Effects of a Low Concentration of Added Plasmin on Ultra-High Temperature Processed Milk

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ABSTRACT

The relationship between proteolysis and gelation was studied in UHT-processed milk following the aseptic addition of the enzyme plasmin at a concentration of .15 mg/L. Individual 250-ml containers of commercially processed (direct steam injection, 134.4°C for 14.2 s) milk were used. The milk was injected with plasmin 1 wk after processing and stored at room temperature (~23°C). Over a 6-mo period, the milk was examined for changes in appearance, pH, apparent viscosity, gel formation, enzymatic activity, and casein breakdown. Control milk samples did not gel during the test period. The milk containers that received the plasmin addition began to form a gel at 90 d of storage, and this gelation was accompanied by an increase in apparent viscosity. In the samples with added plasmin, enzyme activity was detected using the chromogenic substrate, H-D-valyl-L-leucyl-L-lysyl-4-nitroanilide (S-2251), and casein breakdown was apparent as examined by SDS-PAGE. It appeared that the added plasmin preferentially attacked β- and α-caseins over κ-casein. The evidence supports a relationship between a low level of plasmin activity and the gelation of UHT milk.

(Key words: ultra-high temperature milk, gelation, plasmin, proteolysis)

Abbreviation key: DDW = deionized distilled water.

INTRODUCTION

Age gelation in UHT milk is partially attributed to enzyme activity and the resulting proteolysis of milk proteins (4, 5, 13, 14, 15, 17, 19). Proteases present in UHT milk can be native, such as plasmin, or produced by microorganisms prior to processing. Enzymes from both of these sources have been reported to be quite heat stable (1, 11, 16) and capable of partially surviving the UHT processing. Alichandis et al. (2) found that plasmin in skim milk was largely unaffected by pasteurization conditions and that 30 to 40% of plasmin activity remained after UHT processing. In a study conducted recently (9), proteolysis and gelation were investigated in UHT-processed milk following the aseptic addition of combinations of plasmin, plasminogen, trypsin, trypsin inhibitor (Kunitz), and urokinase (plasminogen activator). Control samples did not gel, but samples that received the addition of plasminogen at .3 mg/L did gel. Enzyme activity could not be detected in the gelled milk using an L-lysine-*p*-nitroanilide substrate, but extensive casein breakdown was apparent by SDS-PAGE. These results suggested that plasminogen-derived activity might promote UHT milk gelation. When plasmin was added at .3 or 1.5 mg/L to UHT-processed milk in our previous work (9), there was evidence of extensive proteolysis, but gelation did not occur. That gelation was observed at .3 mg/L plasminogen addition suggested that plasminogen activators converted plasminogen to plasmin to result in proteolysis. Results suggested that the amount of plasmin activity (and resulting proteolysis) is critical with regard to gelation. Limited proteolysis resulting from a low level of active enzyme may play a role in the gelation of UHT milk during storage. The goal of the present study was to determine whether a lower level of plasmin addition would result in gelation of UHT milk.

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MATERIALS AND METHODS

The UHT milk (2% fat, single strength, 250-ml containers) was obtained from Real Fresh (Visalia, CA). The milk had been directly processed at 134.4°C for 14.2 s. The milk was received 4 d after processing and was injected with enzyme 7 d after processing.

Chemicals and Treatments

Plasminogen (zymogen form of plasmin, EC 3.4.21.7) derived from bovine plasma was purchased from the Sigma Chemical Co. (St. Louis, MO). Urokinase (EC 3.4.21.21), a plasminogen activator, was also obtained from Sigma. To obtain active plasmin for injection, 2.0 ml of a urokinase solution (1.67 mg protein/ml .067 *M* phospho-lysine buffer, pH 7.5) was added to a solution of 50.4 mg plasminogen/10 ml deionized distilled water (DDW) and incubated for 20 min at 37°C. The activity of the plasmin was checked according to supplier instructions. The milk containers received a 200-μl injection of the active plasmin solution to obtain .15 mg of plasmin/L milk.

Inoculation Procedures

The enzyme solution was injected aseptically into the milk under a laminar flow hood. Forty-five containers of milk were injected with plasmin solution. The outside upper right corner of each carton was swabbed with ethyl alcohol (95% vol/vol) prior to inoculation. The plasmin solution was cold filter sterilized through a .2 μm filter into a sterile test tube before being 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 732 RTV, Dow-Corning, Midland, MI). 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 (injected milk cartons and controls) were placed in enclosed cabinets for storage (23°C average temperature).

