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Aggregation of Rennet-Altered Casein Micelles at Low Temperatures

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The rennet-induced coagulation of bovine milk at 10 °C was investigated. The rate of change of absorbance at 600 nm was higher in milk renneted at 30 °C than that at 10 °C. The amount of casein sedimented on centrifuging skim milk at 5000*g* for 1 h at 10 °C increased with time after renneting. The viscosity of milk at 10 °C at low shear rates did not change significantly until 10 h after rennet addition, but it increased markedly after 20 h. Smaller particles in milk at 10 °C disappeared slowly over 36 h after rennet addition and aggregated into larger particles. These results suggested that casein micelles in milk aggregate at low temperatures. Reasons for the slow aggregation of milk renneted at 10 °C were investigated by inhibiting chymosin activity by pepstatin A. It is likely that β-casein, or its hydrolysis, plays a role in aggregation of rennet-altered casein micelles at low temperatures.

KEYWORDS: Cold renneting; β-casein; chymosin; coagulation of milk

INTRODUCTION

It is well-known that the conversion of milk to curd during the manufacture of rennet-coagulated cheeses involves four separate but overlapping steps: proteolysis, aggregation/flocculation, gelation, and syneresis (*1*). The first, enzymatic, phase of rennet coagulation essentially involves hydrolysis of the $Phe_{105}-Met_{106}$ bond of κ-casein (although the proteinase of *Cryphonectria parasitica* hydrolyzes the $Ser_{104}-Phe_{105}$ bond rather than $Phe_{105}-Met_{106}$); some rennets also hydrolyze other bonds later to a limited extent. Hydrolysis of the $Phe_{105}-Met_{106}$ bond and factors that affect it are well understood and can be described by Michaelis−Menten kinetics (*2*). The aggregation stage is a biparticle reaction. The aggregates form a three-dimensional particulate gel which is stable for a long time if left undisturbed but contracts (synereses) if cut, broken, or subjected to external pressure.

Aggregation, gelation, and syneresis are probably different phases of the same overall process (*1*). The forces responsible for these reactions have not been defined fully, but hydrophobic bonds are probably major contributors (*3*−*5*). Ca^{2+} and colloidal calcium phosphate (CCP) are essential for aggregation and gelation (*3*−*5*), and probably for syneresis, although it is not possible to investigate the effects of compositional factors on syneresis independently of aggregation and gelation. The mechanism(s) by which Ca^{2+} and CCP affect aggregation and gelation have not been established fully, but they may act by charge neutralization since *para*-casein binds Ca^{2+} more strongly than does casein (*6*). The secondary (aggregation and gelation) phase of rennet-induced coagulation has been reviewed extensively (*2*, *7*−*9*).

The enzymatic phase of rennet action can be monitored by quantifying the products of the reaction, i.e., the caseinomacropeptide (CMP) (as measured by trichloroacetic acid (TCA)-soluble N, TCA-soluble *N*-acetylneuraminic acid (NANA), or directly by HPLC) and *para*-κ-casein as measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) or capillary electrophoresis. The combined enzymatic and aggregation phases are assayed by measuring or visually observing the formation of flecs/flakes of coagulated protein in a film of milk (*10*) or by changes in the scattering of light (*11*−*16*). Turbidity measurements have been made on diluted (*11*, *12*) or undiluted (*7*, *17*−*19*) milk. Various parameters of coagulation can be derived from turbidity−time curves (*19*). Gelation can be studied by several rheological methods (*16*, *20*−*24*).

It is generally held that the rennet-induced coagulation of bovine milk does not occur below about 18 °C (*3*, *5*, *9*, *10*, *25*−*28*). This effect was first reported by Effront (*25*), who showed that the failure to coagulate was due to the influence of temperature on the secondary (nonenzymatic) phase. The effect of temperature on rennet-induced coagulation was investigated further by Berridge (*10*) who showed that the temperature coefficient (Q_{10}) of the enzymatic phase is 2−4 per 10 °C, which is typical for an enzymatic reaction. However, the Q_{10} for the secondary (coagulation) phase is 13−16 (*10*).

As described above, visually observable aggregation and gelation essentially do not occur below ∼18 °C, but it is not known whether some aggregation of renneted micelles occurs at a low temperature. The objective of this study was to investigate the aggregation of milk renneted at 10 °C by rheological techniques and by measuring changes in absorbance at 600 nm, sedimentation of casein aggregates by low-speed centrifugation, and changes in particle size.

