somatic cell count, clotting time, curd firming rate, and final curd firmness. Three age groups from each breed/herd were chosen; first, third, and fourth and later lactations. To avoid lactational and seasonal confounding, two to three cows from each breed/herd were started on the experiment every month during an eight-month period. This gave lactational and seasonal overlap, allowing the two factors to be statistically separated. Plasmin activity was most affected by lactation number after adjusting for somatic cell count and milk production. Milk from fourth- and later-lactation cows had higher plasmin activity than milk from younger animals. Plasmin activity also increased during lactation but was not affected by breed/herd, pH, protein, or fat. Plasminogen averaged 5.4 times the plasmin activity and after adjusting for milk production it was affected only by stage of lactation and season. Plasminogen increased during the first five months of lactation and leveled off through the end. Percent plasmin, [plasmin/(plasmin + plasminogen)], is a measure of plasminogen activation. Percent plasmin increased in milk during late lactation and in milk from fourth- and later-lactation cows. Fourth- and later-lactation cows had a least squares mean of 22% plasmin, and first-lactation cows had a mean of only 10%. Effect of season was different for plasmin and plasminogen. Plasmin activity was higher in milk collected during summer and fall, but plasminogen was higher in milk collected during fall and winter. Percent plasmin in milk was higher in summer and fall. Least squares analysis showed that plasmin activity did not affect any milk clotting

parameters. Somatic cell counts were low with 90% of the samples lower than 300,000. Clotting time was longer in milk from the Holstein herd compared to the Jersey herd and was decreased by lower pH. Increased protein resulted in shorter clotting times. Milk collected during winter had longer clotting times than milk collected during other seasons. Curd firming rate was affected by clotting time, protein, fat, and season. Increased protein and fat improved the firming rate. Milk samples collected in the fall had faster firming rates than milk from other seasons. Stage of lactation did not affect firming rates, and they remained constant during the course of lactation. Cutting time was affected by pH, protein, and fat. Lower pH resulted in shorter cutting times. Higher protein and fat decreased cutting time. Cutting time was different for the two herds of cows. Milk from the Jersey herd had shorter cutting times than milk from the Holstein herd. Longer cutting times were observed in milk collected during winter than in milk collected in other seasons. Cutting time increased to midlactation and then decreased towards the end of lactation in milk from the Holstein herd. It decreased throughout the lactation period in milk from the Jersey herd. Curd firmness was improved by increased protein and fat. Jersey herd milk had firmer curd than Holstein herd milk. Milk collected in the fall had firmer curd than during the other seasons. After statistically adjusting for fat and protein content, lactation number and stage of lactation did not significantly affect curd firmness.

INTRODUCTION

Plasmin (EC 3.4.21.7) is the main proteolytic enzyme in normal milk. It is a blood enzyme which passes into milk, and its activity results in production of γ-caseins and proteose-peptones from β-casein (4). Plasmin is low in normal milk, and most of the enzyme exists as proenzyme, plasminogen (22). Plasminogen activator, which also

is a blood enzyme, has been measured in bovine milk (10, 20). Its action increases plasmin activity during storage, chiefly in pasteurized milk (12, 20). Inhibitors of plasmin and plasminogen activators also pass from blood into milk (8, 10). Although plasmin and plasminogen activators are associated with casein, their inhibitors are found in milk serum (7).

Plasmin activity increases toward the end of lactation (3, 18, 19, 25, 26), during mastitis (18, 19, 24), and in milk from older cows (18, 25, 26). This increases degradation of β-casein in these milks (1, 2). Richardson (21) suggested that increased plasmin activity in late-lactation milk was because more plasmin enters the milk rather than increased plasminogen activation. However, Politis et al. (19) reported plasminogen to plasmin ratios of 6.55 during early lactation and 3.29 at the end of lactation, indicating increased activation of plasminogen to plasmin during late lactation.

Higher plasmin activity has been observed in milk from Holstein Friesian cows compared to Jersey cows (21). A similar trend has been observed in milk from Swedish Friesian and Jersey cattle (25). However, when plasmin activity is adjusted for differences in casein content in milk between these breeds, the difference is removed. Casein interferes with plasmin activity measurements using synthetic substrates by competitively inhibiting plasmin activity (Part 2).

Poor milk clotting properties are often observed in late-lactation milk (longer clotting times and reduced curd firmness) (6, 14, 27). Increased incidence of proteose-peptones (13), γ-caseins, and other casein breakdown products (1, 14) correspond to the observed decreased β– and α_S-casein in such milk. Curd firmness during milk clotting is dependent on β-casein concentration (28). So, plasmin activity could affect the curd firmness of clotted milk.

When Pearse et al. (17) incubated artificial micelle milk with porcine plasmin, 50% of the β-casein was degraded in 3 h, yet a coagulum was formed. After 8 h, even

though a rennet clotting time was observed (clotting time was not delayed until 20 h incubation), fragile curd was formed. Artificial micelle milk incubated with plasmin for longer than 8 h did not form a coagulum. In comparison, Grufferty and Fox (6) found no change in rennet clotting time of casein micelle dispersions before and after incubation for 72 h at 37°C (no plasmin was added). After 96 h, the rennet clotting time doubled.

McMahon and Brown (11) have reviewed factors affecting clotting time in milk. These included pH, temperature, calcium ion activity, and enzyme used. Increased casein concentration has little effect on clotting time because when milk protein is concentrated by ultrafiltration, no change in clotting time occurs. However, curd firmness is affected. Pearse et al.(17) and Grufferty and Fox (6) did not measure curd firmness which would be more affected by plasmin activity than clotting time. Okigbo et al. (15) reported that milk from some individual cows clots quickly but has poor curd firming properties. When pH of such milk was adjusted to 6.3, the clotting time decreased; however, curd firmness showed little improvement. Even pooling good and poor clotting milk samples did not improve the clotting properties of the poor clotting milk.