Analytical Methods

At intervals of 1, 8, 15, 27, 42, 56, 90, 100, 106, 120, 135, 152, and 181 d, the milk was evaluated for changes in appearance, pH, apparent viscosity, enzymatic activity, gel formation, and casein breakdown. Duplicate sample milk cartons were analyzed at each time interval.

Appearance

Color, separation of fat, presence of clots, separation of milk into curd and serum layers, and the formation of a gel (loss of fluidity, custard-like appearance) were recorded for each sample.

pH

The pH of each milk sample was determined using a Corning digital pH meter (model 125, Dow-Corning, Midland, MI).

Apparent Viscosity

The apparent viscosity of each milk sample (at 23°C) was measured using a Brookfield Synchro-Lectric LVT viscometer (Brookfield Engineering Labs, Inc., Stoughton, MA). Spindle 1 was used with a spindle speed of 60 rpm. Duplicate readings were taken directly in centipoise when the spindle had been rotating for 30 s. The Brookfield readings were taken on the entire content of the milk samples.

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 present in the milk regardless of treatment or time. The carton was opened, and the fluid portion gently poured off. The soft, nonfluid gel was placed in a plastic weighing tray, and the weight was recorded in grams.

Enzyme Activity

Two methods were used to measure plasmin activity. In the first method, the chromogenic substrate L-lysine-*p*-nitroanilide (US Biochemical Corp., Cleveland, OH) was used as described previously (9). The assay utilizing S-2251 was based on the procedure of Korycka-Dahl et al. (10). The UHT milk was defatted by centrifugation (model JA2-21, Beckman Centrifuge, Beckman Instruments, Palo Alto, CA;

44,000 × *g*, 4°C, 25 min). ε-Amino caproic acid was added at 6 mg solid/ml defatted milk to ensure separation of plasmin from the casein micelle. The milk was stirred for 2 h at room temperature before being spun in an ultracentrifuge (model L3-50, Beckman) for 1 h at 4°C at 60,000 × *g* to remove the casein proteins from the milk serum that contained the plasmin enzyme.

In the assay procedure, 200 μl of S-2251 (.3 mg/ml above buffer) was added to 900 μl of .1 *M* Tris-HCl buffer, pH 7.4, 110 m*M* NaCl. The contents of the tubes were mixed, and 300 μl of the milk serum was added, followed by the addition of either 20 μl of urokinase solution (1.0 mg solid/ml Tris-HCl buffer) or 20 μl of buffer. The urokinase allowed the detection of any plasminogen present by converting it to plasmin, which in turn acted upon the S-2251 substrate. The contents of the tubes were mixed and incubated at 37°C for 24 h. The reaction was stopped with the addition of 200 μl of 20% trichloracetic acid in DDW. Precipitated proteins were removed by centrifuging for 6 min in an Eppendorf microcentrifuge (Brinkman Instruments, Westbury, NY). The absorbance of the supernatants was read at 410 nm. Plasmin activity was quantified as an increase in absorbance at 410 nm over the appropriate controls. The average of duplicate readings on each of the duplicate samples was recorded.

Casein Hydrolysis

Casein breakdown was observed by SDS-PAGE using the method of Laemmli (12) but with a 15 to 20% gradient. The electrophoresis unit used was the Bio-Rad Protean Dual Vertical Slab Gel Electrophoresis Cell (Bio-Rad, Richmond, CA).

Low molecular weight markers, obtained from Sigma Chemical Co., were myoglobin fragments of 16,950, 14,400, 8160, 6210, and 2510 Da. High molecular weight markers (Pharmacia Laboratory Separations, Piscataway, NJ) were phosphorylase B (94,000 Da), albumin (67,000 Da), ovalbumin (43,000 Da), carbonic anhydrase (30,000 Da), trypsin inhibitor (20,100 Da) and α-lactalbumin (14,400 Da). All molecular weight markers were prepared and used according to supplier instructions.

