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Interaction between Casein Micelles and Whey Protein/κ-Casein Complexes during Renneting of Heat-Treated Reconstituted Skim Milk Powder and Casein Micelle/Serum Mixtures

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ABSTRACT: Casein micelles were separated from unheated reconstituted skim milk powder (RSMP) and were resuspended in the serum of RSMP that had been heated, with and without dialysis of this serum against unheated RSMP. Using size-exclusion chromatography, it was found that the soluble complexes of whey protein (WP) with κ-casein in the serum of the heated milk bind progressively to unheated casein micelles during renneting, even prior to the onset of clotting. Similar trends were noted when casein micelles from RSMP heated at pH values of 6.7, 7.1, or 6.3, each with different amounts of WP coating the micelles, were renneted in the presence of soluble WP/κ-casein complexes. No matter what was the initial load of micelle-bound WP complexes, all micelle types were capable of binding additional serum protein complexes during renneting. However, it is not clear that this binding of WP/κ-casein complexes to the micellar surface is a direct cause of the impaired rennet clotting of the RSMP.

KEYWORDS: Whey protein/κ-casein complexes, heated milk, rennet clotting of milk, casein micelles

INTRODUCTION

Milk clots when its colloidal protein assemblies (casein micelles) are destabilized by the action of rennet. The proteolytic enzyme in rennet, chymosin, cleaves a specific bond of the κ-casein protein on the surface of the micelle and removes the stabilizing "hairy" layer composed of the negatively charged C termini of the protein [caseinomacropeptide (CMP)]. In unheated milk, at its natural pH of 6.7, the casein micelles begin to aggregate only when about 90% of the κ-casein has been hydrolyzed[1] and the onset of gelation requires nearly 95% cleavage.[2] However, in milk that has been heated to over 75 °C, the action of chymosin on κ-casein does not lead to efficient aggregation of casein micelles, so that the curd forms poorly and is not suitable for traditional cheese-making.

Heating milk at temperatures exceeding 75 °C denatures the whey proteins (WP) β-lactoglobulin and α-lactalbumin, which then either bind covalently to κ-casein on the micelle surface or form soluble complexes in the serum, which are composed of the WP and some κ-casein removed from the surfaces of casein micelles; the relative amounts of micelle-bound and soluble WP/κ-casein complexes depend upon the pH at which the milk was heated.[3−6] The soluble WP/κ-casein complexes are approximately spherical in shape and measure between 30 and 70 nm in size depending upon the technique of measurement used.[7−9] The κ-casein associated with serum heat-induced complexes can be completely hydrolyzed by chymosin, but the renneted complexes do not coagulate.[10] It has been suggested that the impairment of rennet clotting in heated milk can be attributed to the binding of heat-denatured WP to the surfaces of casein micelles; this can cause steric hindrance to the aggregation of micelles, although the precise mechanism is not well-defined. It appears to be generally agreed that the effect of heating does not significantly alter the behavior of rennet in the proteolytic phase of the clotting process but rather that the coagulation itself is impaired.[11−13]

Recently,[5] we have studied some details of the impaired rennet clotting of heat-treated milk using reconstituted skim milk powder (RSMP). A series of control "milks" were prepared by exchanging the serum and micellar fractions from heated and unheated RSMP. Rheological measurements showed that the heat-induced soluble complexes of WP and κ-casein can interfere with the enzymatic clotting of a suspension of the native casein micelles from unheated RSMP dispersed in the serum of heated milk. The clotting power was mostly restored by dialyzing this serum against a large excess of unheated milk, to restore the native equilibrium of milk salts, mainly calcium. On the other hand, suspensions of casein micelles from heated milk in the serum of unheated milk clotted poorly, although slightly better than heated milk itself. The impaired rennet clotting properties of heat-treated RSMP appeared to arise from a synergistic effect between three individual milk components, namely, (i) the casein micelles with their heat-modified surfaces, (ii) the WP/κ-casein complexes in the serum, and (iii) other dialyzable components in the serum. The direct role of soluble rather than micelle-bound WP/κ-casein complexes on impaired rennet clotting of heat-treated milk was demonstrated in this way for the first time. The aim of the present study was to examine the interactions between the soluble WP/κ-casein complexes and the casein micelles during the renneting of heat-treated RSMP.

