

Plasmin Activity in Milk

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ABSTRACT

Plasmin and plasminogen-derived activities were measured in bovine and human skim milk with a chromogenic tripeptide H-D-valyl-L-leucyl-L-lysine-p-nitroanilide substrate. One unit of enzyme activity was defined as the amount of plasmin or urokinase-activated plasminogen that produced a change of absorbance at 450 nm of .001 in 1 min at pH 7.4 and 37°C under the described reaction conditions. By this definition, about 4 to 5 units of plasmin were in 1 ml of bovine milk at both early (2 to 3 mo) and late (7 to 8 mo) lactation. However, plasminogen-derived activity was 45.3 ± 13.0 U/ml at the late state of lactation compared to only 26.3 ± 2.7 U/ml at the early stage. Human milk obtained on days 6 to 7 postpartum contained 4.8 ± 4.2 and 12.4 ± 9.9 U/ml of plasmin and plasminogen-derived activity. Upon pasteurization of bovine milk at 72°C for 15 s, both plasmin and plasminogen-derived activities decreased by about 10% whereas a commercial ultrahigh temperature sterilization destroyed plasmin activity to undetectable and plasminogen-derived activity by about 90%. By an assay based on fibrinolysis of iodine-125 labeled fibrin, a plasminogen-activator activity was associated with milk casein micelles whereas an inhibitor of a plasminogen-activator, plasmin, or both were localized in milk serum. Plasminogen-activator activity was diminished in the skim milk by about 80% compared to activity in the casein micelles, apparently due to naturally occurring inhibitors in

milk serum. Quality of milk and dairy products may be influenced by amounts of proteolytic enzymes such as plasmin and factors affecting them.

INTRODUCTION

Milk contains small amounts of trypsin-like serine proteinases responsible for limited proteolysis of milk caseins (7, 8, 9, 15, 18, 20, 21, 29). These proteinases, which are most active at alkaline pH, appear to be bound to casein micelles (7, 8, 18, 23), but small amounts are also in the fat globule membrane (11). The close association of the enzymes with the casein substrate could make casein degradation more efficient. When native whole caseins were prepared from normal milk, some proteolysis of α_{s2} - and β -casein was observed frequently (5, 7, 28). The action of such proteinases on human milk proteins might be responsible for difficulties encountered by numerous researchers in isolation of human milk caseins. A milk alkaline proteinase, thought to be derived from blood, has been identified as plasmin and its inactive proenzyme, plasminogen (7, 9, 13, 23). More recently, thrombin-like activity also has been reported (18). Transformation of plasminogen to active plasmin requires a specific peptide bond cleavage by plasminogen-activators (PA's); the latter are considered to be other serine proteinases that are in many animal tissues and fluids, including the mammary gland and milk (3, 16, 17). The origin, identity, and mechanism of PA action in milk are not understood. The final plasmin activity of milk and, subsequently, milk casein hydrolysis, would depend not only on the amount of plasminogen and PA but also on the quantity of inhibitors. The occurrence in milk of blood serum trypsin inhibitors with both high and low molecular weight is documented (12, 14, 19). They presumably would interfere with the function of serine proteinases and, therefore,

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with plasmin and PA activity. However, other milk constituents, e.g., β -lactoglobulin (13, 24), also could inhibit these enzymes.

Because of their low concentrations, plasmin and plasminogen have been difficult to quantify in milk. Recently, two simple and sufficiently sensitive methods have been elaborated for milk and dairy products (20, 21). In this paper, we measured plasmin and plasminogen-derived activities in bovine and human milk with a chromogenic tripeptide as a substrate (21). Because the endogenous proteinases in bovine milk from late lactation may be contributing factors in the production of poor quality cheese (8), we compared the amounts of plasmin and plasminogen in bovine milk at early and advanced stages of lactation. The influence of different heat treatments on enzyme stability in milk also was investigated. Finally, concentrations and distributions of PA's and inhibitors of PA's and plasmin among milk protein fractions were measured in bovine milk. The importance of these various factors for final proteolytic activity of milk is discussed.

MATERIALS AND METHODS

Enzymes

Porcine blood plasmin, bovine plasma plasminogen, human urine urokinase, and bovine thrombin were purchased from Sigma Chemical Co., St. Louis, MO, and were used without further purification. Ovine plasminogen was purified from blood serum (4).