The aims of this research were to find the percentage of total enzyme (plasmin + plasminogen) present as active plasmin, to examine effects of lactation number and stage of lactation on plasmin and plasminogen activity, and to find relationships between breed of cow, lactation number, season, stage of lactation, pH, SCC, plasmin activity, and milk clotting parameters.

MATERIALS AND METHODS

Sampling

Twenty-one Jersey and twenty-one Holstein cows were sampled once every month during the course of one-lactation period. The Jersey samples came from Richard's dairy in Farmington, Utah, and the Holstein samples from Perkes Brothers' dairy in Hyde Park, Utah. Three age groups were sampled in each herd; first-lactation, third-lactation, and fourth- and later-lactation cows. Composite morning and night samples were drawn each month following DHIA sampling procedures. Samples were immediately cooled to 4°C and held at that temperature while transporting them to the lab for analysis. To avoid lactational and seasonal confounding, groups of two to three cows in each herd were started every month as they freshened from February 1988 thru October 1988. The first group of cows finished ten-month lactations in November 1988 and the last group finished in July 1989. Two cows from each breed/herd did not complete 10 mo of lactation. They were either sold because of mastitis or died, leaving nineteen Holstein and nineteen Jersey cows. A total of 380 samples were analyzed and included in the study.

Chemical Analysis

Samples were analyzed for protein and fat by infrared spectrometry (9). Somatic cells were counted using a Fossmatic cell counter (Foss Electric, Hillerod, Denmark). Plasmin was determined by the method of Schaar and Funke (26) using H-D-valyl-L-leucyl-L-lyslyl-p-nitroanilide (Sigma Chemical Co. St. Louis, MO. catalog # V 7127) as a substrate. Milk was diluted 3:1 with .4 *M* sodium citrate, and after centrifugation at 25,000 *g* for 15 min, 85 µl of supernatant was diluted with assay buffer (50 m*M* Tris, 110 m*M* NaCl, and 3 m*M* ε–amino–n–caproic acid adjusted to pH 7.4 with HCl) and

150 µl of 5 mg/ml substrate solution. Total volume was 850 µl for all assay mixtures. Plasmin + plasminogen or total activity was determined by adding .01 Sigma units urokinase (EC 3.4.23.4) (Sigma Chemical Co. St. Louis, MO. catalog #U 5628) to reaction mixtures. Plasminogen was calculated by difference. Percent plasmin was calculated for each sample by equation 4:

$$\% \text{ plasmin} = 100 \times \frac{\text{plasmin}}{\text{plasmin} + \text{plasminogen}} \qquad (4)$$

Activity was reported as ΔA/min at 405 nm through a 1 cm cell in pH 7.4 buffer at 37°C.

Clotting parameters were estimated using the Formagraph (Foss Electric Hillerod, Denmark). Rennin (EC 3.4.23.4) (New Zealand Rennet Co., Eltham, New Zealand) was diluted 1:100 with distilled water and 200 µl was added to 10 ml milk. Dilutions were at 4°C. Sample pH also was measured and milk production was recorded for each month.

Statistical Analysis

Least squares analysis of covariance was used to estimate effects of breed/herd, lactation number (age), season, and stage of lactation on plasmin, plasminogen, total enzyme (plasmin + plasminogen), and percent plasmin. All two-way interactions were included in the model except month × season which had missing cells. Milk production, pH, SCC, protein, and fat were added to the model as covariates. SCC was linearized by taking the natural log of SCC/1000. If the covariates were not significant, they were removed from the model in a stepwise fashion. Only 10 months of lactation for each cow were used in the model to make it more balanced. Cow was

included as a nested variable (nested in the breed/herd × age interaction) and was used as the error term for testing breed/herd, age, and breed/herd × age effects. Four subclasses for season were used; spring (March, April, and May), summer (June, July, and August), fall (September, October, and November), and winter (December, January, and February). Lactation number had three categories described above. Stage of lactation had ten categories, one for each month. The analysis was run using GLM procedure of SAS.

Least squares analysis of covariance also was done to estimate effects of breed/herd, lactation number, season, and stage of lactation on milk clotting parameters. Plasmin, pH, SCC, milk production, protein, and fat were added as covariates.

RESULTS AND DISCUSSION

Plasmin Activity

Plasmin activity had an overall mean of $1.53 \times 10^{-3} \pm 10.4 \times 10^{-3}$ ΔA/min in 13.3-fold diluted skim milk.This is in agreement with Rollema et al. (23) who found a range of $.40 \times 10^{-3}$–2.6×10^{-3} ΔA/min in 10–fold diluted skim milk. Somatic cell count and milk production were both significant covariates (Table 4). Somatic cell count and plasmin had a correlation coefficient of .42. Politis et al. (18) reported a coefficient of .62; however, they had a more normal distribution of SCC. Somatic cell counts in this study were low, and 90% of the samples had less than 300,000 cells. Low correlations are expected in low SCC milk because there is no linear relationship between plasmin and SCC in the range of 100,000 to 300,000 somatic cells (18). Milk production was negatively correlated with plasmin activity, and least squares means of plasmin activity were adjusted for milk production. Protein, fat, and pH were not significant although pH and plasmin activity had a correlation coefficient of .22.