RESULTS AND DISCUSSION

The purpose of this study was to determine if a low level of plasmin (.15 mg/L) added to a commercially processed UHT milk would result in gelation of the milk during room temperature storage. It has been reported that raw milk normally contains approximately .3 mg/L plasmin and up to nine times more plasminogen (16). Some of the plasmin and plasminogen is inactivated in the processing of the milk. Hence, the addition of .15 mg/L of plasmin to the milk cartons as was done in this study probably represents a reasonable level found in some types of UHT-processed milk. Milk processed by direct steam injection was chosen for use because milk processed in this way is reportedly more susceptible to gelation than indirectly processed milk (5, 7, 14). The UHT milk used in this study had been processed at conditions of 134.4°C for 14.2 s, which is representative of current commercial US processes. Native plasmin could not be detected in the control milk using either of the substrates.

Differences in chemical and physical attributes were observed between control milk samples and milk samples with added plasmin. The first noticeable changes were in the appearance of the milk. Milk with added plasmin could not be distinguished visually from the control milk, which had a typical white milk color for the first three points of analysis (1, 8, and 15 d). At 27 d, the milk with added plasmin was slightly yellow, and some tiny clots were floating at the top. As storage time progressed, the clots increased in size and number; at 56 d, the milk had large pieces of a curd-like material floating at the top. By 100 d, the color was slightly yellow, curd-like pieces floated at the top, and a translucent or transparent, soft and easily disturbed gel appeared at the bottom of the milk container. After 100 d, the control milk was still a milk white color and did not show the changes observed for milk with added plasmin. As storage time continued, the amount of the gel increased. Fat separation was more noticeable in milk with added plasmin as a dispersible creamy layer and as a fat plug. Control milk samples had minimal fat separation at the top of the carton. The milk with added plasmin developed two distinct layers, a gel on the bottom and a liquid portion on the top. Toward the end of this study, the fluid

portion of the UHT milk appeared to be clearing and had a yellow color. These results are consistent with a report by Visser (18), who suggested that continued action of milk proteinase (plasmin) in skim milk results in complete clarification. It cannot be determined if the gel that formed in the milk with added plasmin is the same gel that naturally forms in UHT milk because the control milk samples did not gel. However, the gel resembled what has been described as naturally occurring when milk has, in fact, gelled (4).

In agreement with the results of many others (3, 6, 8, 14), the present study also shows no significant change in pH. Kocak and Zadow (8) concluded that there appears to be no relation between the pH decrease and the onset of gelation in UHT milk. Andrews (3) proposed that the 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 in Maillard-type reactions.

In the unconcentrated UHT milk samples in this study, there is apparently a link between proteolysis, an increase in apparent viscosity, and the formation of a gel. Apparent viscosity readings began to increase at 90 d of storage, and a measurable gel also began to form at the same time in the milk with added plasmin (Figure 1). The viscosities of the control and sample milks were similar until 98 d of storage, when the apparent viscosity approximately doubled to 9.3 cp for the milk with added plasmin. This was also the first analysis time when a noticeable gel was present and could be measured in the milk with added enzyme. From that analysis time, the Brookfield viscosity readings in the milk with added plasmin continued to increase throughout the study compared with control milk samples.

Plasmin activity could be detected using the S-2251 substrate in the milk with added plasmin, but no activity could be measured in control milk samples (data not shown). Plasmin

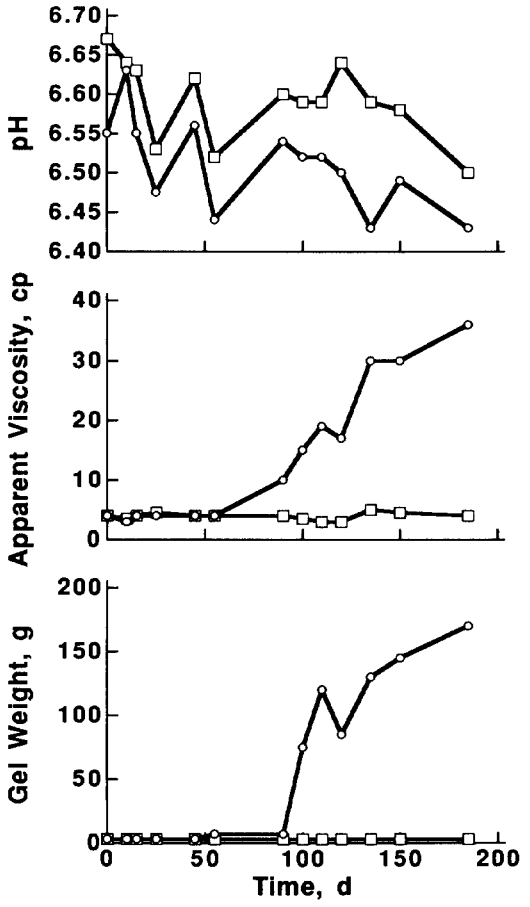

Figure 1. pH change (A), apparent viscosity change (B), and gel formation (C) in UHT milk cartons that received .15 mg/L added plasmin. □ = Controls; ○ = enzyme-added milk.