MATERIALS AND METHODS

Reconstitution of Skim Milk Powder. Low heat skim milk powder was obtained from Gay Lea Foods, Guelph, Ontario, Canada. During the manufacture of this powder, the skim milk was heated at

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75 °C for about 17−18 s and the cutoff temperature before drying was 72 °C. The compositional analysis of this low heat SMP by the manufacturer reports a whey protein nitrogen index (WPNI) value of 6.5−7.0 g of WPN g^{-1} of powder, which is close to the value (7.0 g of WPN g^{-1}) generally measured in fresh milk.[14−16]

The RSMP was prepared by dispersing the powder in Milli-Q water to a final solids content of 11% (w/w). Sodium azide (0.02%) was added as a preservative. The mixture was stirred for 1 h at room temperature, and the reconstituted skim milk powder was left at 4 °C to equilibrate for at least 18 h.

The present study is part of a continuing series on the rennet clotting of heated milk systems. To avoid problems of variation in the composition of fresh milk over extended periods, we used the same batch of low-heat SMP as the raw material throughout the experiments.

Heat Treatment and pH Adjustment. The RSMP samples were heated in a water bath at 90 °C for 10 min in 15 mL aliquots in unstirred test tubes, followed by rapid cooling to room temperature in an ice bath. A 2.5 min temperature come up time was allowed prior to the holding time.

Some experiments required the pH of RSMP samples to be altered from their original pH of 6.7 to pH values of 6.3 or 7.1 before heating the milk. Either 1 M HCl or NaOH was added dropwise to the RSMP with gentle agitation until the required pH had been attained, and the milk samples were allowed to equilibrate with stirring for 2 h. The pH-adjusted samples were then heated at 90 °C, as described above. After the heating step, the pH of these heat-treated RSMP samples was restored to the natural value of 6.7 before any further experimentation. This readjustment of pH has been shown not to cause changes in the distribution of WP/κ-casein between the solution and the micellar surface.[11]

Preparation of Casein Micelle Suspensions in Different Sera. In addition to the RSMP and heated RSMP samples, suspensions of native casein micelles in the sera of heated milk were prepared. For this, unheated and heated samples of RSMP (60 g) were centrifuged (31300*g*, 1 h, 20 °C) using an Optima LE-80K ultracentrifuge (Beckman Coulter Canada, Inc., Mississauga, Ontario, Canada). The sera from the milk samples were separated from the micellar pellets by decantation. The pelleted casein micelles from the unheated RSMP were then redispersed in the serum of the heated milk using the method described by Alexander and Dalgleish.[17] In separate experiments, the serum from heated milk was dialyzed (24 h at 4 °C) against unheated RSMP at a volume ratio of 1:40 before the micelles from the unheated RSMP were added; a Spectra/Por dialysis membrane tubing with a molecular weight cut off of 6000 to 8000 Da was used (Spectrum Laboratories, Inc., Rancho Dominguez, CA).

Similar mixtures were made by suspending the casein micelles from RSMP heated at pH 6.3 in the serum of RSMP that had been heated at its normal pH.

Enzyme Treatment. The milk samples to be renneted were all at pH 6.7, even if heating had previously taken place at a different pH. After preparation, the samples were maintained at a temperature of 30 °C for at least 30 min and were then treated with recombinant chymosin [CHY-MAX ultra, 760−790 International Milk Clotting Units (IMCU) mL^{-1}, Chr. Hansen Company, Milwaukee, WI] to a final enzyme concentration of 0.002% (v/v). All samples were treated with this same amount of rennet even if they differed in the concentration of the substrate, i.e., κ-casein. The present study is based on our previous work,[5] and to allow for meaningful comparisons between treatments, we used similar levels of rennet in all samples.