Table 4. Analysis of covariance for plasmin activity with SCC and milk production included as covariates

Source of variation	df	Sum of squares × 10^{-6}	F value	*P*
Somatic cell count	1	5.0	16.52	.0001
Milk production	1	1.4	4.68	.0313
Plasminogen	1	3.2	10.57	.0013
Breed/herd	1	.8	.41	.5275
Age	2	68.8	17.92	.0001
Breed/herd × Age	2	14.1	3.66	.0369
Cow (Breed/herd × Age)	32	61.4	6.40	.0001
Season	3	5.8	6.42	.0003
Breed/herd × Season	3	1.6	1.77	.1524
Age × Season	6	5.6	3.13	.0055
Stage of lactation	9	11.4	4.21	.0001
Breed/herd × Stage of lactation	9	2.4	.90	.5232
Age × Stage of lactation	18	15.3	2.83	.0001
Error	291	87.2		

Breed/herd was insignificant in affecting plasmin activity; however, lactation number and stage of lactation were highly significant. Season was less significant. This agrees with Politis et al. (18) who showed lactation number and stage of lactation more important than season. In this study, lactation number was the most important factor. Least squares means of plasmin activity for the three age groups during ten-month-lactation periods are shown in Figure 5. During lactation, plasmin activity increased in milk from third-lactation and fourth- and later-lactation cows but remained constant in milk from first-lactation cows. Milk from older cows had the highest activity. Similar trends were observed by Schaar and Funke (26) who showed increased plasmin activity with lactation number and stage of lactation. The reason for such a strong trend with lactation number may be explained by observations that plasmin activity does not return to preinfection levels after mastitis, though SCC returns to preinfection levels (24). So, older cows, having had more mastitis in their lifetime, would exhibit higher plasmin activity than younger animals.

Seasonal effects on plasmin activity can be seen in Figure 6. Milk from first-lactation cows had lower plasmin activity in winter with constant activity during the other seasons. Milk from third-lactation cows had highest activity in summer and fall and milk from fourth- and later-lactation cows had lower activity in spring with constant activities during the other seasons. Richardson (20) observed increased plasmin activity in pasteurized milk during lactation. Plasmin activity increased steadily during August through December and leveled off in January through April, corresponding to summer and fall in New Zealand. Seasonal effects in this study are different than some reports in the literature. This may be explained by lactation and seasonal confounding in other reports. If all cows in an experiment start and end their lactation at the same time, then seasonal and lactational effects are confounded and cannot be separated. In some

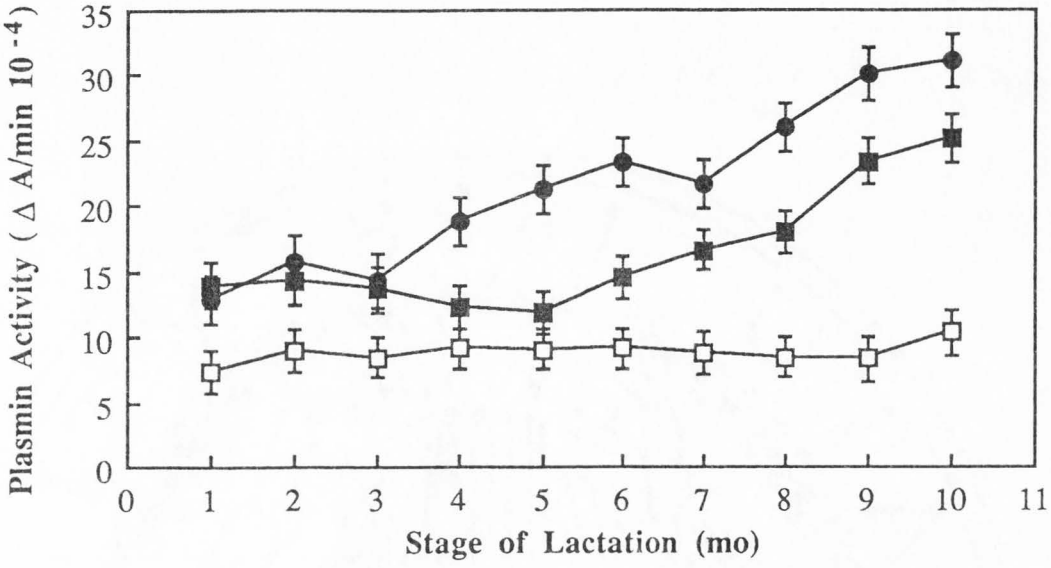

Figure 5. Monthly least squares means (± standard error) of plasmin activity for three age groups; first (□), third (■) and fourth and later lactations (●)

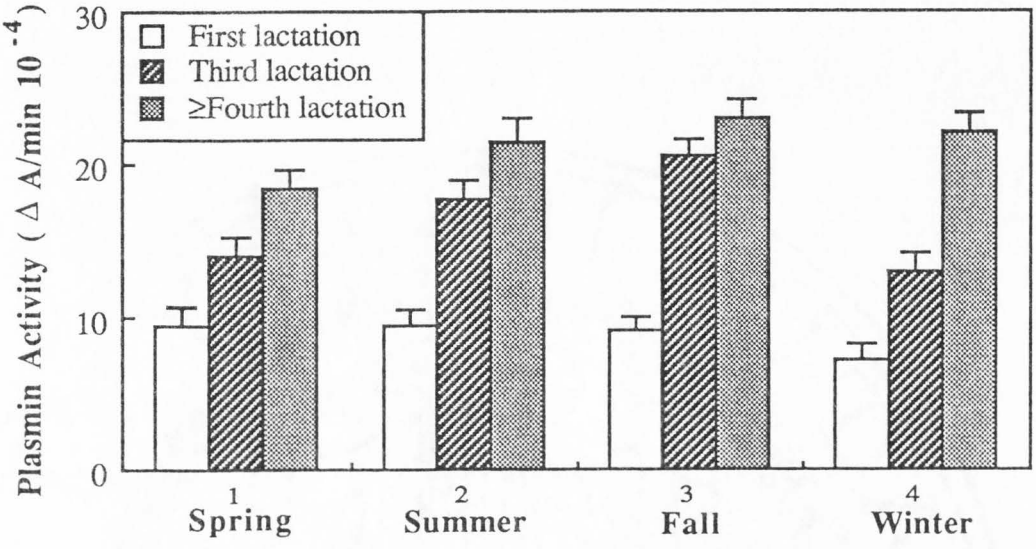

Figure 6. Seasonal least squares means (± standard error) of plasmin activity for three age groups; first, third, and fourth and later lactations

reports, sampling procedures are not detailed enough to determine whether this is the case.

