Effect of Serine Proteolytic Enzymes (Trypsin and Plasmin), Trypsin Inhibitor, and Plasminogen Activator Addition to Ultra-High Temperature Processed Milk

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ABSTRACT
Proteolysis and gelation were investigated in single strength, 2% fat, UHT-processed milk following aseptic addition of combinations of plasmin, plasminogen, trypsin, trypsin inhibitor (Kunitz), and urokinase (plasminogen activator). Individual 230-ml milk containers processed by direct or indirect methods were examined for the following attributes over 10 mo: growth on slants, appearance, pH, apparent viscosity, gel formation, enzymatic activity, and casein breakdown. Control milk samples in the study did not gel. Addition of trypsin at 1.5 or 7.5 mg protein/L of milk or addition of plasmin at .3 or 1.5 mg protein/L did not result in gelation. However, containers with plasminogen at .3 mg protein/L began forming a gel at 5.5 mo. Enzyme activity in plasminogen-treated samples was not detected spectrophotometrically using an L-lysine-p-nitroanilide substrate, but extensive casein breakdown was apparent by SDS-PAGE. The evidence suggests plasminogen-derived activity promotes UHT milk gelation.

INTRODUCTION
Continuing worldwide interest in the application of aseptic packaging in the dairy industry reflects a growing realization that this method of packaging can prove cost effective. Milk, UHT processed, at temperatures of 135 to 149°C with a holding time of 2 to 8 s, has long been a staple for European milk consumers and is becoming a viable option for the American consumer. Although UHT milk is microbiologically stable, it is not shelf-stable for long periods because a gel may form in the milk containers, often accompanied by bitterness. Gelation is the most important single problem facing UHT milk producers because it signifies the final limit of shelf-life (11). The causes of gelation in UHT milk have not been clearly identified, but two theories, both involving proteins and the casein micelle, have been proposed. The first theory is that enzymatic modification of the caseins causes gelation. This involves protease attack and subsequent rearrangement of the milk proteins (1, 25, 35). The second theory is that physical-chemical effects result in the nonenzymatic rearrangement of casein micelles and perhaps the whey protein, β-lactoglobulin (2, 13, 14, 31). Gelation is thought by some to involve a combination of the two theories, in that some proteinases (native, microbial, activated, reactivated) are needed to hydrolyze the caseins, which then leads to the physical-chemical process of gelation (2, 15, 18). This may explain why extensive proteolysis does not correlate with gelation; extensive protein degradation would not allow the physical association of the casein micelles needed for gelation (23).

The presence of proteolytic activity in normal bovine milk has been attributed both to naturally occurring proteinases and to those produced by contaminating bacteria (15, 21, 27, 36). Microbial proteinases have been more extensively studied than indigenous milk.
ENZYME ADDITION TO MILK

proteinas. Some proteinases produced by psychrotrophs can survive UHT treatment (1, 8, 21), and these proteinases can shorten the storage time of UHT milk (6, 35). However, native proteinases apparently are also important, since aseptically drawn UHT milk may coagulate after 3 mo of storage (35). The major native proteinase is identical to the alkaline serine proteinase, plasmin, which is present in blood (7, 16, 17, 26) and it can survive UHT processing (9, 15, 32). Microbial and native proteinases may differ in their mode of casein attack (4, 33) and also in the type of curd formed in milk (36).

Milk contains approximately nine times more plasminogen in the plasminogen form than in the active form (28). This raises the possibility that plasminogen activates that have yet to be isolated in milk may also have an important role in the gelation of UHT milk. The known human plasminogen activators are serine proteinases which cleave a single arginyl-lysine peptide bond to form the active plasmin (29).

Milk, UHT processed, by direct steam injection is reportedly more affected by gelation than that processed by the more severe heating in an indirect method (11). Snoeren (32) assumed that native proteinase plays a role in gelation and suggested that directly and indirectly processed milks may differ in gelation time for two reasons: 1) a larger portion of enzyme is denatured by the more intense indirectly processed milk, or 2) there is more denatured β-lactoglobulin in indirectly processed milk, which inhibits the proteolytic activity of native proteinase (34).