Reagents

Bovine serum albumin (BSA), ϵ -amino-n-caproic acid (EACA), bovine fibrinogen (Fraction I, Type I), and aprotinin were obtained from Sigma. H-D-Valyl-L-leucyl-L-lysine-p-nitroanilide dihydrochloride (S-2251) was purchased from KabiVitrum, Paris, France. Carrier-free Na^{125}I was from New England Nuclear, France. All other chemicals were reagent grade.

Preparation of [^{125}I] Fibrinogen

Commercial fibrinogen further was purified and radioiodinated with Na^{125}I (26).

Preparation of [^{125}I] Fibrin-Coated Plates

Linbro tissue culture multiwell plates (24 wells per plate, each of 3.5 ml capacity) were obtained from Flow Laboratories S.A., Putaux, France. The plates were coated with an [^{125}I]fibrin layer as described by Strickland and Beers (26). However, thrombin solution (40 NIH U/ml in 100 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl) was used instead of fetal bovine serum. Each well contained 20 μg (100,000 cpm) of fibrin that could be solubilized by fibrinolysis. The plates were used immediately after conversion of fibrinogen to fibrin.

Preparation of Milk Samples

Bovine milk was obtained separately from 8 to 10 mastitis-free Holstein cows at approximately the same early (2 to 3 mo) or late (7 to 8 mo) stages of lactation. Samples were collected aseptically (less than 300 bacterial colony counts/ml), cooled to 4°C, defatted by centrifugation, and used immediately for further procedures. Human milk, obtained separately from 5 women at 6 to 7 days after delivery, was defatted and kept frozen at -20°C until used for analysis. Freezing human milk samples once and thawing for assay did not reduce plasmin enzymatic activity. For a study of enzyme thermostability, raw bovine milk (bacterial count <50,000/ml) was obtained from a bulk supply at the I.N.R.A. dairy plant. It was pasteurized at 72°C for 15 s. The ultrahigh temperature (UHT) sterilized milk was obtained from a commercial source. It was prepared by indirect heating in Sordi-Steriplate equipment or by direct heat treatment (APV) at 150°C for 3 s. For study of PA activity and inhibition, bovine milk aseptically collected from individual cows at the 3-mo lactation stage was defatted, and skim milk was ultracentrifuged at 100,000 $\times g$ for 1 h at 4°C. The surface lipid layer was discarded, and a supernatant (milk serum fraction) and a pellet (casein micelles) were recovered. The casein fraction was prepared from casein micelles by their resuspension in pH 8.0 buffer (50 mM Tris-HCl, 110 mM NaCl) at the normal milk concentration (ca 27 g/liter). The terms "casein fraction" and "serum fraction" subsequently used in this paper refer to the milk fractions prepared as described.

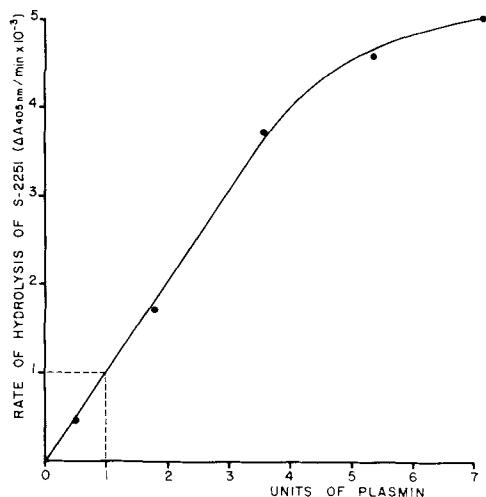


Figure 1. Rate of hydrolysis of S-2251 by plasmin derived from urokinase-activated bovine plasminogen as a function of plasmin activity. The preincubation of plasminogen with ϵ -amino-n-caproic acid and the assay using S-2251 were as described in Materials and Methods. Each point represents the average of two independent samples. ΔA = change of absorbance.