Plasminogen

The overall mean of plasminogen was $8.24 \times 10^{-3} \pm 32.7 \times 10^{-3}$ ΔA/min corresponding to values of $4.9–16.6 \times 10^{-3}$ ΔA/min reported by Rollema et al. (23). The activity of plasminogen (determined after urokinase addition) present averaged 5.4 times the plasmin activity. This is within the range reported by Richardson and Pearce (22) of 2.7–7.3 times the plasmin activity. Statistical analysis for plasminogen can be seen in Table 5.

Plasminogen was not affected by SCC in this study as shown by a correlation coefficient of .28. This conflicts with Politis et al (19) who showed strong correlations between SCC and plasminogen. The discrepancy may be explained by the skewed distribution of SCC in this study. Milk production had more of an effect on plasminogen than plasmin. Plasminogen least squares means were adjusted for milk production. Protein, fat, and pH were insignificant in affecting plasminogen.

Breed/herd, age, and breed/herd × age did not significantly affect plasminogen content in milk (Table 5). Stage of lactation was the most significant factor affecting plasminogen. Plasminogen increased substantially during the first 5 mo of lactation and then reached a plateau (Figure 7). A similar trend was observed by Politis et al. (19). Season was the only other significant factor. The least squares means of season are shown in Table 6. The highest plasminogen values were during fall and winter.

Table 5. Analysis of covariance for plasminogen with milk production included as a covariate

Source of variation	df	Sum of squares $\times 10^{-6}$	F value	*P*
Milk production	1	80.7	23.64	.0001
Breed/herd	1	49.4	1.58	.2176
Lactation number	2	110.7	1.77	.1862
Breed/herd × Age	2	33.6	.54	.5896
Cow (Breed/herd × Age)	32	999.5	9.15	.0001
Season	3	73.6	7.19	.0001
Breed/herd × Season	3	1.6	.16	.9245
Age × Season	6	40.4	1.97	.0695
Stage of lactation	9	277.9	9.05	.0001
Breed/herd × Stage of lactation	9	37.2	1.21	.2881
Age × Stage of lactation	18	74.8	1.22	.2449
Error	293	100.0		

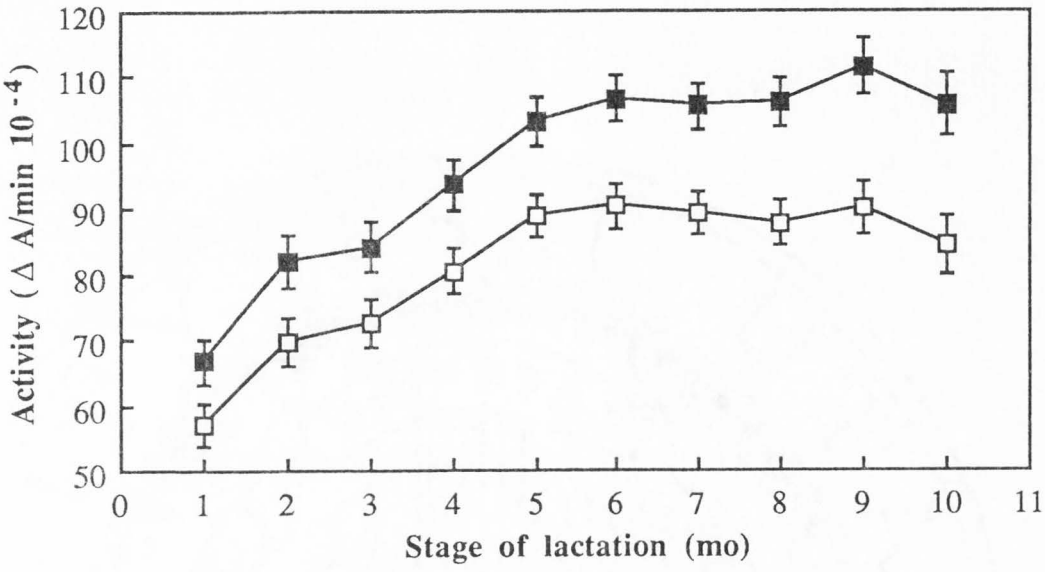

Figure 7. Monthly least squares means (± standard error) of total (plasmin + plasminogen) activity (■) obtained by addition of urokinase to assay mixtures. Plasminogen (□) was calculated by subtracting plasmin activity from total activity

Table 6. Least squares means ($\Delta A/min \times 10^{-4} \pm$ standard error) for plasminogen and total (plasmin + plasminogen). Least squares means (± standard error) for percent plasmin [plasmin/(plasmin + plasminogen)]

Season	Plasminogen	Total	Percent plasmin
Spring	78 ± 2.4	92 ± 2.6	15.6 ± .70
Summer	73 ± 2.4	89 ± 2.6	18.8 ± .71
Fall	85 ± 1.9	103 ± 2.1	17.3 ± .57
Winter	87 ± 2.3	101 ± 2.5	15.5 ± .65

Total (plasmin + plasminogen)

Least squares analysis of total activity gave results similar to plasminogen. Total activity was affected most by stage of lactation and followed the same trend as plasminogen (Figure 7). Seasonal variation is shown in Table 6 and had higher values in fall and winter than spring and summer.

Activation of Plasminogen

To study plasminogen activation, analysis of covariance was used to find factors affecting percent plasmin in milk. Levels of somatic cells were significant in affecting percent plasmin (Table 7). However, this does not suggest a causative relationship because addition of somatic cells followed by incubation at 37°C for 24 h does not affect plasminogen activation; but when the cells are lysed, plasminogen decreases by 5% during the same incubation period (19). Percent plasmin was unaffected by protein, fat, pH, and milk production.