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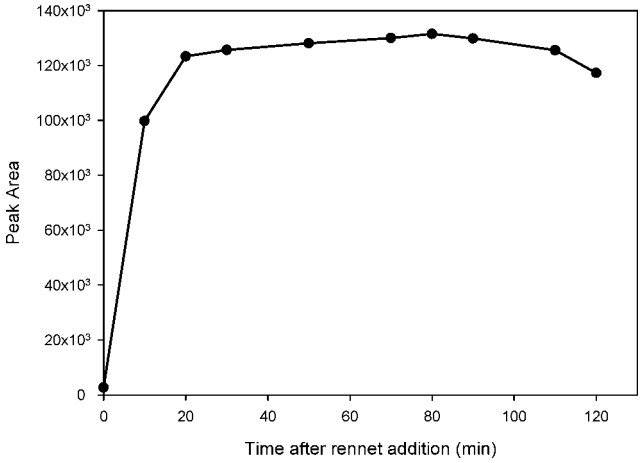

Figure 1. Changes in the area under the peak representing the caseinomacropeptide released in the 12% trichloroacetic acid soluble fraction of milk incubated with 1 μL/mL of coagulant at 10 °C for up to 120 min analyzed using RP-HPLC as a function of time of incubation.

MATERIALS AND METHODS

Milk. Commercial pasteurized skim milk (3.4% protein) was used as substrate. To restrict the growth of microorganisms at 10 °C, sodium azide was added to the milk at a level of 0.05% (w/v). The results reported are the means of three independent trials carried out for each treatment.

Coagulant and Inhibitor. The coagulant used was fermentation-produced chymosin (Maxiren-180, 180 IMCU/mL; DSM Food Specialties, Delft, Netherlands). The concentration of coagulant used was varied with the assay conditions and is indicated in the legend of the figure or table for each experiment. Pepstatin A (Sigma Chemical Co., St. Louis, MO), a competitive inhibitor of aspartyl proteinases, was used in some experiments to inhibit chymosin. Pepstatin was dissolved (1 mg/mL) in absolute ethanol at 60 °C and added to milk at a level of 30 μmol/L.

Measurement of First Phase of Renneting. The first phase of renneting (cleavage of the Phe−Met bond of κ-casein by chymosin) was studied by measuring the release of CMP by reverse-phase (RP)-HPLC. The coagulant was added to milk at 10 °C at a level of 1 μL/mL, and mixed for 10 s. Subsamples (2 mL) of renneted milk were transferred to test tubes and held at 10 °C. The reaction was stopped by adding 4 mL of 12% TCA to 2 mL subsamples of renneted milk at 10 min intervals from 0 to 120 min after rennet addition; as a control, TCA was also added to unrenneted milk. The samples were filtered through Whatman No. 40 filter paper, and the filtrate was analyzed by RP-HPLC (*29*). The area under the peak representing the CMP in the HPLC chromatogram was plotted against time after rennet addition. The first phase of renneting was considered complete at the time (1 h) when the curve reached a plateau (**Figure 1**).

Measurement of Aggregation of Rennet-Altered Casein Micelles by Visible-Wavelength Spectrophotometry. Aggregation of rennet-altered micelles was followed by measuring the absorbance of milk at 600 nm using a CARY UV−Vis 1E spectrophotometer (Varian Australia Pty Ltd., Mulgrave, Victoria, Australia). Pasteurized skim milk was equilibrated at 10 or 30 °C. To complete the hydrolysis of κ-casein in about the same time at 10 or 30 °C, 1 or 0.25 μL of the coagulant was added per milliliter of milk, respectively. A sample of rennet-treated milk was transferred to a 1 mm light path glass microcuvette (Hellma UK Ltd., Southend-on-Sea, Essex, U.K.) and placed in the temperature-controlled compartment of the spectrophotometer (10 or 30 °C). The absorbance of distilled water at the appropriate temperature was set to zero.

Measurement of Aggregation of Rennet-Altered Casein Micelles in Milk by Low-Speed Centrifugation. In preliminary trials, pasteurized skim milk at 10 °C was centrifuged at 500−100000*g* for 1 h using a Sorvall RC5C plus centrifuge (DuPont Company, Stevenage, Herts, U.K.); the protein content of the supernatants was determined by the macro-Kjeldahl method (*30*).

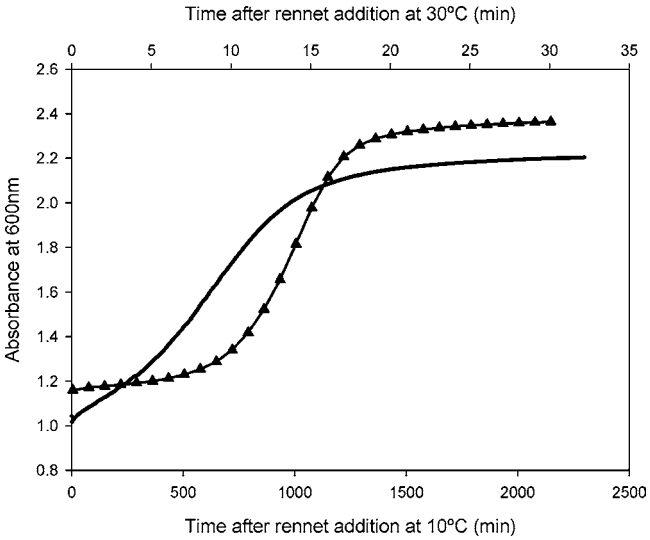

Figure 2. Changes in the absorbance of milk measured in a 1 mm path length glass microcuvette at 600 nm after adding 1 μL or 0.25 μL coagulant per milliliter at 10 (—) or 30 (▲) °C, respectively.