Assay of Plasmin and Plasminogen-Derived Activities in Milk Serum

The assay was based on the method given by Rollema et al. (21) with one modification; plasminogen activation to plasmin was in the presence of both urokinase and the S-2251 substrate. Plasmin and plasminogen were dissociated from casein micelles by incubation of skim milk with 50 mM EACA for 2 h at room temperature. Subsequently, milk serum containing enzymatic activity was freed of casein micelles by ultracentrifugation at $100,000 \times g$ for 1 h at 4°C . Total plasmin plus plasminogen-derived activity (defined as the proteolytic activity that was activated by urokinase) was determined in the reaction mixture (1.5 ml) of 50 mM Tris-HCl buffer, pH 7.4, containing 110 mM NaCl, .6 mM S-2251, 25 to 50 μl milk serum, and about 2 mM EACA. Fifty to 120 Ploug U of urokinase were added at 0 time. The reaction mixture was incubated at 37°C , and absorbance at 405 nm was measured at 30-min intervals. The rate of p-nitroaniline (pNA) formation was calculated from the linear parts of the absorbance versus time curves. After about 30 min lag, the increase in the absorbance was linear at least up to 3 h. Plasmin activity was

measured in a similar reaction mixture without addition of urokinase. Plasminogen-derived activity was calculated by the difference. A similar reaction mixture with urokinase but devoid of milk serum served as a blank. Plasmin and activated-with-urokinase plasminogen activities were expressed in the same units, with one unit being defined as the amount of enzyme that produces a $\Delta A_{405\text{nm}}^{1\text{cm}}$ of .001 in 1 min at pH 7.4 and 37°C when pNA produced from S-2251 substrate is measured in the defined reaction mixture. The rate of pNA formation under these conditions was linear with up to 4 units of enzyme (Figure 1). Reproducibility of the assay was within $\pm 10\%$ of the mean.

Protein concentrations of purified plasminogen or plasmin solutions were measured from their absorptions at 280 nm with $E_{\text{cm}}^{1\%} = 15.3$ (27). Specific activities of purified porcine plasmin and bovine plasminogen solutions in 50 mM Tris-HCl buffer containing 110 mM NaCl were about 50 U/ μg protein. Therefore, one unit of enzyme activity corresponds to about .02 μg of plasmin or plasminogen activated with urokinase.

Assay of PA's and Inhibitors in Milk and Milk Fractions

Assay of PA's and inhibitors was based on the fibrinolysis of insoluble [^{125}I]fibrin by plasmin formed by the reaction of PA on plasminogen (26). The release of soluble [^{125}I]fibrin degradation products (measured from gamma radiation) was proportional to the PA activity over certain ranges of time and enzyme concentration. The PA activity was assayed in .41 ml of 100 mM sodium phosphate buffer, pH 7.4, that contained 150 mM NaCl, 3.3 μg ovine plasminogen, and 0 to 50 μl of skim milk, a casein fraction, or a serum fraction. Parallel control samples with buffer added instead of plasminogen always were prepared to check for fibrinolysis independent of plasminogen. Inhibitory activities of milk fractions on fibrinolysis were measured in the same .41 ml of phosphate buffer that contained 150 mM NaCl, 3.3 μg plasminogen, .15 Ploug mU of urokinase, and 0 to 50 μl of a milk serum or casein fraction. All samples were incubated in duplicate with insoluble [^{125}I]fibrin in the wells of a Linbro tissue culture plate a 37°C for 3 h, which was within the range of linear kinetics. Forty-microliter aliquots were removed

from each well and assayed for soluble [¹²⁵I]-fibrin degradation products by measurement of radioactivity in a Packard Model 5110 gamma counter. The PA activity was expressed in Ploug units of urokinase equivalents. Under our experimental conditions, the release of [¹²⁵I]-fibrin was linear over 0 to .4 Ploug mU of urokinase equivalents.

RESULTS AND DISCUSSION

Measurement of Enzyme Activity

All enzyme assays were on skim milk as fat globule membrane contains only small amounts of plasmin and plasminogen compared to casein micelles (11). Activities of plasmin and urokinase-activated plasminogen were determined in milk serum fractions prepared from milk preincubated with EACA. Under these conditions about 80% of the enzymatic activity was recovered in milk serum whereas in untreated milk it remained associated with casein micelles (Table 1). Preincubation of milk with 50 mM EACA substantially increases sensitivity of plasminogen assay by enhancing rate of plasminogen activation compared to controls without this ligand (22). The EACA is also an