CMP Release and Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC). The release of CMP during renneting was quantified using RP-HPLC according to established methods,[18,19] after some modification. Trichloroacetic acid (TCA, 4%) was added at preset time intervals to aliquots of a rennet-treated sample of RSMP (30 °C) to give a final TCA concentration of 2%. This precipitates the proteins and leaves the CMP in solution. After centrifugation at 4500*g* for 15 min (Eppendorf centrifuge 5415D, Brinkman Instruments, Ltd., Mississauga, Ontario, Canada) and filtration through 0.22 μm pore size filters, the CMP contained in the clear supernatant was quantified using RP-HPLC using a Finnigan Spectra System LC unit (ThermoFinnigan, Burlington, Ontario, Canada) equipped with a UV detector set at 210 nm. A column (μRPC C2/C18 ST 4.6/100, Pharmacia Biotech, Piscataway, NJ) with a 100 μL sample loop was used at a running temperature of 40 °C. A nonlinear gradient was run between solvent A [0.1% (v/v) trifluoroacetic acid (TFA) in water] and solvent B [90:10:1 (v/v/v) acetonitrile/water/TFA]. The total peak area of the CMP was integrated using ChromQuest, version 4.1, software (ThermoFinnigan, Burlington, Ontario, Canada) from approximately 20 to 40 min on the chromatogram. Results were expressed as a percentage of total CMP released once the production of CMP had ceased (3 h for unheated milk and 4 h for heated milk).

The activity of rennet in milk, measured by the rate at which CMP is released, can be closely approximated by first-order reaction kinetics.[20] Accordingly, the first-order rate constant k for each treatment was computed by fitting the measurements of CMP release against time to a reaction of this type. This allows for a comparison of the susceptibility of κ-casein to attack by chymosin in the different mixtures, despite the fact that the enzyme/substrate ratio is different.

Size-Exclusion Chromatography (SEC). After the addition of rennet to RSMP samples (60 mL aliquots in beakers), the reaction was allowed to proceed at 30 °C for times of 15, 30, 45, 60, 75, or 90 min. At the end of each of these times, the chymosin in the sample was inactivated by the addition of a solution of pepstatin A (Sigma-Aldrich Company, St. Louis, MO) containing 5 mg of the protease inhibitor in 7.6 mL of absolute ethanol (dissolved at 60 °C); 1.2 mL of this pepstatin solution was added to 60 mL of the rennet-treated sample, i.e., at a volume ratio of 1:50. This small dilution with ethanol (final concentration of <2%) is unlikely to affect the behavior of the micelles in the milk,[21,22] and quantification of CMP in pepstatin-treated milk following a further incubation for 2 h at 30 °C confirmed that pepstatin A completely inactivated the protease.

The micelle−serum suspensions renneted in this way for different lengths of time were then centrifuged (31300*g*, 1 h, 20 °C) to remove the micellar fraction, and the supernatant sera were analyzed for their content of WP/κ-casein complexes using SEC.[4] A Pharmacia XK 16/70 column with a packed bed height of 68 cm was used. The packing material was S-500 Sephacryl high-resolution gel, with a nominal fractionation range of 40−20 000 kDa (Amersham Biosciences, Inc., Baied'Urfé, Quebec, Canada). Eluted peaks were detected by absorption at 280 nm. Samples (1 mL) were loaded onto the column and eluted at a flow rate of 1 mL min^{-1}, with a mobile phase of 20 mM Bis-Tris propane (pH 7.0) containing 0.02% sodium azide. The total elution time for each sample was 180 min.

Peak areas were measured using a numerical integration routine created in Microsoft Excel.

Statistical Analysis. The percentages of CMP released were expressed as averages of at least three independent replicates, except only for the RSMP heated at pH 7.1, where only two replicates were made. For each treatment, the mean first-order rate constant, k, was calculated and the significance of effects ($\alpha < 0.05$) was determined using analysis of variance (ANOVA) and paired t test (SAS 9.1.3, SAS Institute, Inc.). The SEC peak areas were averages of three independent repetitions. ANOVA and paired t test were also used to test for significant differences ($\alpha < 0.05$) in SEC peak areas obtained at different times of renneting within a treatment as well as between the treatments of interest (i.e., WP/κ-casein complexes in the presence or absence of native casein micelles).

RESULTS AND DISCUSSION

CMP Release and RP-HPLC. The CMP released in the course of renneting was quantified for the unheated and heated RSMP, the serum fraction of heated RSMP (HS), and the milk containing