The goal of this project was to determine the effects of plasmin, proteinase inhibitor, and proteinase activator on the storage quality of 2% fat, commercial single strength UHT milk, processed by either a direct or indirect method. The milk was stored at 25°C and analyzed over 10 mo for various chemical and physical properties.

MATERIALS AND METHODS

The UHT milk (2% fat, single strength, 250-ml containers) was obtained from two companies. Real Fresh (V Visalia, CA) provided milk from the same bulk tank, which was divided and processed by either a direct method at 134.4°C for 14.2 s or an indirect method (indirectly processed milk 2) at 135°C for 13.5 s. Milk obtained from Dairyman, Inc. (Savannah, GA) had been indirectly processed (indirectly processed milk 1) at 137.8°C for 9 s. Milk containers were stored in enclosed cabinets (25.5°C average temperature) prior to and during the study. The milk from Dairyman, Inc. was approximately 2 mo old when the study began while the milks from Real Fresh were 6 wk old.

Chemicals and Treatments

Plasminogen (zymogen form of plasmin, EC 3.4.21.7) derived from bovine plasma was purchased from Sigma Chemical Co., St. Louis, MO. Trypsin (EC 3.4.21.4, Type III), trypsin inhibitor (soybean Kunitz, SBTI), and urokinase (EC 3.4.21.21, plasminogen activator) were also obtained from Sigma. Levels of these enzymes, inhibitor, and activator injected into the individual milk containers are listed in Table 1.

A plasminogen solution of 434.7 mg solid/50 ml deionized distilled water (DDW) was injected into milk containers, using 200 μl or 1 ml to obtain 0.1 or 1.5 mg protein/L milk, respectively. To obtain active plasmin for injection, 4.6 ml of a urokinase solution (3 mg protein/ml 0.067 M phospho-lysine buffer, pH 7.5) was added to a solution of 434.7 mg plasminogen in 45.4 ml DDW and incubated for 20 min at 37°C. The activity of the activated plasminogen was checked according to supplier instructions. Milk containers received 200 μl or 1 ml of the plamin solution to obtain 0.1 or 1.5 mg protein/L milk, respectively. Soybean trypsin inhibitor was used to inhibit both trypsin and plasmin. One milligram of SBTI protein inhibited 1 to 3 mg trypsin activity, respectively (800,000 N-Benzoyl-l-arginine ethyl ester (BAAE) units/mg protein). A solution of 41.5 mg SBTI/50 ml DDW was utilized for milk containers receiving solely SBTI and for the plasmin-inhibited containers with 100 or 500 μl injected to obtain 1.66 or 8.32 mg/L milk, respectively. For the trypsin-inhibited containers, a solution of 203.33 mg SBTI/50 ml DDW was prepared and 100 or 500 μl injected to obtain 1.66 or 8.32 mg/L milk, respectively. After SBTI addition to the trypsin-inhibited containers, trypsin was added by injecting 100
or 500 µl of a 187.5 mg trypsin/50 ml .001 N HCl solution for the 1.5 or the 7.5 mg trypsin protein/L milk, respectively. Urokinase alone was used in a treatment to activate plasminogen which might be naturally present. One milliliter of 2.3 mg urokinase/ml .067 M phospho-lactate buffer, pH 7.5, was added to these milk containers.