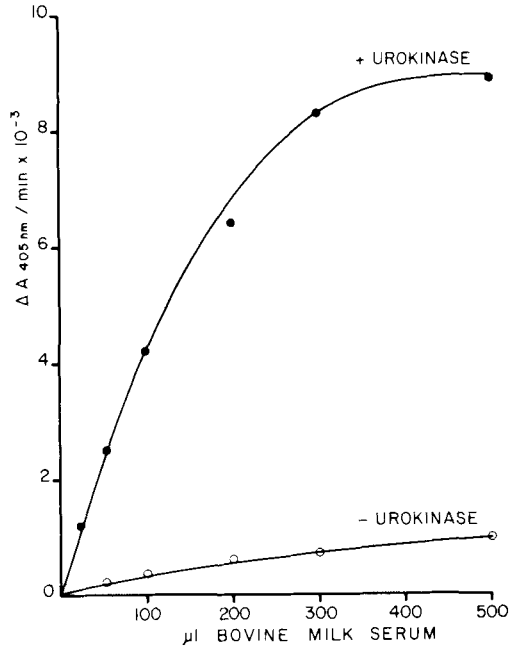


Figure 2. Plasmin and plasminogen-derived activities in ε-amino-n-caproic acid-preincubated milk serum. Each point represents the average of two independent samples. ΔA = change of absorbance.

TABLE 1. Distribution of plasmin plus plasminogen-derived activity between casein and milk serum fractions of bovine milk.

| Sample | Plasmin + plasminogen-derived activity (units/ml) ^a |
|--|--|
| Untreated skim milk ^b | |
| Casein fraction | 46.5 (46.2 - 46.9) |
| Serum fraction | 1.6 (0 - 3.3) |
| Skim milk preincubated for 2 h with 50 mM EACA | |
| Casein fraction | 10.7 (9.4 - 12.1) |
| Serum fraction | 40.8 (34.8 - 46.9) |

^aAverages and ranges calculated from two separate samples with each measured in duplicate.

^bTo control for direct effects of EACA (ε-amino-n-caproic acid) on enzyme activities, casein, and milk serum fractions were not preincubated with EACA but were made 50 mM with EACA immediately before addition to the reaction mixture.

inhibitor (K_i about 80 mM) of plasmin activity (22). The 50 mM concentration of EACA added to milk, however, was diluted sufficiently (30 to 60 times) in the final reaction mixture so that it did not disturb the assay.

Studies with a serine proteinase inhibitor (aprotinin) indicated that measurement of plasmin activity in bovine milk with the S-2251 substrate could be overestimated by approximately 20% (21). This suggests that milk contains nonserine proteinases able to hydrolyze S-2251. However, under our conditions, these proteinases in milk hydrolyzed less than 10% of S-2251; therefore, no correction was made for their presence.

The rate of pNA formation as a function of milk serum quantity is in Figure 2. The rate in the presence of added urokinase exceeded by several times the rate in its absence, indicating that bovine milk contains much more plasminogen than plasmin. This was confirmed subsequently in more systematic studies (Table 2). As shown in Figure 2, the rate of the reaction was directly proportional to the volume of milk

serum added both in the presence and in the absence of added urokinase. Recoveries were good ($93 \pm 6\%$) of purified plasmin and plasminogen added to 50 μ l milk serum at the concentration normally observed in milk. All subsequent assays were with 25 or 50 μ l milk serum volumes, well within the linear range of the assay.

Plasmin and Plasminogen-Derived Activities in Milk

Results for bovine milk at different stages of lactation and for human milk collected on days 6 to 7 postpartum are in Table 2. Mean plasmin activities and standard deviations in bovine milk measured for 8 to 10 cows at early (Group 1) and late (Group 2) lactation stages were $4.2 \pm .8$ and 5.4 ± 2.6 U/ml (not significantly different, $P > .05$). Large individual variations, particularly at the late stage of lactation, may have been caused by ages of animals not being equalized in the experiment. Urokinase-activated plasminogen activities were 26.3 ± 2.7 and 45.3 ± 13.0 U/ml for Groups 1 and 2. The observed increase in plasminogen-derived activity for the late lactation milk was significant ($P < .01$). The average plasminogen-derived activity in bovine milk at the two stages of lactation exceeded average plasmin activity by about 6 to 8 times, but individual variations were marked.