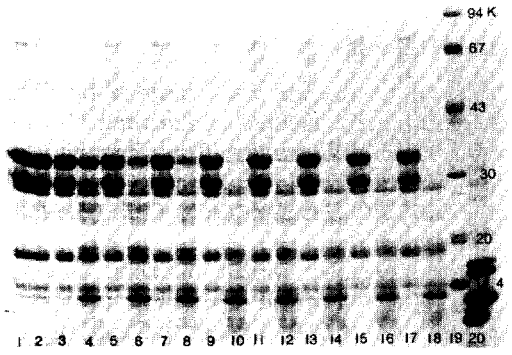

Figure 2. Gradient SDS-PAGE of stored UHT milk with added plasmin, .15 mg/L. Lane 1, control milk (CM) no added enzyme 1 d; lane 2, plasmin-added milk (PAM) 1 d; lane 3, CM 8 d; lane 4, PAM 8 d; lane 5, CM 15 d; lane 6, PAM 15 d; lane 7, CM 27 d; lane 8, PAM 27 d; lane 9, CM 56 d; lane 10, PAM 56 d; lane 11, CM 90 d; lane 12, PAM 90 d; lane 13, CM 106 d; lane 14, PAM 106 d; lane 15, CM 135 d; lane 16, PAM 135 d; lane 17, CM 181 d; lane 18, PAM 181 d; lane 19, high molecular weight standards; lane 20, low molecular weight standards.

activity (using S-2251) was relatively constant throughout the study. This was indicated by an increase in absorbance of approximately .10 in milk with added plasmin over the control milk. The added plasmin was active over the test period; similar results were found in the previous study (9). The L-Lys-*p*-nitroanilide substrate-based assay was not sensitive enough to detect the plasmin added to the milk.

In milk samples with added enzyme, proteolysis was observed (visualized by SDS-PAGE) at 1 wk of storage and continued gradually over the storage period (Figure 2). Little, if any, proteolysis occurred in the control milk during the 23 wk of storage at room temperature. In the milk with added enzyme, β-casein was hydrolyzed more rapidly than α, but both α- and β-casein were preferentially attacked over κ-casein by the added plasmin. At 23 wk of storage, both α- and β-caseins were essentially hydrolyzed, but κ-casein was still present. This is quite consistent with reports concerning the action of plasmin on casein proteins (16). In the present study, at 12 wk of storage, when there were increases in apparent viscosity and onset of gelation, only a small amount of β- and α-casein remained, but κ-casein was present and remained visible (by SDS-PAGE) throughout the study. Earlier work (9) showed that UHT milk to which .3 mg/L plasminogen was added had gelled in 8 wk.

Results obtained in this study are consistent with current thought on the cause of gelation in unconcentrated UHT milk. Some proteolysis is apparently necessary to hydrolyze the caseins partially before the modified caseins physically associate to form a gel structure (4, 5, 14). The mechanism of gelation is proposed to be different for unconcentrated versus concentrated UHT milk (4). In unconcentrated UHT milk, a good correlation was found between plasminogen or plasmin activity and time of gelation; when the protease inhibitors diisoproplyphosphofluoridate and aprotonin were added, no gel formed (4). However, concentrated UHT milk gelled with no evidence of proteolysis. Electron microscopy was used to determine that the gel was different between concentrated and unconcentrated UHT milks in this same study. In UHT skim milk, the gel was formed by thread-like structures, but the casein micelles were almost fully disintegrated. The concentrated milk showed the aggregation of partially deformed casein micelles into a three dimensional network. The scope of this present work covered only unconcentrated UHT milks in which proteases are thought to be required in order to initiate gelation.

