Chymosin Activity Against α_{s1}-Casein in Model Systems: Influence of Whey Proteins[1]

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ABSTRACT

The influence of native or heat-denatured α-lactalbumin (LA) and β-lactoglobulin (LG) on chymosin activity against α_{s1}-casein (CN) in buffered and simulated milk ultrafiltrate model systems was evaluated. The α_{s1}-CN solution (2.5 mg/ml) was adjusted to pH 5.5 using glucono-Δ-lactone; α-LA or β-LG, either native or heat-denatured (100°C for 15 min), was then added. Sufficient chymosin was added to hydrolyze 99% of the α_{s1}-CN in 4 h at 20°C in an uninhibited system. Fast protein liquid chromatography was used to quantify intact α_{s1}-CN at 0, 0.5, 1, 2, 3, and 4 h and to evaluate chymosin activity. Rate constants for each reaction were determined. Simulated milk ultrafiltrate alone did not influence the rate of α_{s1}-CN hydrolysis, and, in the absence of milk salts, only denatured β-LG reduced the rate of α_{s1}-CN hydrolysis significantly. When simulated milk ultrafiltrate was added to reaction mixtures, both native and heat-denatured β-LG significantly inhibited chymosin activity. Native α-LA did not influence hydrolysis of α_{s1}-CN; heat-denatured α-LA inhibited chymosin only in the presence of simulated milk ultrafiltrate.
(**Key words**: whey proteins, chymosin, α_{s1}-casein, inhibition)

Abbreviation key: **BTP** = bis-tris-propane, **SMUF** = simulated milk ultrafiltrate.

INTRODUCTION

Casein and whey proteins are the two major protein fractions in milk, but, during cheese manufacture, virtually all whey proteins are removed from cheese curd. Ultrafiltration and heat treatment can be used to retain whey proteins in cheese. Although denatured whey proteins can be incorporated into cheese using heat treatments, retention of native whey proteins requires UF technology. Many cheese varieties have been manufactured from UF milk (2, 4, 6, 12, 13, 14, 15, 18, 22, 23, 24). However, UF cheese exhibits slower proteolysis during cheese ripening, which influences the development of cheese flavor and texture. Many theories have been proposed to explain the influence of whey proteins on cheese maturation. Both native and denatured whey proteins could alter proteolysis by interrupting the casein matrix and by blocking substrate binding to enzymes (5). Furthermore, increasing the viscosity of the aqueous phase in cheese would reduce the rate of enzyme diffusion (32). The inhibition of plasmin by whey proteins has been one reason for the lack of maturation in UF cheese (1, 21, 31). Whey proteins may have a similar inhibitory effect on chymosin (17).

Chymosin, usually the preferred milk-clotting enzyme, hydrolyzes the Phe_{105}-Met_{106} bond of κ-CN specifically and rapidly (32). In solution at pH 6.7, Phe_{23}-Phe_{24} and Leu_{192}-Tyr_{193} bonds in α_{s1}-CN and β-CN also are susceptible to chymosin. In cheese systems, hydrolysis of casein by chymosin differs from that in milk and model protein systems because pH is reduced to 5.2, the temperature is reduced to 8°C, salt is added to achieve about 5% salt-in-moisture, and casein is in an aggregated state (10). One of the main hydrolytic products of α_{s1}-CN in cheese is fragment 24 to 199, commonly known as α_{s1}-I-CN (11). By 2 to 3 mo, a substantial portion of the Phe_{23}-Phe_{24} bond of the α_{s1}-CN has been hydrolyzed, resulting in a softening of the cheese structure and the formation of the desired texture in Cheddar cheese and in many other cheese varieties (8, 9).

Understanding the consequences of incorporating whey proteins into cheese is important for processors who are manufacturing cheese fortified with whey protein. One example is the manufacture of low fat cheese using fat mimetics made from partially denatured whey proteins. The objective of this study was to evaluate the influence of native and heat-denatured α-LA and β-LG on chymosin activity

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against α_{s1}-CN in buffered and simulated milk ultrafiltrate (**SMUF**) model systems.