The coagulant was added to pasteurized skim milk at a level of 1 μL/mL at 10 °C. Samples of milk were taken at 0, 12, 18, 24, 36, 42, or 48 h after rennet addition and centrifuged at 5000*g* for 1 h at 10 °C. A sample of unrenneted milk was also centrifuged at 5000*g* for 1 h at 10 °C.

Measurement of the Viscosity of Milk Using a Low-Shear Viscometer. The coagulant was added at a level of 1 μL/mL to milk at 10 °C in the concentric cylinder of a Contraves Low Shear 30 viscometer (Contraves, Zurich, Switzerland); the samples were held for 0, 5, 10, 15, or 20 h after rennet addition, and the torque was then measured at shear rates ($\dot{\gamma}$) from 1.7 to 69.5 s^{-1}. The viscosity (η) of the samples was plotted as a function of $\dot{\gamma}$.

To study the effect of adding pepstatin A (30 μmol/L) on the viscosity of milk renneted (1 μL/mL) at 10 °C, samples of renneted milk were transferred to the viscometer after adding pepstatin A at the completion of the first phase of renneting (1 h), the samples were held at 10 °C for 0, 9, 14, or 24 h after the addition of pepstatin A, and the torque was measured at $\dot{\gamma}$ from 1.7 to 69.5 s^{-1}.

Measurement of Aggregation of Rennet-Altered Casein Micelles in Milk by Laser Light Scattering. The coagulant was added to pasteurized skim milk at 10 or 30 °C at a level of 1 μL/mL and the particle size distribution determined at 5 min intervals at 30 °C or at 2 h intervals at 10 °C by laser light scattering, using a Mastersizer model S (Malvern Instruments Ltd., Malvern, Worchestershire, U.K.) equipped with a He−Ne laser (λ = 633 nm). Synthetic milk ultrafiltrate (SMUF) (*31*) at 10 or 30 °C was used to dilute the milk samples. The presentation parameters used were as follows: refractive index of milk and diluent, 1.3993 and 1.3300, respectively, and absorption = 0.1.

To study the significance of hydrolysis of β-casein by chymosin on the coagulation of milk at 10 °C, the coagulant was added to milk at a level of 1 μL/mL at 10 °C. After completion of the first phase of renneting (1 h), pepstatin A was added to the renneted milk at a level of 30 μmol/L and changes in the particle size distribution were determined by Mastersizer at 2 h intervals for 36 h. The effect of adding a heat-inactivated (70 °C for 10 min) coagulant to milk at 10 °C at a level of 1 μL/mL on particle size distribution was also determined. In a further experiment, the coagulant (1 μL/mL) and pepstatin A (30 μmol/L) were added simultaneously to milk at 10 °C and the changes in particle size distribution determined at 4 h intervals for 24 h. To allow observations at evenly spaced time intervals over 24 or 36 h, the samples were divided in 3 batches, which were renneted at different times.

Urea-Polyacrylamide Gel Electrophoresis. Proteolysis of rennet-treated milk samples was assessed by urea-polyacrylamide gel electrophoresis (PAGE) (12.5% T, 4% C, pH 8.9) according to the method of Andrews (*32*), as modified by Shalabi and Fox (*33*). The gels were

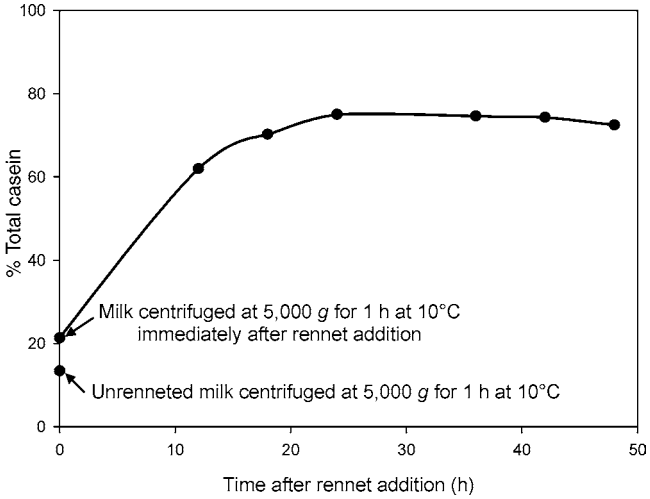

Figure 3. Changes in the percentage of total casein sedimented (measured by macro-Kjeldahl method) at 5000*g* for 1 h in unrenneted milk or in milk incubated with 1 μL/mL of coagulant at 10 °C for up to 48 h.