INOCULATION PROCEDURES

The enzyme and inhibitor solutions were injected into the milk cartons under a laminar flow hood. Thirty-six containers (12 of each type of processed milk) were placed in the hood at a time and comprised one treatment group (Table 1). The outside upper right corner of each carton was swabbed with ethyl alcohol (95% vol/vol) prior to inoculation. All solutions were cold filtered sterilized through a 0.2 µm filter into a sterile test tube. The solutions were injected into the upper right corner of each milk carton using sterile disposable needles and syringes. The cartons were immediately sealed with an adhesive (Silastic 722 RTV, Dow-Corning, Midland, MI). To milk containers receiving both enzyme and inhibitor, the inhibitor was injected first, but not more than 3 h before the enzyme solution. After the adhesive had set (approximately 1 h), each container was gently rotated by hand for about 30 s. When the adhesive had hardened (24 h), all containers were placed in enclosed cabinets (25°C average temperature) for storage.

### Table 1

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<th>Inhibitor</th>
<th>Activator</th>
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Analytical and Statistical Methods

At intervals of 1, 7, 14, 28, 56, 70, 84, 112, 155, 196, 240, and 281 d, milk was evaluated for growth on slants, appearance, pH, apparent viscosity, enzymatic activity, gel formation, and casein breakdown. One milk carton of each type of milk was analyzed per treatment at each of the above time points. Treatment that yielded interesting results or required confirmation were repeated using either the levels given in Table 1 or slightly modified levels. These additional smaller experiments were conducted using duplicate milk cartons of each type of milk per treatment at each time point. Data from all experiments were analyzed using SAS (Cary, NC) procedures. Data normality and variance homogeneity were checked, allowing an ANOVA to be run.

Growth on Slants

To check for contamination of the milk during the inoculation procedure, microbiological slants were prepared. The slants were made with standard plate count agar. At each time interval, the milk containers were opened under the laminar flow hood, and a loopful of milk from each container was streaked onto a slant. The slants were capped, stored at 25°C for 7 d, and examined for growth at d 2 and 7. If growth on the slants indicated contamination of the milk cartons, data were not used.

ENZYME ADDITION TO MILK

Appearance
Color, separation of fat, presence of clouds and clots, separation of milk into a curd and a serum layer, and the formation of a gel (loss of fluidity, custard-like appearance) were the visible characteristics recorded in the milk samples.

pH
The pH of each milk container was determined using a Corning digital pH meter (model 125).

Apparent Viscosity
Apparent viscosity of each milk container (at 25°C) was measured using a Brookfield Synchro-Lectric LVT viscometer. Spindle 1 was used with a spindle speed of 60 rpm. Readings directly in centipoise (cp) were taken in duplicate when the spindle had been rotating for 30 s.

Gel Formation
When a gel was present in the milk container, it was weighed. This gel differed in appearance from the less than 2 g of thick sediment that was present in the milk regardless of treatment or time. The container was opened, and the fluid portion poured off. The nonfluid gel was placed in plastic weighing trays, and the weight was recorded in grams.

Enzyme Activity
The methods for measuring trypsin activity and plasmin activity were identical except for the chromogenic substrate and for the incubation time of the assay. The substrate solution for trypsin was 20 mg/ml α-N-benzoyl-DL-arginine-p-nitroanilide hydrochloride (BAPNA) in dimethyl sulfoxide (DMSO). To 1 ml of milk, 1 ml of a 0.1 M Tris-Cl, 0.02 M CaCl₂, pH 8.2 buffer was added, and the tubes were warmed 5 min at 37°C. A 200-μl aliquot of the BAPNA substrate was added, and the tubes incubated 30 min at 37°C. The reaction was stopped with the addition of 1 ml 20% (wt/vol) TCA. From each tube, 1.4 ml was centrifuged for 6 min in an Eppendorf microcentrifuge (model 5414). The absorbance of the supernatant was read at 400 nm in a Perkin Elmer spectrophotometer (model 571, Perkin Elmer, Norwalk, CT). For determination of plasmin activity the procedure was the same but the substrate solution was 200 μl of a 20mg/ml L-lysine-phenylalanyl chloride (US Biochemical Corp., Cleveland, OH) in DMSO, and samples were incubated for 24 h at 37°C.

Casin Breakdown
The SDS-PAGE was carried out using the method of Laemmli (20) but with a 15 to 20% gradient.