Table 7. Analysis of covariance for percent plasmin [plasmin/(plasmin + plasminogen)] with SCC and milk production included as covariates

Source of variation	df	Sum of squares	F value	*P*
Somatic cell count	1	.027	9.14	.0027
Breed/herd	1	.002	.09	.7713
Age	2	.849	19.09	.0001
Breed/herd × Age	2	.063	1.42	.2564
Cow (Breed/herd × Age)	32	.712	7.57	.0001
Season	3	.039	4.46	.0044
Breed/herd × Season	3	.007	.77	.5141
Age × Season	6	.031	1.74	.1114
Stage of lactation	9	.032	1.22	.2804
Breed/herd × Stage of lactation	9	.038	1.44	.1702
Age × Stage of lactation	18	.078	1.47	.0994
Error	293	.861		

Breed/herd, and stage of lactation were not significant in the statistical model. However, lactation number was highly significant. Milk from fourth- and later-lactation cows had higher percent plasmin than milk from younger cows (Table 8), suggesting increased plasminogen activation in milk from older cows. Because plasminogen activators have been shown to be synthesized in mouse mammary tissue (16) and isolated from human mammary tissue (5), mammary plasminogen activator is probably present in bovine species. Its increased secretion would lead to increased percent plasmin. It is possible that in response to increased incidence of mastitis, plasminogen activator synthesis increases in bovine mammary tissue resulting in higher plasmin activity in aged cows and during late lactation.

Table 8. Least squares means (± standard error) of percent plasmin, [plasmin/(plasmin + plasminogen)], for lactation number

Lactation number	Mean (%)	Standard error
First lactation	10.3	.47
Third lactation	17.7	.49
≥Fourth lactation	22.4	.56

Season was slightly significant in affecting percent plasmin in milk. Milk collected during summer and fall had higher percent plasmin (Table 6) than milk collected during the other seasons. Although stage of lactation was not significant overall, percent plasmin increased during the last 2 mo of lactation (Figure 8), suggesting more plasminogen activation during late lactation, a trend observed in other work (18, 19).

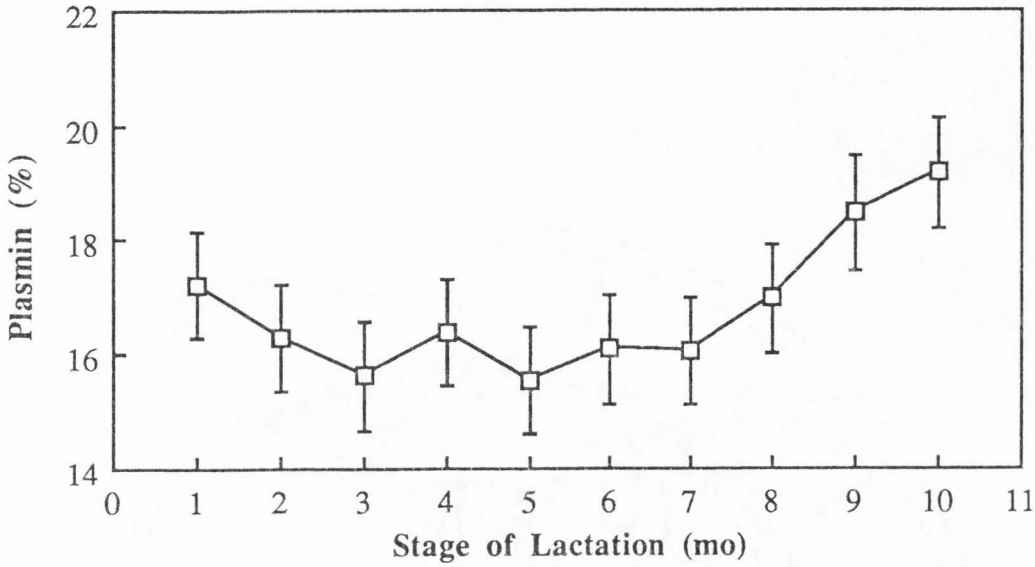

Figure 8. Monthly least squares means (± standard error) of percent plasmin [plasmin/(plasmin + plasminogen)]

Milk Clotting Parameters

A typical formagraph tracing is shown in Figure 9. Rennet clotting time and K_{20}, are shown as time measurements. Clotting time is the time from rennet addition until the lines begin to diverge. Rennet clotting time + K_{20} represents the time until cutting the curd in normal cheese making procedures. Final curd firmness is estimated as the width of the diverging lines at three times the rennet clotting time.

Clotting Time. Statistical analysis for rennet clotting time is shown in Table 9. Protein was less significant than pH in affecting clotting time. Plots of raw data for pH versus clotting time showed a nonlinear response curve. So, pH^2 and pH were added to the model as covariates. Protein had an inverse, and pH had a direct relationship with clotting time. Fat, SCC, and plasmin activity were insignificant in affecting clotting time. The level of somatic cells was low in this study. Only six samples had over three million somatic cells and 90% of the samples had less than 300,000. Politis et al. (19) reported high correlations between plasmin activity and somatic cells above 300,000 but showed no plasmin activity increase in the range of 100,000 to 300,000. This shows that high quality milk was produced by cows in this study and a full range of SCC milks was not obtained.