CONCLUSIONS

Added plasmin promotes gelation in directly processed UHT milk over a 6-mo storage period at ambient temperatures. The addition of plasmin at .15 mg/L resulted in gel formation and casein breakdown products observable by SDS-PAGE. The added plasmin preferentially attacked α- and β-casein, consistent with the mode of action reported for native milk plasmin. Plasmin activity could be detected in milk samples with added enzyme by using the chromogenic S-2251 substrate. Control milk samples did not gel or exhibit detectable proteolysis with the S-2251 assay or by SDS-PAGE. This study adds support to the enzyme-based theory for the cause of gelation in unconcentrated UHT-processed milk.

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REFERENCES

1 Adams, D. M., J. T. Barach, and M. L. Speck. 1975. Heat resistant proteases produced in milk by psychrotrophic bacteria of dairy origin. J. Dairy Sci. 58:828.

2 Alichandis, E., J.H.M. Wrathall, and A. T. Andrews. 1986. Heat stability of plasmin (milk proteinase) and plasminogen. J. Dairy Res. 53:259.

3 Andrews, A. T., B. E. Brooker, and D. G. Hobbs. 1977. Properties of aseptically packed ultra-heat-treated milk. Electron microscopic examination of changes during storage. J. Dairy Res. 44:283.

4 de Koning, P. J., J. Kaper, H. S. Rollema, and F. M. Driessen. 1985. Age-thinning and gelation in unconcentrated and concentrated UHT-sterilized skim milk. Effect of native milk proteinase. Neth. Milk Dairy J. 39:71.

5 Harwalkar, V. R. 1982. Age gelation of sterilized milks. Page 229 *in* Developments in dairy chemistry—1 Proteins. P. F. Fox, ed. Appl. Sci. Publ., London, Engl.

6 Harwalkar, V. R., D. C. Beckett, R. C. McKellar, and D. B. Emmons. 1983. Age thickening and gelation of

sterilized evaporated milk. J. Dairy Sci. 66:735.
7 Hostettler, H. 1972. Appearance, flavour, and texture aspects in IDF monograph on UHT milk. FIL-IDF Annu. Bull., Part V. Int. Dairy Fed., Brussels, Belgium.
8 Kocak, H. R., and J. G. Zadow. 1985. Age gelation of UHT milk as influenced by storage temperature. Aust. J. Dairy Technol. 40:14.
9 Kohlmann, K. L., S. S. Nielsen, and M. R. Ladisch. 1988. Effect of serine proteolytic enzymes (trypsin and plasmin), trypsin inhibitor, and plasminogen activator addition to ultra high temperature processed milk. J. Dairy Sci. 71:1728.
10 Korycka-Dahl, M., B. R. Dumas, N. Chene, and J. Martel. 1983. Plasmin activity in milk. J. Dairy Sci. 66: 704.
11 Law, B. A. 1979. Reviews of the progress of dairy science: enzymes of psychrotrophic bacteria and their effects on milk and milk products. J. Dairy Res. 46: 573.
12 Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature (Lond.) 227:680.
13 Manj, B., and Y. Kakuda. 1988. The role of protein denaturation, extent of proteolysis, and storage temperature on the mechanism of age gelation in a model system. J. Dairy Sci. 71:1455.
14 Manj, B., Y. Kakuda, and D. R. Arnott. 1986. Effect of storage temperature on age gelation of ultra-high temperature milk processed by direct and indirect heating systems. J. Dairy Sci. 69:2994.
15 Payens, T.A.J. 1978. On different modes of casein clotting; the kinetics of enzymatic and non-enzymatic coagulation compared. Neth. Milk Dairy J. 32:170.
16 Richardson, B. C. 1983. The proteinases of bovine milk and the effect of pasteurization on their activity. N.Z. J. Dairy Sci. Technol. 18:233.
17 Snoeren, T.H.M., C. A. van der Spek, R. Dekker, and P. Both. 1979. Proteolysis during the storage of UHT-sterilized whole milk. 1. Experiments with milk heated by the direct system for 4 seconds at 142°C. Neth. Milk Dairy J. 33:31.
18 Visser, S. 1981. Proteolytic enzymes and their action on milk proteins. A review. Neth. Milk Dairy J. 33:65.
19 Zadow, J. G., and F. Chituta. 1975. Age gelation of ultra-high temperature milk. Aust. J. Dairy Technol. (Sep):104.