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Investigation of the Colloidal Interactions at Play in Combined Acidification and Rennet of Different Heat-Treated Milks

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The properties of gels obtained by combined acidification and rennet were investigated for milks heated at different temperature−time regimes using a high temperature short time (HTST) pilot plant system. Increasing amounts of heat-induced whey protein complexes were found in the soluble phase as a function of heating time/temperature, and only in the most extensively heated milk (i.e., 85 °C/300 s), these complexes were in quantities comparable to those reported in previous studies. Two levels of rennet were studied, and at the gelation pH, the amount of CMP released was 11 ± 1% and 26 ± 5% in the low and high rennet experiments, respectively. These two levels of rennet caused profound changes in the gelation behavior and in the structure development of the network. When a small amount of rennet was used, different heating temperature−time regimes did not affect the first stage of renneting. Increasing the extent of milk heat treatment and/or the level of rennet increased the pH of gelation and the stiffness of the gels. This work is the first to compare the effect of heating (using a pilot plant setup) and amount of rennet on the destabilization and interaction of casein micelles during aggregation by combined rennet and acidification.

KEYWORDS: Rennet milk gels; acid milk gels; dairy gels; diffusing wave spectroscopy; heat-induced interactions

INTRODUCTION

Heat treatment of milk is a necessary processing step in the dairy industry to destroy microorganisms and inactivate spoilage enzymes. Temperatures above 70 °C cause whey protein denaturation. The denatured whey proteins have increased susceptibility to association reactions, and the formation of disulfide-linked complexes between β-lactoglobulin and κ-casein is of technological importance (*1*). Industrial heat treatment is typically carried out with heating regimens involving rapid heat transfer and the use of shear (e.g., HTST), and these processes greatly differ from batch heating in the laboratory in terms of the length of heat treatment, the rates of heating and cooling, and the presence of shear.

Many dairy products rely on the destabilization of the casein micelle through acidification and/or renneting. Both processes reduce the steric and electrostatic stability of the casein micelle through elimination or charge neutralization of the hairy polyelectrolyte layer of κ-casein that exists on the micellar surface (*2*), albeit through different mechanisms. Through titration of the negative charges in the κ-casein hairy layer, acidification reduces the electrostatic repulsion and causes the hairy layer to collapse onto the micelle, subsequently eliminating steric repulsion and leading to micelle aggregation at pH ∼4.9 (*3*). Alternatively, in rennet coagulation, the stability of the casein micelle is reduced by enzymatic cleavage of the $Phe_{105}-Met_{106}$ bond in κ-casein, which releases the hydrophilic caseinomacropeptide (CMP) moiety into the serum, leaving a hydrophobic patch on the surface of the casein micelle (*4*). At the natural pH of milk, cleavage of 85−90% of the CMP significantly reduces steric repulsion and the destabilized micelles aggregate to form a particle gel (*4*, *5*).

Heat treatment also affects the two destabilization processes differently. Soluble heat-induced whey protein complexes associate with the micelle upon acidification (*1*,*6*,*7*) and the interaction results in the formation of an acid gel at higher pH, with increased gel stiffness and reduced syneresis (*3*, *7*). Alternatively, soluble complexes are able to be renneted and to associate with the casein micelles (*8*, *9*). In contrast to acid-gelation, the associated complexes sterically hinder the aggregation of renneted micelles, causing longer gelation times and reduced gel stiffness (*10*).

Although there have been great advances in our understanding of the effects of heat treatment on rennet and acid-induced coagulation of milk, when taken as separate reactions, much less is understood about how heat treatment affects gelation when milk is subjected to combined acidification and rennet, which is very often employed in cheese production. In combined gelation, the micelles are destabilized to levels of κ-casein hydrolysis and pH insufficient to independently cause micelle aggregation, and the resulting gels show increased stiffness (*11*−*14*). Milk heat treatment has been shown to reduce this trend in prerenneted acid gels (*12*).

Previous reports on combination gels have described the rheological behavior of the gels (*11*−*14*). Using diffusing wave spectroscopy (DWS), it is possible to better describe the pregelation behavior of the milk treated with different amounts of rennet during acidification. This has been previously shown using a back scattering geometry (*15*). The use of transmission DWS will provide additional insight into the behavior of micelles during

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destabilization and enhance our understanding of these complex systems. The objective of this research was to characterize the interactions in milk which lead to the aggregation of casein micelles using DWS, rheology, and microscopy. Milk with a different processing history and two (low) levels of rennet were employed in this study.