MATERIALS AND METHODS

Materials

For these studies, α_{s1}-CN, α-LA, β-LG, and bis-tris-propane (**BTP**) were purchased from Sigma Chemical Co. (St. Louis, MO). Glucono-Δ-lactone was purchased from Eastman Kodak Company (Rochester, NY). Double-strength fermentation-derived chymosin (Chymax®) was kindly donated by Pfizer Inc. (Milwaukee, WI). The filters (pore size 0.8 and 0.2 μm; diameter 0.47 μm) that were used to filter buffer solutions were obtained from Fisher Scientific (Norcross, GA). Syringe filters (0.2 μm) from Chrom Tech, Inc. (Apple Valley, MN) were used to filter samples before they were injected into the FPLC® (Pharmacia Biotech, Uppsala, Sweden). Mono Q HR 5/5 columns were purchased from Pharmacia Biotech.

Preparation of Reaction Solutions

Reaction solutions contained 2.25 mg/ml of α_{s1}-CN and were acidified to pH 5.5 with 0.3 mg/ml of glucono-Δ-lactone. We chose pH 5.5 because α_{s1}-CN was insoluble below that pH. The concentration of α_{s1}-CN in these model systems was one-tenth the concentration of α_{s1}-CN in milk because it was insoluble at higher concentrations. Acidified substrate solution (4.5 ml) was added to five beakers for five different experimental conditions: no whey protein (control), native α-LA, native β-LG, denatured α-LA, and denatured β-LG. The concentrations of whey proteins in reaction solutions were 1 mg of α-LA/ml and 2 mg of β-LG/ml. Whey proteins were dissolved separately in deionized water before heat treatment and addition to reaction mixtures. Denatured α-LA and β-LG were obtained by heating individual solutions of whey protein in a water bath at 100°C for 15 min. To adjust the total volume of reaction mixtures to 5 ml, either 0.5 ml of whey protein solution or distilled water was added to 4.5 ml of substrate solution.

Assay solutions for Experiment 2 were identical to those of Experiment 1 except that 0.133% (wt/vol) SMUF was added. The SMUF was mixed as a dry mixture according to the formulation given by Jenness and Koops (19) with one modification. Magnesium chloride was used as the source of Mg instead of magnesium citrate because of availability. The mixture was kept in a desiccator until use because magnesium chloride is hygroscopic. The amount of added SMUF was based on the ratio of total salt to α_{s1}-CN in milk. Both Experiments 1 and 2 were duplicated.

Enzyme Activity

Chymosin was diluted (1:100, vol/vol) with 0.01 *M* acetate buffer (pH 5.5), and 0.65 ml of dilute enzyme was added to each beaker to start the reaction. Beakers were covered to prevent evaporation. The amount of chymosin (0.31 rennin units/ml of assay solution) was 12 times the amount normally added to cheese milk and was sufficient to hydrolyze 99% of α_{s1}-CN in 4 h at 20 ± 1°C. At 0, 0.5, 1, 2, 3, and 4 h, 0.5 ml was removed from each beaker and was immersed in a boiling water bath for 3 min to stop the reaction. Amounts of unhydrolyzed α_{s1}-CN, at each interval, were determined according to methods of Davies and Law (3) with a minor modification. The BTP-urea buffer (pH 7.0, 0.005 *M* BTP, 3.3 *M* urea, with and without NaCl) was prepared with deionized water. Buffer solutions were filtered through a membrane filter, stored at 4°C in glass containers, warmed to 20°C, and degassed before use.

Determination of Reaction Rate

The typical analysis of Michaelis-Menten kinetics requires measuring initial rates of enzyme reaction solutions that contain different substrate concentrations. Because substrate concentrations could not be increased above 2.25 mg/ml (solubility was limited at pH 5.5), typical analysis of Michaelis-Menten kinetics was not possible. The validity of this assumption was checked by attempting to estimate maximum velocity and Michaelis constant by fitting the time course of reactions to the integrated form of the Michaelis-Menten equation. Estimates for both parameters approached infinity, indicating that the substrate concentration was too low to make the transition from first order to zero order. Also, the data were fitted to zero-order, second-order, and third-order equations, but the correlation coefficients were lower than for the first-order fit. Therefore, for low substrate concentration and high enzyme activity, first-order kinetics were assumed (25). The rate constants for all reactions were determined by using the reaction kinetics program (26). The basic equation for determination of first-order rate constants is

$$-dA/dt = kA \quad [1]$$

where

dA = change in concentration of α_{s1}-CN,
t = time, and
k = rate constant, which is dependent on time.