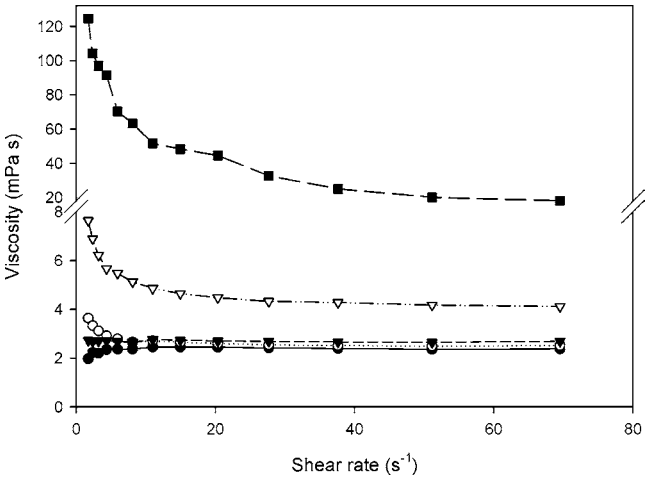

Figure 4. Changes in the viscosity of milk measured using a low shear viscometer at a shear rate ($\dot{\gamma}$) in the range from 1.7 to 69.5 s^{-1} after incubating with 1 μL/mL of coagulant at 10 °C for 0 (●), 5 (○), 10 (▼), 15 (▽), or 20 (■) h.

stained directly with Coomassie Brilliant Blue G250 (*34*), destained in several changes of distilled water, and scanned on a flatbed scanner. The total viable bacterial count of the samples was determined on plate count agar (Oxoid Ltd., Basingstoke, Hampshire, U.K.) by the pour-plate technique, with aerobic incubation at 30 °C for 3 days.

RESULTS AND DISCUSSION

Changes in Absorbance. The coagulant was added to a sample of pasteurized skim milk equilibrated at 10 or 30 °C, and the aggregation of casein micelles was observed by measuring absorbance at 600 nm (A_{600}). As renneting progressed, the A_{600} of renneted milk samples increased to a plateau value and remained constant thereafter (**Figure 2**). The A_{600} reached a plateau value 20 or 1500 min after rennet addition in milk renneted at 30 or 10 °C, respectively; the rate of change of A_{600} was higher in milk renneted at 30 °C than in milk renneted at 10 °C but the plateau value was similar in both cases. This suggests that the milk renneted at 10 °C aggregates in a similar way as at 30 °C, but at a slower rate. The results obtained for milk renneted at 30 °C are consistent with those reported in previous studies (*7*, *11*, *12*, *17*−*19*); changes in the A_{600} of milk renneted at 10 °C have not been reported previously.

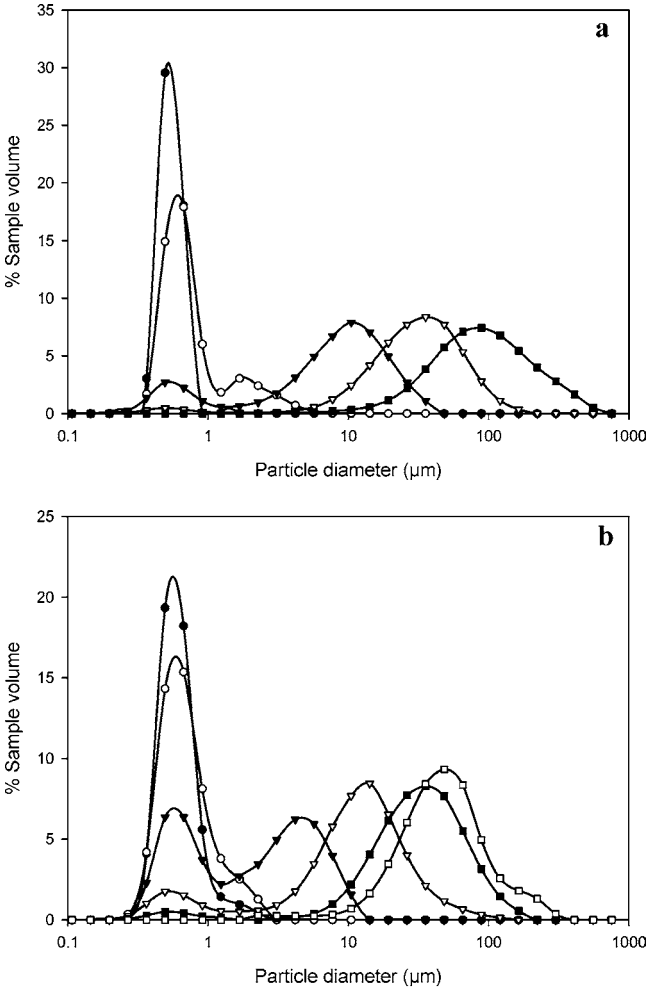

Figure 5. Changes in particle size distribution in milk measured by laser light scattering using a Mastersizer after incubating with 1 μL/mL of coagulant for (**a**) 0 (●), 25 (○), 30 (▼), 35 (▽), or 50 (■) min at 30 °C or (**b**) 0 (●), 10 (○), 16 (▼), 20 (▽), 24 (■), or 36 (□) h at 10 °C.