Although age was insignificant, breed/herd and season significantly affected clotting time (Table 10). Milk from the Jersey herd had a shorter clotting time than milk from the Holstein herd, and milk samples collected during winter had longer clotting times than milk collected during other seasons. The breed/herd and stage of lactation differences can be seen in Figure 10. Plotted least squares means have been adjusted for pH, pH^2 and protein. Protein and fat increased at the end of the lactation period in milk from both breed/herds. This resulted in shorter clotting times for Jersey herd and Holstein herd milks at the end of lactation. However, the shortest clotting time for milk from the Holstein herd was in the first month and was not different from the Jersey herd

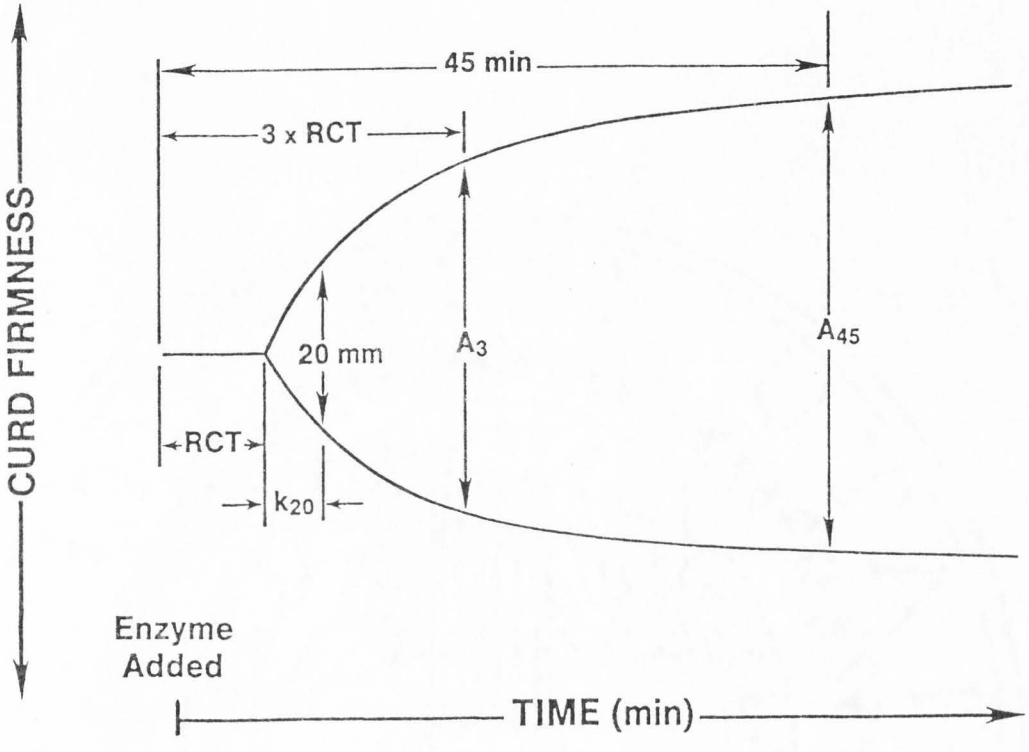

Figure 9. Formagraph tracing of milk coagulation. RCT is the rennet clotting time. K_{20} is the time from start of coagulation until the curd reaches a strength of 20 mm. A_3 is the curd firmness at three times RCT and A_{45} is the curd firmness observed 45 min after enzyme addition

Table 9. Analysis of covariance for clotting time. Protein, pH^2, and pH were included as covariates

Source of variation	df	Sum of squares	F value	*P*
pH × pH	1	61.4	29.45	.0001
pH	1	57.0	27.37	.0001
Protein	1	11.5	5.53	.0192
Breed/herd	1	174.2	10.97	.0023
Age	2	24.3	.77	.4735
Breed/herd × Age	2	56.5	1.78	.1850
Cow (Breed/herd × Age)	32	508.4	7.62	.0001
Season	3	109.4	17.48	.0001
Breed/herd × Season	3	17.2	2.76	.0424
Age × Season	6	10.7	.86	.5238
Stage of lactation	9	42.4	2.26	.0186
Breed/herd × Stage of lactation	9	56.7	3.02	.0018
Age × Stage of lactation	18	38.3	1.02	.4359
Error	291	607.0		

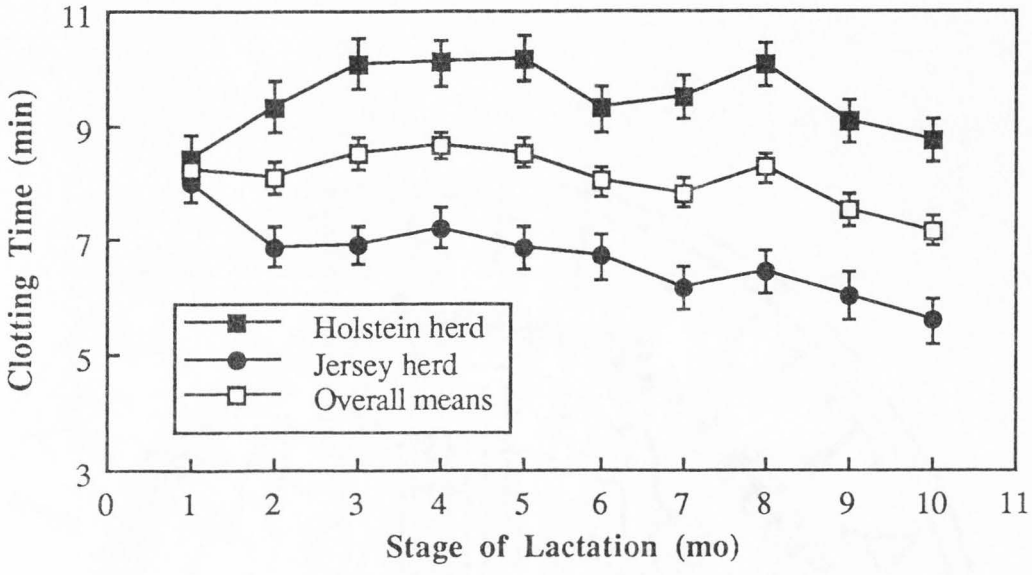

Figure 10 Least squares means (± standard error) of clotting time in milk from the Holstein herd (■) and the Jersey herd (●) during 10-mo lactation periods

Table 10. Least squares means (± standard error) of rennet clotting time for breed/herd and season

Factor	Least squares mean (min)	Standard error
Breed/herd		
Holstein	9.5	.17
Jersey	6.7	.17
Season		
Spring	7.9	.19
Summer	7.2	.21
Fall	7.9	.16
Winter	9.3	.18

for that month. Jersey herd milk clotting time was more constant than Holstein herd.