For human milk (Group 3) at 6 to 7 days postpartum, the mean and standard deviation for plasmin activity were 4.8 ± 4.2 U/ml and

for plasminogen-derived activity were 12.4 ± 9.9 U/ml. Thus, plasmin activity in human milk was not different from bovine milk, but plasminogen-derived activity was significantly lower ($P < .05$) than that in early lactation bovine milk. Because of the small number of individual human milks available to us and the large standard deviations from means, these results should be confirmed with a larger number of samples.

Based on our calculations that one unit of enzyme activity corresponds to .02 μ g of plasmin or plasminogen (see Materials and Methods), bovine milk contains about .1 μ g plasmin and from .5 to .9 μ g plasminogen/ml, and human milk contains about .1 μ g plasmin and .2 μ g plasminogen/ml. Similar results for bovine milk have been reported (9, 20, 21) except that one group found a concentration of plasmin about 50 times smaller than that of plasminogen (21). This could be related to different concentrations of PA's or PA inhibitors in the investigated milk samples. Our results indicate that human milk has no more plasmin or plasminogen-derived activity than bovine milk. This appears to disagree with (10, 25) in which as much as 3 to 5 times greater proteinase activities were in human milk than in cow's milk. However, because the latter authors measured overall proteolytic activity, the observed increase could be related to proteolytic enzymes other than plasmin in human milk, e.g., thrombin. Compared to plasmin, bovine thrombin

TABLE 2. Plasmin and plasminogen-derived activities in bovine and human milks at different stages of lactation.

| Group no. | Milk source | Stage of lactation | No. of donors | Plasminogen | | | |
|-----------|-------------|--------------------|---------------|-------------------------|-----|-------------------|------|
| | | | | Plasmin | | Plasminogen | |
| | | | | (Units/ml) ^a | | | |
| | | | | \bar{X} | SD | \bar{X} | SD |
| 1 | Bovine | 2-3 mo | 8 | 4.2 | .8 | 26.3 | 2.7 |
| 2 | Bovine | 7-8 mo | 10 | 5.4 ^b | 2.6 | 45.3 ^c | 13.0 |
| 3 | Human | 6-7 days | 5 | 4.8 ^d | 4.2 | 12.4 ^e | 9.9 |

^aDuplicate samples from each donor were analyzed. Activity is expressed as mean and standard deviation.

^bNot significantly different from Groups 1 or 3.

^cSignificantly different ($P < .01$) from Groups 1 and 3.

^dNot significantly different from Group 1.

^eSignificantly different ($P < .05$) from Group 1.

hydrolyzed the S-2251 substrate at a negligible rate (1).

Increase in plasmin and plasminogen-derived activities in bovine milk at advanced stages of lactation compared to early lactation might be expected, as these constituents originate in milk from blood and late lactation is characterized by increased permeability of blood vessels in mammary glands. The possibility that these enzymes were of bacterial or leukocytic cell origin was eliminated by using milk of low bacterial count from cows free of mastitis. In studies of Barry and Donnelly (2), a marked decrease in β -casein content with the concomitant appearance of γ -caseins (characteristic products of β -casein proteolysis by plasmin) was found in cow's milk of late lactation. Our observations that proteinase activities are higher in such a milk compared to early lactation milk agree with their report.

Plasmin and Plasminogen Thermostability in Milk

Pasteurization of milk at 72°C for 15 s decreased plasmin or plasminogen-derived activities by about 10% (Table 3). In (15), milk pasteurization led to a 30 to 40% increase in proteolytic activity, possible caused by an inactivation of milk proteinase inhibitors. That we were unable to detect this effect may be due to the specificity of our assay for plasmin and plasminogen-like activities. Alternatively, differences in milk composition could be responsible.

Commercial UHT sterilization almost completely destroyed milk plasminogen; about 90% of its potential activity was lost. The assay was not sensitive enough to detect if plasmin remained. Other workers showed that in UHT-heated milk, α_{s2} - and β -caseins gradually dis-