Table 1. Particle Size Distribution Parameters [*D*(*v*, 0.5) and *D*(*v*, 0.9)] Measured by Laser Light Scattering in Milk Incubated with 1 μL/mL of Coagulant at 30 °C (Milk-1) and Milk Incubated with 1 μL/mL of Coagulant at 10 °C (Milk-2)[a]

sample	time after rennet addition	*D*(*v*, 0.5) μm	*D*(*v*, 0.9) μm
milk-1	0 min	0.5	0.6
	25 min	0.6	1.8
	30 min	8.0	19.0
	35 min	29.0	67.0
	50 min	82.0	239.0
milk-2	0 h	0.5	0.8
	10 h	0.6	1.2
	16 h	2.0	6.0
	20 h	11.0	27.0
	24 h	30.0	72.0
	36 h	43.0	107.0

[a] Results of trial 1 are shown; similar results were obtained for trials 2 and 3.

Changes in the Sedimentability of Casein at 5000*g*. When pasteurized skim milk at 10 °C was centrifuged at 5000*g* for 1 h, ∼13% of the total casein was sedimented. This force and time of centrifugation were used in further experiments. When samples of pasteurized skim milk at 10 °C were renneted and centrifuged at 5000*g* for 1 h at various time points after rennet addition, the amount of casein sedimented increased with time

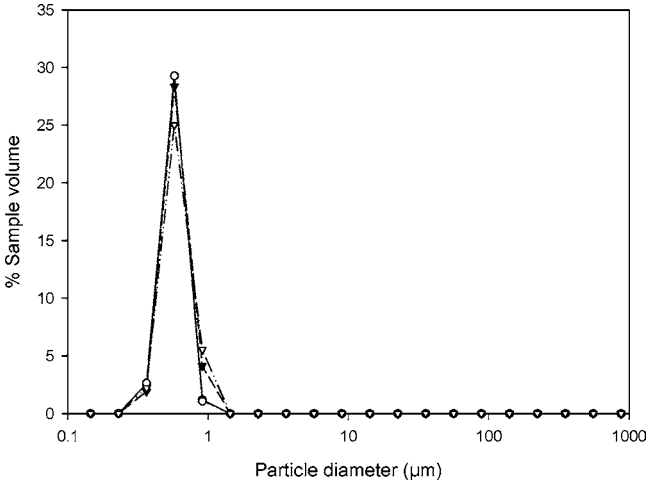

Figure 6. Changes in particle size distribution in milk measured by laser light scattering using a Mastersizer after incubating with 1 μL/mL of heat-inactivated coagulant for 0 (●) or 36 (○) h and after incubating with 1 μL/mL of pepstatin A-inactivated coagulant for 0 (▼) or 36 (▽) h at 10 °C.

after renneting (**Figure 3**). For the milk sample centrifuged immediately after rennet addition, 21% of the total casein was sedimented, probably due to chymosin activity during centrifugation (1 h). Twelve and 24 h after rennet addition, ~62% and 75%, respectively, of the total casein in milk was sedimentable; the amount remained constant thereafter. These results suggest that as renneting progressed the casein micelles formed aggregates large enough to be sedimented by 5000*g* for 1 h. To the best of our knowledge, data on the sedimentability of rennet-induced casein aggregates at 5000*g* have not been published previously.

Changes in Viscosity. Pasteurized skim milk at 10 °C was renneted in the concentric cylinder of the Contraves viscometer and viscosity measured at different time points after rennet addition. The viscosity of unrenneted milk at low shear rates (<3 s^{-1}) was 2 mPa s (**Figure 4**) and did not change significantly until 10 h after rennet addition, suggesting that no significant aggregation of casein micelles occurred during this period. Thereafter, the viscosity increased to 8 mPa s 15 h after rennet addition, indicating the beginning of aggregation of casein micelles into larger particles. The viscosity increased 12-fold (125 mPa s) after 20 h. The viscosity of all the samples decreased markedly with increasing shear rate, displaying typical thixotropic characteristics.

It is well known that the viscosity of milk increases during renneting. The measurement of the increase in the viscosity of milk renneted at temperatures greater than than 18 °C using various rheological methods has been used extensively previously to study coagulation of milk by chymosin (*16*, *20*−*24*). However, the rennet-induced coagulation of milk at low temperatures by low shear viscometry has not been studied previously.