RESULTS
Only two of the over 400 slant tubes prepared from milk containers in this study showed microbial growth at 2 and 7 d storage at 25°C. Data corresponding to these two containers were not further used since the slants indicated possible contamination.

Enzyme Activity
During storage, fat separated to the top of the milk carton (approximately 0 to 2 g) in some of the containers, but there was no regularity with which this separation occurred. The control milks changed slightly from chalk-white to off-white but retained an acceptable milk color. The color of milk that received urokinase or SBT1 (both levels) alone was similar to the controls. There seemed to be no difference in color between the three types of milk included in the study.

Milk with plasminogen addition was quite similar in appearance to the controls until 5.5 mo (155 d) of storage. At that time a gel (curd appearance, loss of fluidity) began to form in the bottom of the containers of the directly processed milk with 3 mg/L added plasminogen. At 7 mo (196 d) this gel structure was apparent in the indirectly processed milk 1.

When plasmin was added, the milk became slightly yellow in color by the 2nd wk for the 1.5 mg/L treatment and by the 1st mo for the 3 mg/L treatment. Clots that developed on the milk surface became larger and more numerous over time. In the milk where inhibitor had been added prior to addition of active enzyme, the color also became yellow, and clots developed. These changes occurred approximately 1 to 2 mo slower than when no inhibitor was present. There did not appear to be a difference in clot formation between milk types.

Milk with added trypsin (both levels, active and inhibited) showed great changes in appearance compared with that of the controls, but no gel structure appeared in any of the containers. The milk turned yellow by the 2nd or 3rd wk of storage. In containers with active trypsin, clots developed and became larger and more numerous within the 1st mo of storage. At 1 mo there existed two layers (approximately equal in volume) in the milk, a top layer of large white clots, and a bottom layer of clear yellow serum. As time progressed, the serum layer increased considerably in volume. Containers with inhibited trypsin exhibited these changes but were about 2 to 4 wk slower in development. The changes occurred more rapidly with the higher level of trypsin than with the lower, and no differences were apparent between milk types. Some containers at the last three time periods analyzed had a slightly sour odor.

pH

pH dropped during storage in all types of milk for all treatments, and differences were significant between certain treatments for this pH decrease. Four of the 14 treatments were not significantly different from the controls at the end of storage: SBTI (both levels), urokinase, and plasminogen (3 mg/L). The pattern of pH decrease during storage is shown for three of the treatments against the control treatment in Figures 1A, 2A, and 3A. Milk with plasminogen added at .3 mg/L showed the least pH drop (Figure 1A). The pH decrease was larger when plasmin was added at .3 mg/L (Figure 2A) and was the greatest with trypsin added at 1.5 mg/L (Figure 3A). In the latter two cases, values were significantly different from the controls.

Apparent Viscosity

Figure 1B represents results that show the viscosity changes for plasminogen-treated samples with .3 mg/L. Apparent viscosity of the directly processed milk increased beginning at about 112 d. This milk, along with other plasminogen-treated milks, began forming a gel structure in the bottom of the containers at about the same time. A Brookfield reading on the gelled milk after 196 d was not possible since not enough liquid remained for a determination. Apparent viscosity for milk with 1.5 mg/L added plasminogen remained between 4 and 6 cp.

There was no increase in apparent viscosity in milk that contained active plasmin (both levels) or active plasmin and SBTI (both levels). Figure 2B shows this pattern for plasmin added at .3 mg/L. No apparent viscosity increase was found for SBTI alone or urokinase alone.