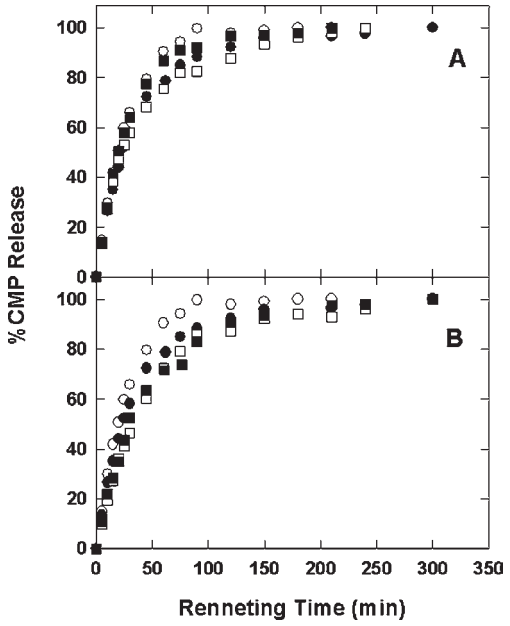

Figure 1. Amount of CMP released as a function of time after rennet addition, expressed as a percentage of the total available κ-casein in the different samples: (A) (○) unheated RSMP, (●) heated RSMP, (□) serum of heated RSMP (HS), and (■) unheated micelles dispersed in HS and (B) (○) unheated RSMP, (●) RSMP heated at natural pH, (□) RSMP heated at pH 7.1, and (■) micelles from milk heated at pH 6.3 dispersed in HS.

unheated casein micelles dispersed in HS (Figure 1A). A comparison of the mean first-order rate constants, k (range of 0.028−0.039 s^{-1}), of all of these treatments showed no significant differences ($\alpha = 0.18$); a further pairwise comparison of unheated and heated RSMP was also not significant ($\alpha = 0.056$). However, there was a significant decrease ($\alpha = 0.001$) in the rate of CMP release when the milks had been heated at pH 7.1 or 6.3 (Figure 1B). This differs from the observation by Anema and coworkers,[11] who found no significant effect of heating milk at pH values from 6.5 to 7.1 on the rate of enzymatic hydrolysis. When milk is heated at its natural pH, the literature reports either no significant effect[11] or only a marginal effect[12,13] of heating on the activity of rennet. It has also been demonstrated that this small decrease in enzymatic activity (rate of CMP release) is not the reason for the poor rennet clotting properties of heated milk, and impairment in clotting was mainly attributed to the heat-induced binding of denatured WPs to the surfaces of casein micelles.[13] It is important to note that the κ-casein in WP/κ-casein complexes is as susceptible to the action of chymosin as the κ-casein in the casein micelles. These results agree with those by Mollé et al.[10]

Interaction between Casein Micelles and WP/κ-Casein Complexes during Renneting. A typical SEC profile of the serum obtained from the mixture containing the casein micellar pellet from unheated RSMP (UP) suspended in the serum of heated RSMP (HS) is shown in Figure 2. Five peaks appear in the chromatogram. It has previously been shown[8,9,23−26] that peak 1 is composed of nonprotein material, peak 2 contains the soluble WP/κ-casein complexes produced during the heating of the milk, peak 3 is a mixture of native whey proteins and monomeric caseins, and peaks 4 and 5 contain small molecules of nonprotein material. The centrifugation conditions used to separate the serum and micellar fractions have been shown to completely

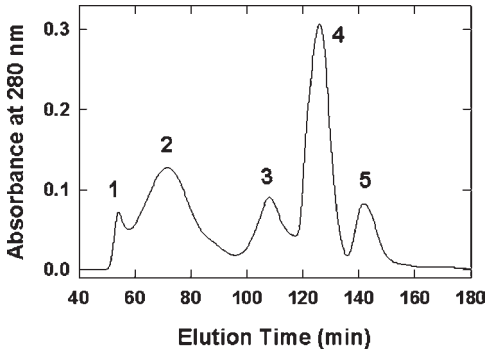

Figure 2. Typical elution profile obtained by SEC. The sample was the serum fraction from UP/HS, a suspension of native casein micelles from unheated RSMP in the serum of heated RSMP.

exclude casein micelles from the serum fraction.[9,24] It is the behavior of peak 2 that is of importance in our experiments. All of the other peaks remain the same during renneting, but peak 2 was seen to change in some samples, as described below.