Statistical Analysis

Reaction rate data were analyzed using a split-plot design (28, 30); SMUF was the whole-plot factor, and treatment was the subplot factor. Tukey's honestly significant difference test was used for separation of means. The following model was utilized:

$$Y_{ijk} = \mu + S_i + \epsilon_{j(i)} + T_k + ST_{ik} + \epsilon_{k(ij)} \quad [2]$$

where

Y_{ijk} = rate constant ijk;
μ = overall mean;
S_i = SMUF (i = 0, without and 1, with);
$\epsilon_{j(i)}$ = whole-plot error, the mean square of which was used to test SMUF effect where j = effect of replicate (j = 1 and 2), and subscript j(i) indicates that replicate (j) is nested within SMUF (i);
T_k = without or with addition of native or denatured α-LA and β-LG (k = 0, 1, 2, 3, and 4);
ST_{ik} = interaction of SMUF by treatment; and
$\epsilon_{k(ij)}$ = subplot error, and its means square is used to test the effects of treatment and SMUF by treatment interaction.

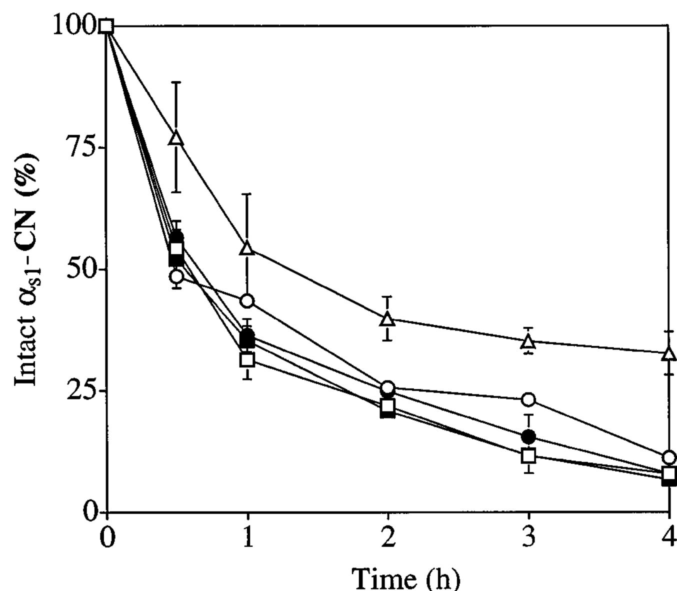

Figure 1. Percentage of intact α_{s1}-CN remaining after specific reaction times in the presence and absence of native (n) and heat-denatured (d) whey proteins (α-LA and β-LG). Each point represents the mean (±SD) of two trials. Simulated milk ultrafiltrate was not added in this experiment (□ = α_{s1}-CN, ■ = α_{s1}-CN plus n α-LA, ○ = α_{s1}-CN plus n β-LG, ● = α_{s1}-CN plus d α-LA, and △ = α_{s1}-CN plus d β-LG).

RESULTS AND DISCUSSION

Figures 1 and 2 show the percentages of intact α_{s1}-CN at different intervals during hydrolysis of α_{s1}-CN by chymosin in the presence or absence of native and denatured whey proteins (α-LA and β-LG) with and without addition of SMUF. Each data point shown in Figures 1 and 2 is a mean of duplicate determinations of α_{s1}-CN concentration as determined from FPLC® ion-exchange chromatography; 120 FPLC® analyses were done.

In the absence of SMUF, denatured β-LG inhibited chymosin so that 33% intact α_{s1}-CN remained after 4 h; the other reaction mixtures had <12% intact α_{s1}-CN remaining after 4 h (Figure 1). Upon addition of SMUF (Figure 2), the reaction mixtures containing native and denatured β-LG had the highest percentage of intact α_{s1}-CN after 4 h of hydrolysis, 27 and 35%, respectively. Statistical analysis of rate constants confirmed these observations (Table 1). In the absence of SMUF, only denatured β-LG reduced reaction rates of α_{s1}-CN hydrolysis ($P < 0.05$), but, when SMUF was included in the reaction mixture, both native and denatured β-LG reduced the reaction rate ($P < 0.05$).

The only inhibitory effect that was attributable to α-LA was when it was heat-denatured and added to reaction mixtures containing SMUF. Also, neither α-LA nor β-LG (native or denatured) was hydrolyzed by chymosin, which is consistent with the observations of Harper et al. (17) on cheese slurries.

Within each form of α-LA and β-LG and under the conditions of these experiments, the heat-denatured form appeared to have a greater inhibitory effect on the rate of α_{s1}-CN hydrolysis than did the native form, but this inhibitory effect was not statistically significant. Side chains, which are normally buried in the native structure, are exposed by denaturation and may interfere with the access of chymosin to α_{s1}-CN.