Changes in the Size of Aggregates of Casein Micelles. Pasteurized skim milk was renneted at 30 °C and the particle size distribution measured at 5 min intervals. At 0 min, i.e., in unrenneted milk, the size of particles was in the range of 0.3−1 μm (**Figure 5a**). A total of 50% of the particles [$D(v, 0.5)$] were less than 0.5 μm, and 90% [$D(v, 0.9)$] were less than 0.6 μm (**Table 1**). There were no changes in particle size distribution until 20 min after rennet addition. At 25 min after rennet addition, the $D(v, 0.5)$ and $D(v, 0.9)$ increased to ~0.6 and 1.8 μm, respectively, indicating that the proportion of smaller particles decreased and larger aggregates were formed. The $D(v, 0.5)$ and $D(v, 0.9)$ increased further to ~8 and 19 μm, respectively, 30 min after rennet addition. Also, very large aggregates (up to 48 μm) were observed at this time. The particles in the range of 0.3−1 μm had almost disappeared by 35 min after rennet addition, and the size of the largest aggregates had increased to ~190 μm. Within 50 min of

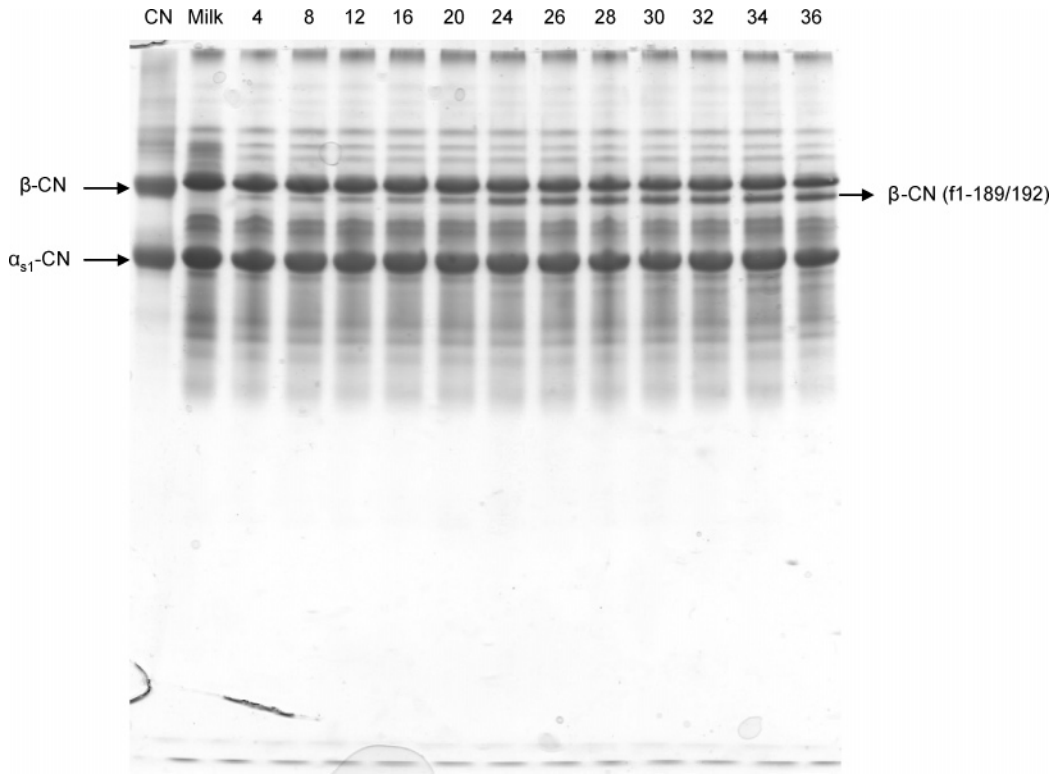

Figure 7. Urea-polyacrylamide gel electrophoretograms (12.5% T, 4% C, pH 8.9) of bovine sodium caseinate (CN), milk, and milk incubated with 1 μL/mL of coagulant at 10 °C for 4, 8, 12, 16, 20, 24, 26, 28, 30, 32, 34, or 36 h.

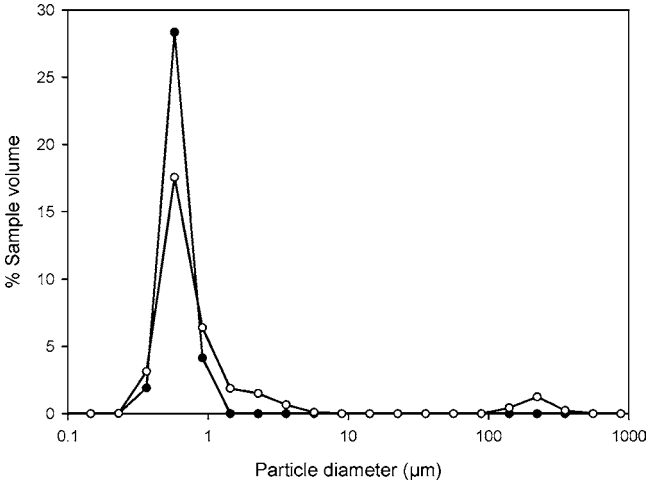

Figure 8. Changes in particle size distribution measured by laser light scattering using a Mastersizer after adding pepstatin A (30 μmol/L milk) at the end of the first phase of renneting (1 h) to milk incubated with 1 μL/mL of coagulant for 0 (●) and 36 (○) h at 10 °C.

renneting, the $D(v, 0.5)$ and $D(v, 0.9)$ increased to ~82 and 239 μm, respectively. The increase in $D(v, 0.5)$ indicates that during renneting the proportion of smaller particles (0.3−1 μm) decreased, and the increase in $D(v, 0.9)$ indicated that the proportion of the large aggregates increased continuously; the maximum size of the aggregates increased (to ~555 μm) over time.