Figure 3B presents the changes in apparent viscosity in samples treated with trypsin at 1.5 mg/L. Viscosity increased over storage, but a gel did not form in any of the cartons. The indirectly processed milk 2 (trypsin 1.5 mg/L) showed a dramatic increase in viscosity at 28 d and 56 d, but this trend did not continue. Data for 7.5 mg/L trypsin addition was similar, but
ENZYME ADDITION TO MILK

Figure 2. pH change, apparent viscosity change, and gel formation in UHT milk cartons that received .3 mg/L added plasmin. a) Indirectly heated milk 1 (o), b) directly heated milk (9), c) indirectly heated milk 2 (b); d) control (average of the three milk types) (V).

there was no dramatic increase in viscosity for both the indirectly processed milks. The dramatic increase, then decrease, in apparent viscosity for the indirectly processed milk with 1.5 mg/L added trypsin was most likely due to continued proteolysis since extensive casein breakdown was shown by SDS-PAGE.

Apparent viscosity readings seemed to provide little information, possibly because readings were taken only on the liquid portion of the milk container. In a separate experiment, Brookfield readings were taken on the entire liquid and gel portion. The gel was broken, resulting in a homogeneous product with higher Brookfield readings (66 cp) than in the case where the Brookfield was done on the liquid portion of a milk container with partial gelation.

Gel Formation

Control samples and milk cartons that received SBTI, urokinase, plasmin, trypsin, plasmin plus SBTI, or trypsin plus SBTI did not gel. In some of the plasminogen-treated milk samples a gel formed in the bottom of the cartons (Figure 1C). The gel was soft and translucent. Just prior to gelation of plasminogen (3 mg/L) treated milk, there appeared to be granules dispersed throughout the milk as has been described by Harwalker et al. (12). At the higher plasminogen level (1.5 mg/L), gel

formation was sporadic and never exceeded 15 g. This pattern was also true for plasmin addition at 3 mg/L (Figure 2C) and for inhibited plasmin at 3 mg/L, but gel weight never exceeded 9 g. No gel was noticed in containers with trypsin (Figure 3C).

Enzyme Activity

Enzyme activity data, expressed as absorbance at 400 nm, are shown in Figures 4 and 5 for two treatments. Figure 4 presents data from the addition of plasmin at the 1.5 mg/L level. Plasmin activity (at both levels) remained steady with a slight decrease over extended storage. In milk that contained plasmin and SBTI, activity was not completely inhibited. Containers with plasminogen, SBTI, or urokinase showed no increase in absorbance over controls. Trypsin activity increased over time in all three types of milk for both 1.5 and 7.5 mg/L treatments. This increase occurred primarily between 30 and 100 d of storage, when activity in containers with trypsin added at 1.5 mg/L stabilized until about 150 d (Figure 5). At this point it was no longer possible to determine trypsin activity, since TCA addition after the BAPNA assay would not precipitate the proteins, presumably because the proteins had been cleaved to small peptides. Where SBTI had been added with trypsin (both levels), no increase in absorbance over the control was seen in the assay. Milk with SBTI alone (both levels) or urokinase alone also showed no increase in absorbance over the controls (data not shown).

Casein Breakdown

Casein breakdown in samples from three treatments is visualized by SDS-PAGE in Figures 6 to 8. Controls (all three milks) showed little if any casein attack during storage. This was also true for SBTI-treated milks (both levels) and for the urokinase-treated milks. In the only milk that gelled upon storage (plasminogen at 3 mg/L), there was evidence of a gradual casein breakdown (Figure 6). In this treatment the indirectly processed 2 milk showed less casein proteolysis than the directly processed milk from 2 mo of storage through the remaining storage period. Plasminogen added at 1.5 mg/L showed a faster rate of proteolysis, as did any samples with plasmin or trypsin. Milk with 3 mg/L of added plasmin showed gradual casein breakdown during storage, but some higher molecular weight caseins remained at 3 mo of storage (Figure 7). Milk with the 1.5 mg/L plasmin showed complete breakdown by 2 mo of storage. Trypsin-treated milks (both levels) had even faster proteolysis, with complete casein degradation by 1 wk of storage.