The structure of β-LG is known to be affected by pH. Hambling et al. (16) reported that, at pH <3.5, β-LG dimers dissociated into monomers, and, in the pH range of 3.7 to 6.5, β-LG reversibly octamerized to produce aggregates with a molecular mass of 144 kDa. In our model systems, native β-LG probably existed as a mixture of dimers and octamers that apparently did not interact with chymosin or influence its activity unless milk salts were included in the reaction mixture.

Although a complete understanding of how the various milk salts influence whey protein structure is not known, researchers have shown that divalent cations interact with whey proteins. Jeyarajah and Allen

(20) found that Ca bound weakly to β-LG, and, when bound, caused shifts in protein structure that can be observed using the fluorescent probe, anilinonaphthalene-sulfonic acid, which binds to nonpolar, hydrophobic sites on proteins and indicates the level of exposed hydrophobic amino acid residues. In their study, Jeyarajah and Allen (20) found that the hydrophobic regions of β-LG were more available for binding to the fluorescent probe when calcium concentration was increased to 20 m*M*. We observed those addition of SMUF to reaction mixtures increased the ability of native β-LG to inhibit chymosin activity against α_{s1}-CN, a result that is probably related to the interaction of Ca with β-LG. Calcium also binds very strongly to α-LA; if Ca is removed from α-LA, conformation shifts occur that are equivalent to that occurring during acid denaturation (7). Because α-LA inhibited chymosin activity only when it was denatured and added to SMUF, we hypothesized that heating, and the release of bound calcium from α-LA during heating, generated several different molecular configurations of α-LA. We think that when these molecular species were then added to buffers containing SMUF, interactions between α-LA, calcium salt and possibly other milk salts, and either α_{s1}-casein or chymosin resulted in reduced hydrolysis of α_{s1}-casein.

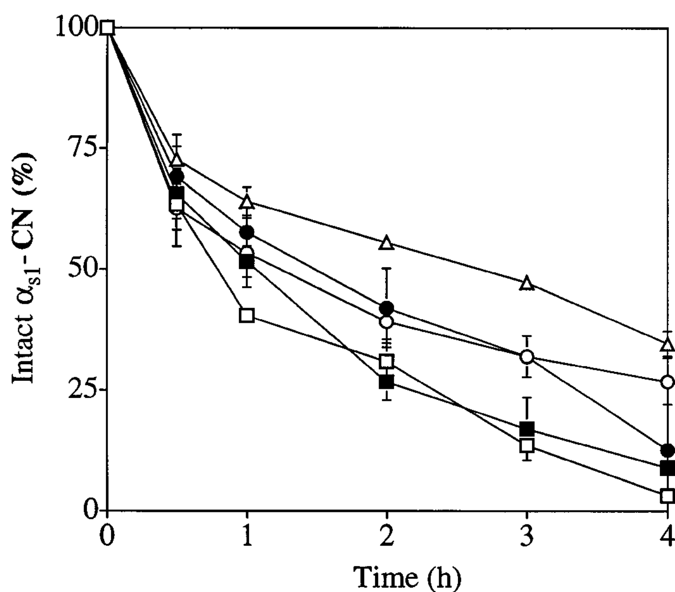

Figure 2. Percentage of intact α_{s1}-CN remaining after specific reaction times in the presence and absence of native (n) and denatured (d) whey proteins (α-LA and β-LG). Each point represents the mean (±SD) of two trials. Simulated milk ultrafiltrate was added in this experiment (□ = α_{s1}-CN, ■ = α_{s1}-CN plus n α-LA, ○ = α_{s1}-CN plus n β-LG, ● = α_{s1}-CN plus d α-LA, and △ = α_{s1}-CN plus d β-LG).

TABLE 1. Rate constant, coefficients of determination (r^2) and 95% confidence limits (CL) resulting from fitting data from hydrolysis of α_{s1}-CN by chymosin in the absence and presence of SMUF and native (n) or denatured (d) whey proteins to first-order reaction kinetics.