In the milk renneted at 10 °C, the particle size distribution was measured at 2 h intervals. A similar change in the size distribution of aggregates was observed, but much more slowly than at 30 °C (**Figure 5b**). At 0 min, i.e., in unrenneted milk, the size of particles was in the range of 0.27−2.28 μm, the $D(v, 0.5)$ and $D(v, 0.9)$ were 0.5 and 0.8 μm, respectively (**Table 1**). At 10 h after rennet addition, $D(v, 0.5)$ and $D(v, 0.9)$ had increased slightly to ~0.6 and 1.2 μm, respectively. After 16 h, $D(v, 0.5)$ and $D(v, 0.9)$ had increased to ~2 and 6 μm, respectively, and the size of the largest aggregates had increased to ~12 μm. The smaller particles had disappeared completely 36 h after rennet addition. $D(v, 0.5)$ and $D(v, 0.9)$ had increased to ~43 and 107 μm, and the size of the largest aggregates had increased to ~302 μm. At this stage, the aggregates formed a weak gel (as observed visually). The milk renneted at 10 °C formed a weaker gel at 36 h (at which time all the smaller particles had disappeared) than the gel formed at 30 °C after 50 min. No previous work appears available on the measurement of size of aggregates formed during the rennet coagulation of milk. On the basis of the results of this study, measurement of the particle size distribution appears to be a useful tool for studying the aggregation of renneted milk.

The pH and the total plate count of the samples renneted at 10 °C did not change significantly over 36 h (results not shown), indicating that the aggregation and weak gelation observed were not due to microbial growth. Also, there was no change in the particle size in milk incubated with heat- or pepstatin A-inactivated coagulant at 10 °C (**Figure 6**), indicating that active chymosin was responsible for the changes in particle size in milk renneted at 10 °C.

Mechanism of Aggregation of Rennet-Altered Casein Micelles in Milk at 10 °C. The results of this study indicated