Plasmin samples with added SBTI resembled their active counterparts in protein hydrolysis.
Figure 6. Gradient SDS-PAGE of stored UHT milk with added plasminogen, 3 mg/L. Indirectly processed 1, directly processed, and indirectly processed 2 milks were applied in 25 μl constant volumes. Lane 1, control milk (no added enzyme) d; lane 2, milk 1 d; lane 3, milk 7 d; lane 4, milk 56 d; lane 5, milk 70 d; lane 6, milk 112 d; lane 7, milk 155 d; lane 8, control milk, 135 d; lane 9, milk 196 d. An asterisk indicates gelation of the milk.

Figure 7. Gradient SDS-PAGE of stored directly processed UHT milk with added plasmin, 3 mg/L. Milk samples were applied in 25 μl constant volumes. Lane 1, control milk (no added enzyme) d; lane 2, milk 1 d; lane 3, milk 7 d; lane 4, milk 56 d; lane 5, milk 70 d; lane 6, milk 112 d; lane 7, milk 155 d; lane 8, control milk, 155 d; lane 9, milk 196 d; lane 10, milk 240 d.

(Figure 8). Plasmin or trypsin were not completely inhibited by the SBTI. Casein hydrolysis by trypsin or plasmin did proceed in the presence of SBTI. Trypsin-treated milks with SBTI had casein breakdown products present at one week of storage, and degradation was complete by 2 mo. Casein degradation by trypsin in the presence of SBTI may have occurred because SBTI was not combined with the trypsin prior to addition, so trypsin was not inhibited at the time of addition. Therefore, a separate small experiment was designed in which SBTI and trypsin were mixed and incubated 20 min at 37°C and then added to the milk. Results were similar to those observed in the larger experiment; caseins were degraded during storage but somewhat more slowly than when SBTI and trypsin were added uncombined.

Because the levels of plasmin used in the large experiment caused too extensive and rapid proteolysis to allow gelation, a separate 10-wk experiment was conducted with 15 mg/L added plasmin. At 8 wk of storage, the milk had gelled completely and SDS-PAGE showed α- and β-casein breakdown with limited κ-casein

breakdown. At the time of gelation, Brookfield apparent viscosity readings were approximately 66 cp, and no plasmin activity could be detected.

DISCUSSION

This study investigated some of the changes related to gelation in UHT milk after addition of serine proteinases, urokinase activator, and SBTI. The pH results obtained in this study with stored UHT milk agree with literature reports for storage of UHT milk. Harwalkar et al. (12) reported that at 12 mo of storage the pH of gelled milk samples was similar to those that did not change. Kocak and Zadow (18) reported that the onset of gelation could not be correlated to the level or extent of the pH decrease. This pH drop is more pronounced as storage temperature increases (3, 18, 23, 37). Andrews (3) proposed that this decrease in pH during storage is due to loss of positive charges on the protein molecule caused by the reaction of free ε-amino groups of lysine in Maillard-type reactions.

Higher levels of proteinase than normally present in milk were chosen for addition to milk in this study to accelerate the proteolytic effects. Age gelation has been reported to occur unpredictably beginning from 6 mo to greater than 1 yr (11). Gelation has been described as loss of fluidity and apparent viscosity readings exceeding 10 cp (18, 23). It was thought that if a proteinase is involved in such gelation, specific amounts of added proteinase would give information relating proteinase action to age gelation. Plasmin at .3 mg/L was used since this is the level reported to be present naturally in bovine milk (28, 30). No gels formed in milks that received active enzyme or active enzyme and inhibitor. However, a gel did form in milks

ENZYME ADDITION TO MILK

with the zymogen, plasminogen, at the 3 mg/L level. Gel characteristics were consistent with literature descriptions, as the gel formed in the container bottom (12), was translucent or transparent (19), and was easily dispersed by agitation into a homogeneous liquid. To the best of our knowledge, no prior studies on the age gelation of UHT milk have tested the effect of added plasminogen, but the difference in gelation onset between directly and indirectly processed UHT milk found in this study with added plasminogen fits with previous literature reports for stored UHT milk (10, 23, 32, 35).