The sera from heated RSMP, undialyzed (HS), or dialyzed against unheated RSMP (HSD), without the addition of the micellar fraction, were treated with rennet and analyzed by SEC. Although these sera contain only about a third of the total κ-casein present in the original RSMP, they were treated with similar amounts of rennet (0.002%, v/v) as the RSMP. In HS, renneting of the protein complexes showed only a small loss of peak 2 in the chromatogram (Figure 3A) even at the end of 75 min of rennet reaction, and this small decrease was not statistically significant ($\alpha = 0.18$). The integrated area of the peak is shown as a function of the extent of κ-casein breakdown in Figure 3C, where it can be seen that, even when around 85% of CMP has been released, there is very little loss of the complexes. Self-aggregation of the complexes would result in a diminution of peak 2 in the chromatogram, because aggregated particles would be removed from the serum by centrifugation during preparation of the sample for chromatography, or, if they aggregated only slightly, the peak would shift to shorter elution times. Thus, it was evident that the WP/κ-casein complexes in the heated serum did not self-aggregate during the length of the renneting experiment. Even after extended periods of enzyme reaction (≥95% CMP release), i.e., up to 3 h after rennet addition, the HS showed no visible signs of clotting or turbidity (results not shown). Similar observations on the chymosin sensitivity of WP/κ-casein complexes and their inability to clot have been reported by other authors,[10] but under different experimental conditions (temperature of 37 °C and more than 5 times the enzyme concentration used here).

When the HS fraction was dialyzed against unheated RSMP before being renneted (Figure 4A), a somewhat greater decrease in peak 2 occurred than in the undialyzed HS sample. The relative decrease was 15 ± 5% when about 85% of the κ-casein had been destroyed, 90 min after rennet addition ($\alpha < 0.0001$). The aggregation of the renneted WP/κ-casein complexes is obviously improved by the dialysis but remains low even at the longest renneting times used.

In contrast to the behavior of the isolated WP/κ-casein complexes, when a dispersion of unheated casein micelles in heated serum (UP/HS) was renneted (Figure 3B), peak 2 was seen to progressively decrease in area over the course of renneting from 0 to 90 min. The decrease was significant overall and in all pairwise comparisons; α ranged between 0.0006 and 0.042.

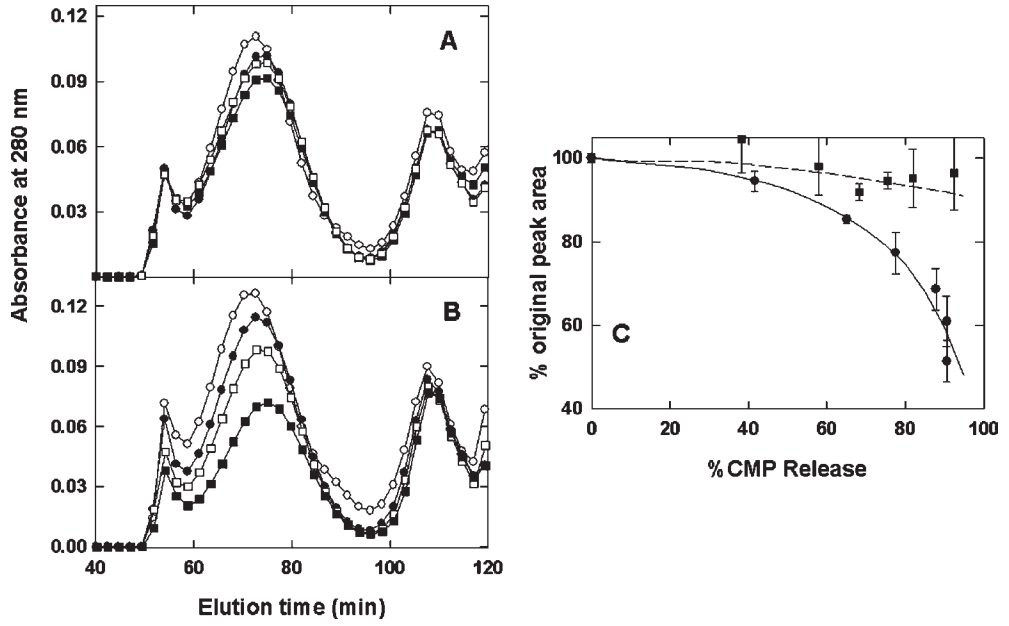

Figure 3. WP/κ-casein complex peak in the elution profile obtained by SEC. (A) Serum of heated milk (HS) during renneting and (B) centrifugal supernatant obtained from a renneted suspension of native casein micelles from unheated RSMP in HS (UP/HS). Renneting times were (○) 0 min, (●) 30 min, (□) 60 min, and (■) 75 min. (C) Relative area of the WP/κ-casein complex peak plotted as a function of the percentage of CMP released during renneting for (●) UPHS and (■) HS.