Milk from a few of the cows had significantly increased clotting times at the end of lactation. However, some of these cows went more than 10 mo and the data could not be included in statistical analysis. Two cows sold early because of mastitis had milk that exhibited increased clotting times including one sample that did not clot in 30 min.

Curd Firming Rate. Curd firming rate was evaluated in two ways. First, K_{20} was adjusted for the clotting time in the statistical model. The statistical analysis for K_{20} with clotting time included as a covariate is shown in Table 11. Protein was highly significant in reducing K_{20} or increasing the curd firming rate. Increased fat slightly reduced K_{20}. Plasmin and SCC did not affect K_{20}. Correlation coefficients between these two variables and K_{20} were both –.08. Breed/herd, age, and breed/herd × age were not significant in changing K_{20}. Adjusting for protein and fat removed breed/herd differences. pH did not affect K_{20} after adjusting for protein and fat. Stage of lactation was not significant, and least squares means for both breed/herds were constant through

Table 11. Analysis of covariance for K_{20}. Clotting time, protein and fat were included as covariates

Source of variation	df	Sum of squares	F value	*P*
Clotting time	1	276.6	179.51	.0001
Protein	1	48.5	31.46	.0001
Fat	1	8.8	5.70	.0176
Breed/herd	1	32.7	2.34	.1359
Age	2	37.1	1.33	.2795
Breed/herd × Age	2	55.8	2.00	.1523
Cow (Breed/herd × Age)	32	447.3	9.07	.0001
Season	3	21.5	4.66	.0034
Breed/herd × Season	3	9.5	2.05	.1072
Age × Season	6	14.0	1.51	.1747
Stage of lactation	9	21.2	1.53	.1370
Breed/herd × Stage of lactation	9	46.9	3.38	.0006
Age × Stage of lactation	18	42.6	1.54	.0768
Error	291	448.4		

the lactation period. Samples from fall season had lower K_{20} compared to the other seasons.

The second method for estimating curd firming rate was to transform the data using equation 5:

$$\text{firming rate} = \frac{\text{clotting time}}{\text{clotting time} + K_{20}} \quad (5)$$

Although K_{20} is inversely proportional to firming rate, this transformation is directly proportional and gave slightly different results compared to K_{20}. Protein and fat had similar effects on firming rate and K_{20}. However, breed/herd became significant for the transformed data. Milk from the Holstein and Jersey herds had least squares means of .641 ± .005 and .712 ± .005. Firming rate was higher in milk collected during the fall than during other seasons.

Cutting Time (clotting time + K_{20}). pH was a significant covariate affecting the cutting time (Table 12). Lower pH resulted in shorter cutting times. Protein and fat affected cutting time less than pH. Higher protein and fat decreased cutting time (correlation coefficient = -.41). Plasmin activity did not affect cutting time. Cutting time was different for the two herds of cows. Least squares means for the Jersey and Holstein herds were 9.6 ± .21 min and 15.3 ± .21 min. Although lactation number did not affect cutting time, season affected it significantly. Longer cutting times were observed in milk collected during winter than in milk collected in other seasons. Least squares means of cutting times for the two breed/herds during 10 mo lactation can be seen in Figure 11. Cutting time increased to midlactation and then decreased towards the end of lactation.

Curd Firmness. Curd firmness was adjusted for clotting time and K_{20} (Table 13). Higher protein significantly increased curd firmness accounting for most of the monthly

Table 12. Analysis of covariance for cutting time. pH, protein, fat, and plasmin were included as covariates

Source of variation	df	Sum of squares	F value	*P*
pH	1	400.9	57.69	.0001
Protein	1	130.4	18.76	.0001
Fat	1	30.1	4.34	.0382
Plasmin	1	21.4	3.08	.0802
Breed/herd	1	620.1	7.86	.0085
Age	2	227.8	1.44	.2510
Breed/herd × Age	2	424.4	2.69	.0832
Cow (Breed/herd × Age)	32	2524.1	11.35	.0001
Season	3	241.3	11.57	.0001
Breed/herd × Season	3	108.9	5.22	.0016
Age × Season	6	64.9	1.56	.1598
Stage of lactation	9	240.7	3.85	.0001
Breed/herd × Stage of lactation	9	171.0	2.73	.0045
Age × Stage of lactation	18	121.3	.97	.4955
Error	290	2015.5		

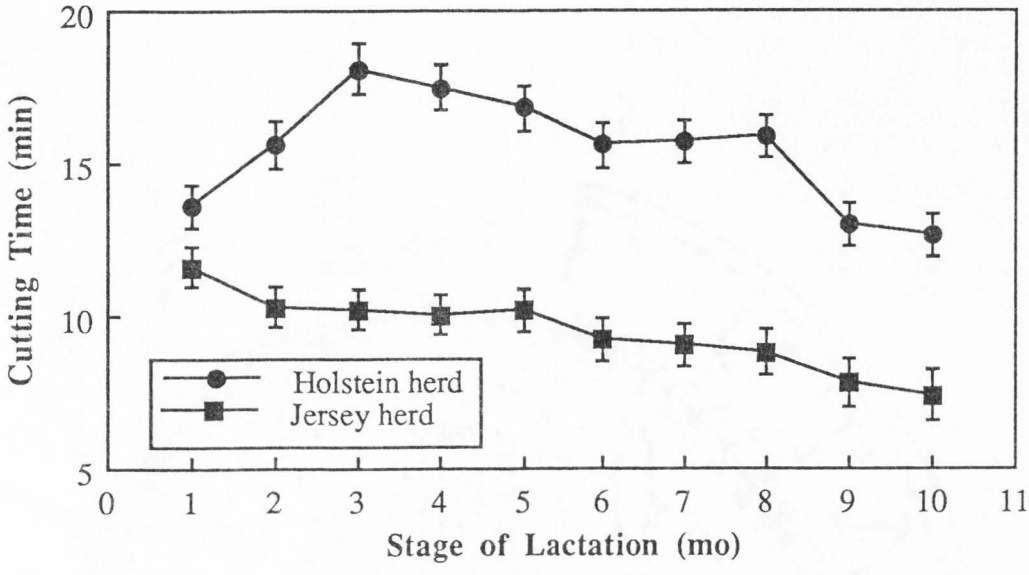

Figure 11. Least squares means (± standard error) of cutting time for two breed/herds during 10 mo lactation periods