Treatment	Rate	r^2	95% CL
Without SMUF			
α_{s1}-CN	0.61^{ab}	0.955	0.17
α_{s1}-CN + n α-LA	0.65^{ab}	0.968	0.16
α_{s1}-CN + n β-LG	0.47^{bc}	0.928	0.18
α_{s1}-CN + d α-LA	0.60^{ab}	0.972	0.13
α_{s1}-CN + d β-LG	0.28^{c}	0.860	0.15
With SMUF			
α_{s1}-CN	0.80^{a}	0.940	0.27
α_{s1}-CN + n α-LA	0.59^{ab}	0.969	0.15
α_{s1}-CN + n β-LG	0.30^{c}	0.916	0.13
α_{s1}-CN + d α-LA	0.46^{bc}	0.937	0.16
α_{s1}-CN + d β-LG	0.23^{c}	0.932	0.09

[a,b,c]Means in the same column and section (with or without SMUF) and without a common superscript differ ($P < 0.05$).

A better understanding of the structure of whey proteins and their interaction with casein in cheese may help to overcome the lack of proteolysis and flavor development in cheese fortified with whey protein. Our work addresses the potential impact on UF cheese in which native whey proteins are in the soluble form and may be applicable to cheese containing whey proteins that were heat-denatured in the absence of casein. If whey proteins were heated in the presence of casein before cheese manufacture, the resulting structures would be different than in our model systems because α-LA and β-LG were heat-denatured in the absence of α_{s1}-CN and milk salts. That whey proteins could inhibit the action of chymosin in such systems is possible but must be verified by further research.

Another factor that limits the extrapolation of this research to cheese is that α_{s1}-CN was in a soluble state, but, in cheese, α_{s1}-CN associates with the other caseins to form an insoluble protein matrix. However, McSweeney et al. (27) have shown that many of the chymosin cleavage sites of α_{s1}-CN are hydrolyzed in both cheese and soluble systems, indicating that the accessibility of those peptide linkages in α_{s1}-CN to chymosin is similar in cheese or aqueous systems.

We did not add a denaturing agent to our model systems because we wanted to approximate conditions in the aqueous phase of cheese. Urea has been used by Mulvihill and Fox (29) to study the chymosin action on α_{s1}-CN in the isoelectric region. They (29) found that casein could be solubilized in the isoelectric region using 5 *M* urea; however, optimum proteolysis occurred at pH 2.8.

CONCLUSIONS

The inhibitory behavior of α-LA and β-LG on chymosin activity toward α_{s1}-CN in buffered model systems depended on the structure of these whey proteins (native or denatured) and on the presence of SMUF. α-Lactalbumin inhibited chymosin activity against α_{s1}-CN only when α-LA was denatured and added to reaction mixtures that contained SMUF. In the buffered system, only denatured α-LG inhibited the rate of α_{s1}-CN hydrolysis. In the presence of SMUF, inhibitory effects were significant when native and denatured β-LG were included in reaction mixtures.