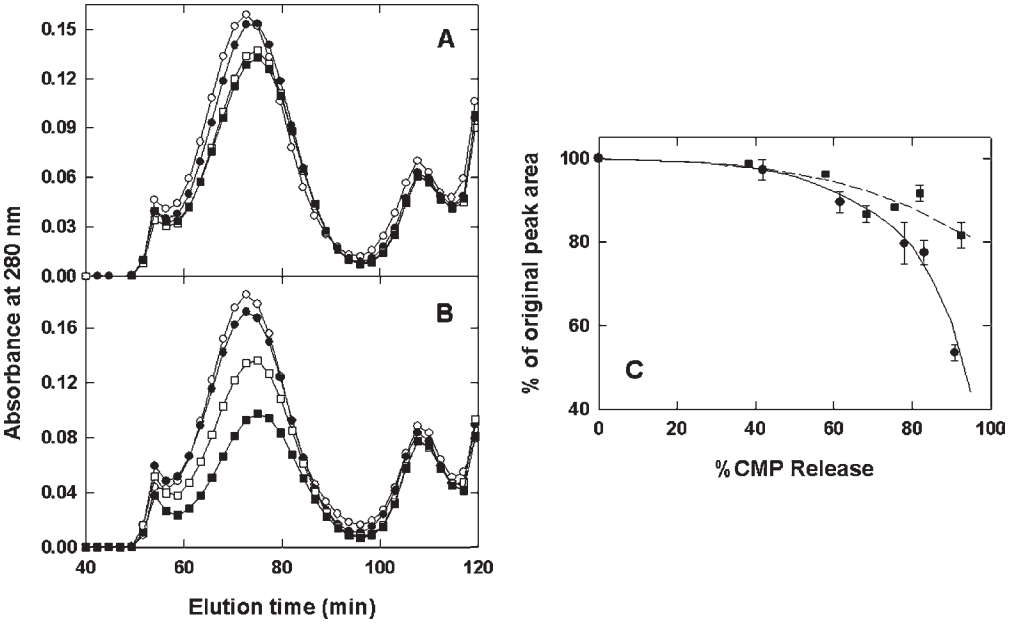

Figure 4. (A) WP/κ-casein complex peak of HSD, the serum of heated RSMP that had been dialyzed (4 °C, 24 h) against unheated RSMP and (B) WP/κ-casein complex peak of the centrifugal supernatant obtained from UP/HSD, a suspension of native casein micelles from unheated RSMP in HSD. Renneting times were (○) 0 min, (●) 30 min, (□) 60 min, and (■) 75 min. (C) Relative area of the WP/κ-casein complex peak plotted as a function of the percentage of CMP released during renneting for (●) UPHSD and (■) HSD.

Results obtained beyond 75 min are not presented because they became unreliable as a result of strong curd formation, which made extraction of the serum harder to reproduce. The rheological clotting time (defined as the time when the phase angle δ equals 45°) of the UP/HS suspension was known to be 80−85 min in these samples.[5] Although 90% of the CMP in UP/HS has been released in about 70−75 min, the micelles did not clot until after 80 min of renneting. Measurement of the peak areas (Figure 3C) showed that the loss of WP/κ-casein complexes from the serum was not linear with the extent of release of the CMP; it was slow until about 60% of the κ-casein had been destroyed, after which the peak size decreased rapidly.

The differences in the peak areas between UP/HS and HS were significant at 60 min ($\alpha = 0.005$) and 75 min ($\alpha = 0.01$) of renneting but did not show any significant difference in the first 30 min after rennet addition ($\alpha = 0.245$).