Table 13. Analysis of covariance for curd firmness. Clotting time, K_{20}, protein and fat were included as covariates

Source of variation	df	Sum of squares	F value	*P*
Clotting time	1	23.2	3.06	.0813
K_{20}	1	783.6	103.43	.0001
Protein	1	721.0	95.16	.0001
Fat	1	42.5	5.62	.0185
Breed/herd	1	185.3	5.74	.0226
Age	2	51.9	.80	.4562
Breed/herd × Age	2	72.6	1.13	.3371
Cow (Breed/herd × Age)	32	1032.5	4.26	.0001
Season	3	87.9	3.87	.0097
Breed/herd × Season	3	111.4	4.90	.0024
Age × Season	6	34.0	.75	.6121
Stage of lactation	9	84.6	1.24	.2699
Breed/herd × Stage of lactation	9	33.4	.49	.8812
Age × Stage of lactation	18	163.0	1.20	.2636
Error	290	2197.1		

variability within each cow. Higher fat increased firmness slightly. Plasmin activity, SCC, and pH had no significant effect on curd firmness. This does not mean that plasmin activity does not affect curd firmness because SCC was low in this study, and with a correlation coefficient for plasmin and SCC of .42, effects of high plasmin activity on curd firmness are still debatable. A higher correlation coefficient of .62 was observed by Politis et al.(19), but they had more normal distribution of somatic cells. Isolated cases of milk samples with high somatic cells (>3 million) in this study had poor clottability. These milk samples appeared to have some relationship between reduced curd firmness and increased plasmin activity, but this could not be tested in the statistical model because their frequency was low.

Individual cow selection would be the best way to improve the problem of poor clotting late-lactation milk. The high variability among cows, not only for curd firmness but for all dependent variables in this study, suggests that selection and genetic improvement is possible for milk clotting characteristics. Another reason for individual cow selection is that mixing poor clotting milk with good clotting milk does not improve the poor clotting milk (15). This could affect cheese industry in countries where lactational overlap is common.

Breed/herd significantly affected curd firmness. Milk from the Holstein herd had weaker curd than milk from the Jersey herd even after adjusting for protein and fat (Table 14). Seasonal effects on curd firmness also can be seen in Table 14. Highest curd firmness was in milk collected during the fall and each breed/herd showed a similar seasonal effect.

Table 14. Least squares means (± standard error) of curd firmness for breed/herd and season

Factor	Least squares mean (mm)	Standard error
Breed/herd		
Holstein	43.7	.41
Jersey	47.4	.39
Season		
Spring	45.1	.37
Summer	44.9	.41
Fall	46.5	.31
Winter	45.6	.39

CONCLUSIONS

Lactation number was the most important factor affecting plasmin activity and percent plasmin, suggesting that higher plasmin activity in older cows is a result of increased plasminogen activation. Plasmin and plasminogen increased during lactation. Plasminogen increased at the beginning of lactation and then levelled off. Plasmin was constant at the beginning of lactation and increased toward the end of lactation. This means more plasminogen was activated at the end of lactation than the beginning. Somatic cell counts were low in this study; therefore, the effect of high somatic cell count on plasmin activity was not determined. Effect of season on plasmin and plasminogen content of milk was different. Plasmin was highest in summer and fall, and plasminogen was highest during fall and winter. Protein, pH, and fat did not affect plasmin or plasminogen activity.

Plasmin activity and somatic cells did not significantly affect any clotting parameters in this study. However, since somatic cells and plasmin are correlated and 90% of samples had fewer than 300,000 somatic cells, effects of high somatic cell count and plasmin activity on milk coagulability could not be estimated. We suggest that clotting of high quality milk is not affected by plasmin activity.

Clotting time was directly proportional to pH and inversely proportional to protein. Breed/herd, season, and stage of lactation affected clotting time. Milk from the Holstein herd had longer clotting times than milk from the Jersey herd. Milk samples collected during the winter season had longer clotting times than during other seasons. Holstein herd milk clotting time increased at the beginning of lactation and decreased slightly at the end. Jersey herd milk clotting time decreased throughout the lactation period.

Curd firming rate was affected by clotting time, protein, fat, and season. Increased protein and fat improved the firming rate. Milk samples collected in the fall

had higher firming rates than milk from other seasons. Stage of lactation did not affect firming rates and they remained constant during the course of lactation in milk from the Holstein and Jersey herds.

Cutting time was affected by pH, protein, and fat. Lower pH resulted in shorter cutting times. Higher protein and fat decreased cutting time. Plasmin activity did not affect cutting time. Cutting time was different for the two herds of cows. Milk from the Jersey herd had shorter cutting times than milk from the Holstein herd. Lactation number did not affect cutting time. Longer cutting times were observed in milk collected during winter than in milk collected in other seasons. Cutting time increased to midlactation and then decreased towards the end of lactation in milk from the Holstein herd. It decreased throughout the lactation period in milk from the Jersey herd.

Curd firmness was improved by increased protein and fat. Jersey herd milk had firmer curd than Holstein herd milk. Milk collected in the fall had firmer curd than during other seasons. After statistically adjusting for protein and fat, lactation number and stage of lactation did not significantly affect curd firmness.

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