Results of SDS-PAGE for samples with added plasminogen (both levels) suggested that plasmin activity was present. It seems as if plasminogen activators must be present naturally in the milk and perhaps be at least partially responsible for the gel formed. Further work will be concentrated on the plasminogen activator in milk. Milk samples with plasminogen and inhibitor added will be included as an additional treatment in activator studies.

As no gel formed with added active enzyme, perhaps a rapid casein breakdown does not allow for association of enzyme-modified proteins into a gel network. With enzyme levels used, clear yellow serum was formed upon prolonged storage, but no caseins were detected at this point by SDS-PAGE. Snoeren (33) noticed this clear solution without formation of a typical gel structure which he attributed to native proteinase. Extensive proteolysis not leading to gelation has also been noticed by others (18, 23). Kocak and Zadow (18) suggested that age gelation follows a two-stage mechanism. The first stage involves some proteolysis of milk proteins followed by a second stage where storage-induced physicochemical changes effect the aggregation of depectinized micelles. Continued proteolytic attack during storage may result in denaturation of the gel protein structure and finally in a reduced apparent viscosity.

The presence of active enzyme in the presence of SBTI during the storage period was confirmed by the enzyme assay procedures. The SBTI was not able to completely inhibit plasmin, although it was inhibited in nonmilk preliminary tests. The ability of trypsin inhibitors to inhibit milk proteinases has been questioned. Some groups have found plasmin to be completely inhibited by SBTI (5, 16) while others have found only partial inhibition of proteinase activity in milk by trypsin inhibitors (17).

It is difficult to measure low proteinase activity in milk. Fox (9) suggested that proteinases are not detected in UHT milks or creams because the level present is too low to be detected by assay methods but high enough to modify the casein sufficiently for coagulation after prolonged storage. In the present study no plasmin activity was found in plasminogen, urokinase, or SBTI (alone)-treated samples using the substrate L-lysine-p-nitroanilide. With this substrate the 3 mg/L of added plasmin could be detected. This was the lower limit of sensitivity for this assay. Plasmin in unheated milk is reported to be 3 mg/L, and the amount of plasminogen can be 2 to 50 times greater (28). Heat treatment is known to inactivate partially plasmin and plasminogen, but it is not known how the heat treatments used on UHT milks in this study affected the natural levels of plasmin and plasminogen. As our study was being completed, Manji et al. (23) reported the effect of direct and indirect UHT heat treatments on the plasmin and plasminogen levels in milk, using the more sensitive substrate, H-D-valyl-L-leucyl-L-lysine-p-nitroanilide. Detecting the very low plasminogen-derived activity possibly associated with gel formation would require the use of such a sensitive substrate. Further studies are required to relate these levels of plasminogen-derived activity to the amount of casein hydrolysis possibly associated with gel formation. Although plasmin activity could not be measured, the extent of casein breakdown was followed by SDS-PAGE. This study adds support to previous findings that the extent of proteolysis does not correlate well with the onset of gelation in UHT milk (18, 22, 23, 24).

CONCLUSIONS

Plasminogen-derived activity may promote gelation in UHT milk over 10 mo of storage at ambient temperatures. Addition of plasminogen to UHT milk at 3 mg/L results in gel formation and the appearance of casein breakdown products observable by SDS-PAGE, even though plasmin activity is not detectable by the proteinase assay using L-lysine-p-nitroanilide as
the substrate. In comparison, addition of plasmin results in extensive proteolysis of the casein in UHT milk but no gel formation. The rate and extent of casein hydrolysis may be too large to allow the enzyme modified caseins to form a gel observable after long periods. This leads to the hypothesis that both directly and indirectly processed UHT milks contain heat stable plasminogen activators. Activators could convert plasminogen to plasmin, thus resulting in a gentle casein hydrolysis to give a gel with casein fragments detectable by SDS-PAGE.

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