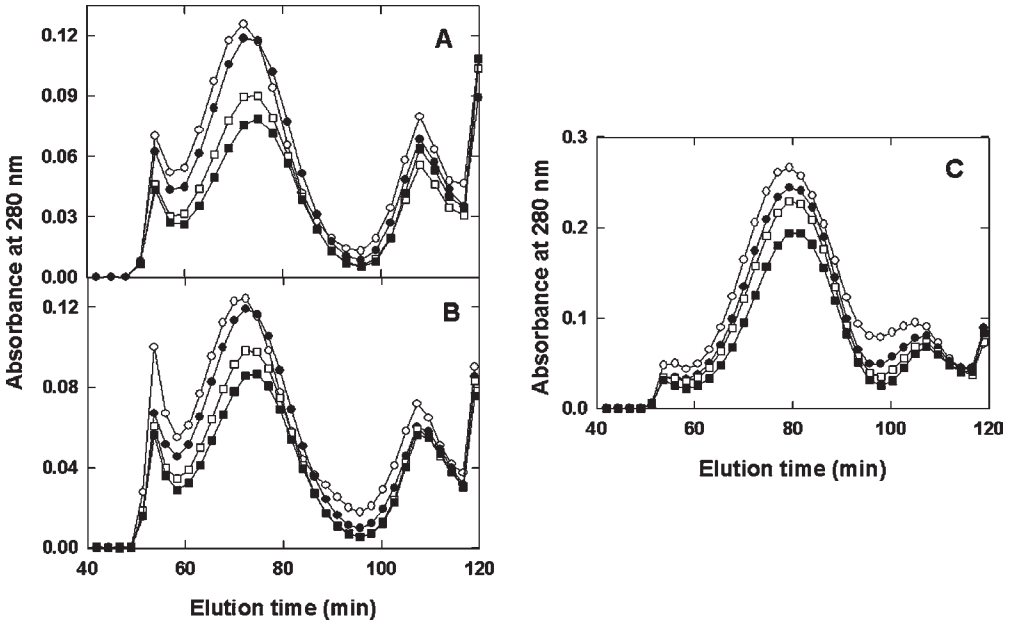

Figure 5. WP/κ-casein complex peak of the centrifugal supernatants of (A) RSMP heated at native pH of 6.7, (B) micelles from RSMP heated at pH 6.3 suspended in serum of RSMP heated at pH 6.7, and (C) RSMP heated at pH 7.1. Renneting times were (○) 0 min, (●) 30 min, (□) 60 min, and (■) 75 min.

A similar trend was observed when unheated casein micelles were suspended in HSD, the serum that had been previously dialyzed against unheated milk (Figure 4B); the dialysis was performed against an excess of unheated RSMP primarily to restore the ionic equilibrium and should not affect the protein-based components. Even in the UP/HSD suspension, the decrease in peak 2 with renneting time showed overall significance ($\alpha < 0.0001$) and significance between all of the time pairs that were compared (α ranged from <0.0001 to 0.0013). In both cases, whether the HS was dialyzed or not, the peak area decreased by about 30 ± 5% at about 85% κ-casein cleavage, i.e., nearly 60 min after rennet addition (Figures 3C and 4C). Near the time (around 70−75 min in UP/HSD but even later in UP/HS) when micelles began to clot, i.e., after about 90% of the CMP had been released, the peak area had decreased by 50 ± 4% in the two samples. It is evident, therefore, that, in the presence of casein micelles, some reaction is causing the WP/κ-casein complexes to disappear from the solution and that this reaction is related to the extent of renneting of the system.

Again, the differences in the peak areas between UP/HSD and HSD were significant at 60 min ($\alpha = 0.018$) and 75 min ($\alpha < 0.0001$) of renneting but did not show any significant difference in the first 30 min after rennet addition ($\alpha = 0.086$).

The gradual loss of WP/κ-casein complexes from the serum during renneting of UP/HS can most likely be attributed to their binding to the casein micelles, possibly via hydrophobic interactions between the exposed *para*-κ-casein residues on the surfaces of the casein micelles and of the complexes. The disappearance of the complexes starts well before the onset of clotting of the casein micelles; therefore, it does not appear to result from simple entrapment of the complexes by the curd formed by the coagulating micelles. Renneting of HSD alone caused a more significant loss of WP/κ-casein complexes (15 ± 5% after 90 min) than the renneting of HS, but the decrease was much less than in the suspension UP/HSD when casein micelles were present (Figure 4C). We conclude that, as the breakdown of κ-casein on the micellar surface and in the WP/κ-casein complexes increases, the tendency for the complexes to bind to the micellar surface also increases. Thus, as the density of the hairy layer on the casein micelle surface decreases and small gaps appear in it, the small complexes can find their way through the surface layer and bind to the denuded regions of the micellar surface.