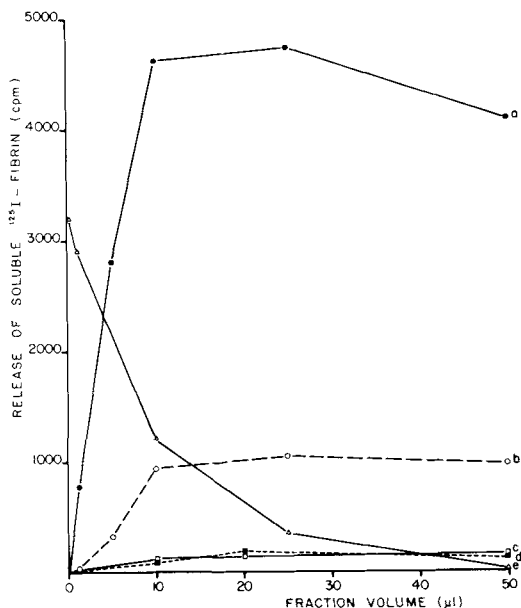


Figure 3. Plasminogen activators and inhibitors of plasmin and plasminogen activators in bovine milk fractions. Assays are based on fibrinolysis of [125 I]-fibrin with the following added to the reaction mixture: a) 0 to 50 μ l casein fraction plus 3.3 μ g plasminogen; b) 0 to 50 μ l casein fraction; c) 0 to 50 μ l serum fraction; d) 0 to 50 μ l serum fraction plus 3.3 μ g plasminogen; e) 0 to 50 μ l serum fraction plus 3.3 μ g plasminogen and .15 Ploug mU urokinase. Background counts of about 500 to 600 cpm for the controls with buffer only or buffer with plasminogen were subtracted from the corresponding samples. Each point represents the average of two separate samples, each prepared from milk pooled from two cows.

appeared upon prolonged storage, and a protein fraction containing plasmin-derived activity was isolated (6, 24). Our results provide direct quantitation of plasminogen-derived activity in UHT-heated milk.

TABLE 3. Effects of heat treatments on plasmin and plasminogen-derived activities in bovine milk.

| Sample | Plasminogen-derived activities | |
|--|--------------------------------|--------------------|
| | Plasmin | Plasminogen |
| | (Units/ml) ^a | |
| Raw milk | 4.8 (4.0 - 6.0) | 23.9 (20.8 - 26.1) |
| Pasteurized raw milk | 4.3 (3.6 - 5.2) | 22.3 (20.1 - 23.4) |
| Commercial ultrahigh temperature sterilized milk | ND ^b | 2.5 (.7 - 4.0) |

^aAverages and ranges from three separate samples.

^bND, Not detectable.

PA's and Inhibitors in Milk

Results pointed out that a majority of the proteolytic activity in freshly drawn milk was in the form of inactive plasminogen. Further gradual transformation to active plasmin is expected during milk storage if active PA remains in milk. To measure small quantities of PA in milk, we used a sensitive method based on hydrolysis of radioactive fibrin in the presence of saturating amounts (with respect to PA) of added ovine plasminogen. Inhibitors were measured under similar conditions but with urokinase used to replace a milk activator. We were able to measure PA activity independently of inhibitors because we found that they were located in different milk fractions. Activity of PA in bovine milk was associated with the milk casein fraction (Figure 3a) but not with the milk serum fraction (Figure 3d), whereas inhibitors for PA, plasmin, or both were in the milk serum fraction (Figure 3e). The parallel samples deprived of plasminogen (Figure 3b,c) indicated little plasminogen-independent fibrinolytic activity in the casein fraction but only residual activity in the serum fraction. Milk plasmin, thrombin, and possibly other unidentified proteinases could contribute to this activity.

A linear increase in PA activity, measured as release of soluble [¹²⁵I]fibrin, was observed only for small volumes of the casein fraction (Figure 3a). The subsequent decrease in linearity and plateau were possibly due to a marked increase of casein in the incubation mixture. Casein would interfere with the assay by competing with [¹²⁵I]fibrin for an active site on plasmin or by absorbing and immobilizing the enzyme. After subtraction of controls for plasminogen-independent proteolysis, PA activities of .035, .210, and .002 Ploug mU (expressed as urokinase equivalents) were measured in 10- μ l volumes of skim milk, casein fraction, and milk serum fraction, respectively. Activity of PA was diminished by about 80% in skim milk compared to activity in the casein fraction. The lower measurements of PA activity in skim milk were apparently due to the presence of naturally-occurring inhibitors in milk serum; the assay used would not differentiate between inhibitors of PA or plasmin.

Further studies on the nature and activities of PA's and inhibitors as well as on other proteinases not measured by our assays will pro-

vide better understanding of the proteolytic changes in milk and their effects.

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