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REFERENCES

1 Bastian, E. D., K. G. Hansen, and R. J. Brown. 1993. Inhibition of plasmin by β-lactoglobulin using casein and a synthetic substrate. J. Dairy Sci. 76:3354.
2 Chapman, H. R., V. E. Bines, F. A. Glover, and P. J. Skudder. 1974. Use of milk concentrated by ultrafiltration for making hard cheese, soft cheese and yoghurt. J. Soc. Dairy Technol. 27: 151.
3 Davies, D. T., and A.J.R. Law. 1987. Quantitative fractionation of casein mixtures by fast protein liquid chromatography. J. Dairy Res. 54:369.
4 de Boer, R., and P.F.C. Nooy. 1980. Low-fat semi-hard cheese from ultrafiltrated milk. Nordeuropaeisk Mejeritidsskrift 46:52.
5 de Koning, P. J., R. de Boer, P. Both, and P.F.C. Nooy. 1981. Comparison of proteolysis in a low-fat semi-hard type of cheese manufactured by standard and by ultrafiltration techniques. Neth. Milk Dairy J. 35:35.
6 Ernstrom, C. A., B. J. Sutherland, and G. W. Jameson. 1980. Cheese base for processing. A high yield product from whole milk by ultrafiltration. J. Dairy Sci. 63:228.
7 Farrell, H. M. 1988. Physical equilibria: proteins. Page 461 *in* Fundamentals of Dairy Chemistry. 3rd ed. N. P. Wong, ed. Van Nostrand Reinhold, New York, NY.
8 Fox, P. F. 1988. Rennets and their action in cheese manufacture and ripening. Biotechnol. Appl. Biochem. 10:522.
9 Fox, P. F. 1989. Proteolysis during cheese manufacture and ripening. J. Dairy Sci. 72:1379.
10 Fox, P. F., and J. Guiney. 1973. Casein micelle structure: susceptibility of various casein systems to proteolysis. J. Dairy Res. 40:229.
11 Fox, P. F., and B. F. Walley. 1971. Influence of sodium chloride on the proteolysis of casein by rennet and by pepsin. J. Dairy Res. 38:165.
12 Friis, T. 1981. Production of Mozzarella cheese based on ultrafiltration. Nordeuropaeisk Mejeritidsskrift 47:220.
13 Goudedranche, H., J. Maubois, L. Ducruet, and M. Mahaut. 1981. Utilization of the new mineral membranes for making semi-hard cheeses. Desalination 35:243.
14 Green, M. L., F. A. Glover, E.M.W. Scurlock, R. J. Marshall, and D. S. Hatfield. 1981. Effect of use of milk concentrated by ultrafiltration on the manufacture and ripening of Cheddar cheese. J. Dairy Res. 48:333.
15 Green, M. L., A. Turvey, and D. G. Hobbs. 1981. Development of structure and texture in Cheddar cheese. J. Dairy Res. 48:343.
16 Hambling, S. G., A. S. McAlpine, and L. Sawyer. 1992. β-Lactoglobulin. Page 141 *in* Advanced Dairy Chemistry. Vol. 1. Proteins. P. F. Fox, ed. Elsevier Appl. Sci., New York, NY.
17 Harper, J., M. Iyer, D. Knighton., and J. Lelievre. 1989. Effects of whey proteins on the proteolysis of Cheddar cheese slurries (a model for the maturation of cheeses made from ultrafiltered milk). J. Dairy Sci. 72:333.
18 Iyer, M., and J. Lelievre. 1987. Yield of Cheddar cheese manufactured from milk concentrated by ultrafiltration. J. Soc. Dairy Technol. 40:45.
19 Jenness, R., and J. Koops. 1962. Preparation and properties of a salt solution which simulates milk ultrafiltrate. Neth. Milk Dairy J. 16:153.
20 Jeyarajah, S., and J. C. Allen. 1994. Calcium binding and salt-induced structural changes of native and preheated β-lactoglobulin. J. Agric. Food Chem. 42:80.
21 Kaminogawa, S., H. Mizobuchi, and K. Yamauchi. 1972. Comparison of bovine milk protease with plasmin. Agric. Biol. Chem. 36:2163.
22 Kealy, K. S., and F. V. Kosikowski. 1986. Cottage cheese from ultrafiltered skim milk retentates in industrial cheese making. J. Dairy Sci. 69:1479.
23 Kosikowski, F. V., A. R. Masters, and V. V. Mistry. 1985. Cottage cheese from retentate-supplemented skim milk. J. Dairy Sci. 68:541.
24 Kyle, S., and M. W. Hickey. 1993. Feta cheese with ultrafiltration retentate. Aust. J. Dairy Technol. 48:47.
25 Labuza, T. P. 1985. An integrated approach to food chemistry: illustrative cases. Page 913 *in* Food Chemistry. 2nd ed. O. R. Fennema, ed. Marcel Dekker, Inc., New York, NY.
26 Labuza, T. P., K. A. Nelson, and G. J. Nelson. 1991. Water Analyzer Series-Reaction Kinetics Program Version 2.10. Univ. Minnesota, St. Paul.
27 McSweeney, P.L.H., S. Pochet, P. F. Fox, and A. Healy. 1994. Partial identification of peptides from the water-insoluble fraction of Cheddar cheese. J. Dairy Res. 61:587.
28 Montgomery, D. C. 1991. Design and Analysis of Experiments. 3rd ed. John Wiley & Sons, New York, NY.
29 Mulvihill, D. M., and P. F. Fox. 1976. Selective denaturation of milk coagulants in 5 *M*-urea. J. Dairy Res. 44:319.
30 Ott, L. 1984. An Introduction to Statistical Methods and Data Analysis. 2nd ed. PWS Publ., Boston, MA.
31 Snoeren, T.H.M., J.A.M. van Riel, and P. Both. 1980. Some properties of a milk proteinase isolated from UHT milk. Zuivelzicht 72:42.
32 van Hooydonk, A.C.M., C. Olieman, and H. G. Hagedoorn. 1984. Kinetics of the chymosin-catalyzed proteolysis of κ-casein in milk. Neth. Milk Dairy J. 38:207.