We have previously found that simply dialyzing the serum of heated milk against unheated milk mostly restored the rennet coagulation of native casein micelles suspended in this serum.[5] This beneficial effect of serum dialysis was not simply the result of restored serum ions, because casein micelles were found to clot well in the permeate of heated milk, which was completely devoid of serum protein complexes but which had its native ionic equilibrium altered by heat. The results described above show that dialyzing the complex-containing serum of heated milk against unheated milk does not greatly alter the amount of WP/κ-casein complexes binding to the native casein micelles during renneting. Therefore, the improved enzymatic clotting seen in the previous work when native casein micelles were dispersed in HSD cannot be a result of different extents of interactions between the casein micelles and WP/κ-casein complexes. It therefore becomes questionable whether the binding of WP/κ-casein complexes to the micellar surface creates sufficient steric effects to cause impaired rennet clotting of heat-altered casein micelles. Alternatively, the WP/κ-casein complexes formed on the casein micellar surface during heating may not be the same as those released into the serum, so that there is a difference between the surfaces of heat-modified casein micelles and the casein micelles in our experiments, where the surface is modified by the addition of the complexes during renneting.

Interaction of WP/κ-Casein Complexes with Casein Micelles Heated at Different pH Values. It is well-established that heating milk at pH values lower or higher than its natural value of 6.7 causes different extents of binding of WP/κ-casein complexes with the casein micelle.[3,4,6,25] However, the detailed amounts of the complex bound at a defined pH differ between researchers. Our previous study[5] using this milk powder showed that casein micelles from the RSMP that we used, heated at 90 °C

for 10 min at its natural pH, have approximately 30% of the original WP bound to the casein micelle, while casein micelles from RSMP heated at pH 6.3 have about 80% of the total WP bound to them, and those from RSMP heated at pH 7.1 have nearly complex-free surfaces (≤5% of total WP). To study whether all of these types of casein micelles with different amounts of surface-bound WP were capable of binding even more WP/κ-casein complexes during renneting, RSMPs heated at pH 6.7 or 7.1 (in the latter case, pH being brought back to 6.7 before renneting) were treated with rennet similarly to the UP/HS mixtures and the loss of complexes from the serum was measured by SEC. The renneting of the RSMP heated at pH 6.3 could not be performed so simply, because its serum contains only small amounts of free WP/κ-casein complexes; therefore, micelles from the RSMP heated at pH 6.3 were isolated by centrifugation and were resuspended in the serum of milk heated at pH 6.7 (HS). All of these heated milk systems showed similar trends of decreasing amounts of WP/κ-casein complexes as renneting proceeded (Figure 5). No matter what the initial load of WP/κ-casein complexes on the micelle surface was, all micelle types seemed capable of binding more serum protein complexes in the course of the reaction with rennet. Thus, even the casein micelles from milk heated at pH 6.3, which already possess a large amount of bound WP, are capable of binding more. Indeed, it appeared that the casein micelles from milks heated at pH 6.3 and 6.7 absorbed the same amount of whey protein. On the other hand, the casein micelles from milk heated at pH 7.1 appeared to interact with the serum WP/κ-casein complexes to a greater extent than those from the milks heated at pH 6.7 or 6.3, as might be expected because the casein micelles heated at that pH have very low coverages of WP.[3−5]

Previously, the impairment of rennet clotting of native casein micelles suspended in the serum of heated milk was partly attributed to the presence of heat-induced WP/κ-casein complexes in the serum.[5] Our present results show that the serum protein complexes can bind to the surfaces of native casein micelles during renneting. In addition, we have shown that this binding process of WP/κ-casein complexes to casein micelles from unheated milk seems to be unaffected by the change from undialyzed (HS) to dialyzed serum (HSD) as the suspending medium. However, it was shown in our previous study that the clotting of native casein micelles can be almost completely restored when suspended in HSD.[5] This raises the question as to whether steric hindrance from micelle-bound WPs is the main cause of impaired rennet clotting of casein micelles in heated milk. It was also interesting to note that the serum protein complexes can bind to the surfaces of renneted casein micelles independent of the amount of WP already bound to the micelle, as was found in milks heated at pH values of 6.7, 7.1, or 6.3. It remains to be determined what importance these interactions between casein micelles and WP/κ-casein complexes have on the overall renneting behavior of heated milk systems.

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