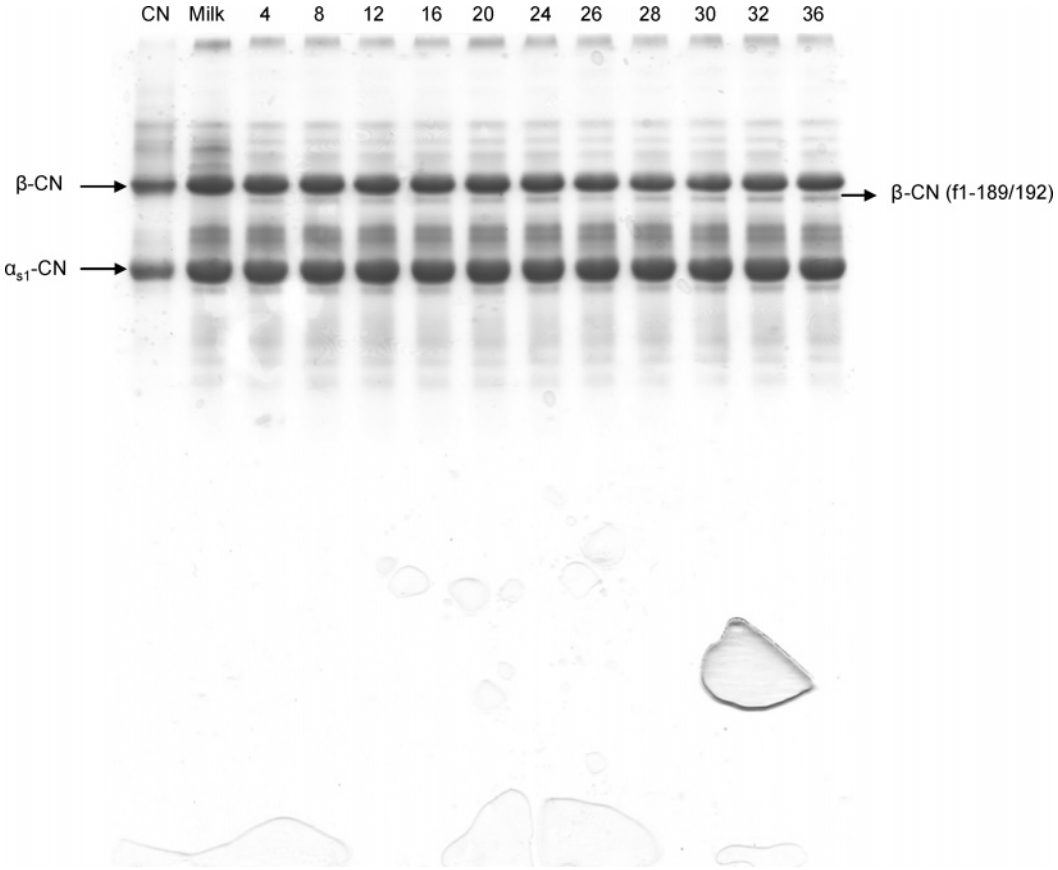

Figure 9. Urea-polyacrylamide gel electrophoretogram (12.5% T, 4% C, pH 8.9) of bovine sodium caseinate (CN), milk, and after adding pepstatin A (30 μmol/L milk) at the end of the first phase of renneting (1 h) to milk incubated with 1 μL/mL of coagulant for 4, 8, 12, 16, 20, 24, 26, 28, 30, 32, and 36 h at 10 °C.

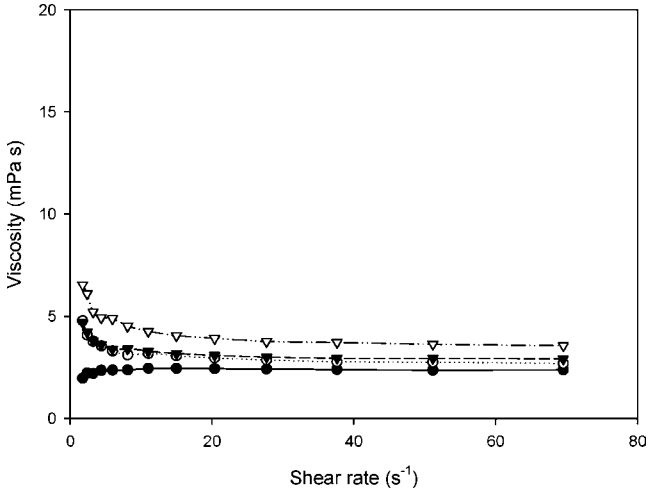

Figure 10. Changes in the viscosity measured using a low shear viscometer at a shear rate ($\dot{\gamma}$) in the range from 1.7 to 69.5 s^{-1} after adding pepstatin A (30 μmol/L milk) at the end of the first phase of renneting (1 h) to milk incubated with 1 μL/mL of coagulant for 0 (●), 10 (○), 15 (▼), or 25 (▽) h.

that casein micelles aggregate slowly to form large particles on renneting milk at 10 °C. Urea-PAGE of milk samples renneted at 10 °C showed that extensive hydrolysis of β-casein occurred during the long period of renneting (**Figure 7**). The principal breakdown product had the same electrophoretic mobility as β-casein (f1-189/192), suggesting that chymosin was responsible for its formation (*35*−*37*). The dissociation of β-casein from the casein micelles at low temperatures is well known (*38*−*40*); hence, β-casein becomes more susceptible to hydrolysis by chymosin than α_{s1}-casein (*41*).

To investigate if the hydrolysis of β-casein by chymosin was responsible for the aggregation observed in milk renneted at 10 °C, pepstatin A was added to renneted milk after 1 h, i.e., when the first phase of renneting at 10 °C was complete, to prevent hydrolysis of β-casein. Samples were taken at 2 h intervals and the particle size distribution and the level of proteolysis determined. The $D(v, 0.5)$ and $D(v, 0.9)$ changed little over time (**Figure 8**), and urea-PAGE of these samples indicated that very little hydrolysis of β-casein occurred (**Figure 9**). The viscosity of these milk samples at 10 °C was measured 10, 15, and 25 h after rennet addition. There was little change in the viscosity even 25 h after rennet addition (**Figure 10**). These results suggest that at 10 °C in the absence of active chymosin, the casein micelles failed to aggregate and form a weak gel, suggesting that slow proteolysis of β-casein at 10 °C may be associated with the aggregation and gelation of rennet-altered casein micelles at 10 °C.

Conclusions. It is generally held that renneted bovine milk does not coagulate below about 18 °C. However, it was found that the absorbance of milk at 600 nm, the amount of casein sedimentable at 5000*g* for 1 h, the viscosity of milk samples, and the particle size in milk renneted at 10 °C all changed over 36 h of renneting. These results suggest that casein micelles in milk do aggregate at 10 °C, but at a slow rate. When chymosin activity was inhibited after the completion of first phase of renneting by adding pepstatin A, aggregation of rennet-altered casein micelles did not occur even 36 h after rennet addition. These results suggest that β-casein, or its hydrolysis, plays a role in aggregation of rennet-altered casein micelles at low temperatures. Further studies to investigate this hypothesis are ongoing.

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