MATERIALS AND METHODS

Heating of Milk. Bulk skim milk was obtained from Gay Lea Foods (Guelph, ON, Canada), and sodium azide (0.02% w/w) was added to inhibit bacterial growth. A portion of the milk was heat-treated with pilot plant HTST equipment (Microthermics, Raleigh, NC, USA). Heat treatments included 74 °C, 80 °C, 85 °C, and 90 °C for 20 s (denoted 74 °C/20 s, 80 °C/20 s, 85 °C/20 s, and 90 °C/20 s, respectively) and 80 and 85 °C for 300 s (denoted 80 °C/300 s and 85 °C/300 s, respectively). The preheat temperature was 50 °C, and the homogenization pressure was 1000 psi with back pressure of ~100 psi. The flow rate was 1 L/min. The heated milk, which exited the system at ~20 °C, was collected and equilibrated for 2 h at room temperature. The pH of the unheated and heated milks was also measured.

As the changes induced by increasing the extent of heating were gradual, results from only some of the treatments are presented for clarity in the figures.

Characterization of Heat-Induced Soluble Complexes. To separate the soluble and colloidal materials, the heated and unheated milks were centrifuged at 25000*g* for 1 h (*16*) at 20 °C in a Beckman Coulter Optima LE-80K ultracentrifuge, with rotor type 70.1 Ti (Beckman Coulter Canada Inc., Mississauga, ON, Canada). The supernatant was carefully removed with a syringe and filtered using a 0.45 μm filter (Millipore Corporation, Bedford, MA, USA). Supernatants were stored at refrigeration temperatures (4 °C) until time of analysis. The supernatants of the heated and unheated milks were analyzed by size exclusion chromatography (SEC) using an AKTA system (GE Healthcare Biosciences, Uppsala, Sweden) equipped with a 1.0 mL sample loop. A Pharmacia XK 16/70 column (Piscataway, NJ, USA) was used, with a packed bed height of 67 cm. The packing material was S-500 Sephacryl high-resolution gel (Amersham Biosciences Inc., Baie d'Urfe, QC, Canada). The mobile phase was 20 mM bis-Tris-propane at a pH of 7.0 with 0.2 g/L of sodium azide. The flow rate was 1 mL/min, and the total elution time per sample was 180 min. Eluted peaks were detected at 280 nm. Fractions of 5 mL were collected for analysis by electrophoresis.

Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was performed using a Bio-Rad electrophoresis unit (Bio-Rad Laboratories, Hercules, CA, USA). Samples collected from SEC were concentrated 10× using a Centrivap cold trap (Labconco, Kansas City, MO, USA) for ~3.5 h at 60 °C. The concentrated SEC samples and samples of the centrifugal supernatants were diluted (1:2 ratio) with sample buffer (0.5 M Tris-HCl, pH 6.8, 20 g/kg SDS, 190 g/kg glycerol, 0.5 g/kg β-mercaptoethanol, and 0.1 g/kg bromophenol blue) and heated for 5 min at 95 °C. Similar samples were analyzed in nonreducing conditions with sample buffer that did not contain β-mercaptoethanol to determine the involvement of disulfide-linkages.

The resolving gel contained 18% acrylamide in 0.75 M Tris-HCl at pH 8.9, and the stacking gel contained 4% acrylamide in 0.1 M Tris-phosphate buffer at pH 6.7. The electrophoresis buffer was 0.7 M Tris-HCl and 0.45 M glycine at pH 8.3. The volume of sample loaded onto the gels was 5 and 7 μL for the centrifugal supernatant and concentrated SEC samples, respectively. The electrophoretic separation was performed at 200 V for 40 min.

The gels were stained with Coomassie blue in 50% (v/v) methanol and 10% (v/v) acetic acid for 40 min with shaking. Destaining was performed for 1 h with a 45% (v/v) methanol and 10% (v/v) acetic acid solution and then for 12 h with a 22.5% (v/v) methanol and 5% (v/v) acetic acid solution. The scanned (Sharp JX-330 scanner, Pharmacia Biotech) gels were analyzed qualitatively for protein composition.

Gelation by Simultaneous Acidification and Rennet. Heated milk was gelled by a combination of glucono-δ-lactone (GDL, 1.5% w/v) and rennet. Rennet (Chymostar, Danisco, Cranberry, NJ, USA) was added to milk in a ratio of 0.5 and 2 μL diluted rennet per mL of milk, yielding a final rennet concentration of 3.14×10^{-4} international milk clotting units (IMCU) per mL and 1.26×10^{-3} IMCU/mL for low and high rennet concentrations, respectively. The level of rennet in the low-rennet treatment is similar to that used for cottage cheese manufacture (*17*). Control experiments were also carried out using only 1.5% GDL. The acidification kinetics of GDL did not vary with heating treatment (results not shown). A control treatment of milk gelled by only rennet was not included because the activity of rennet is pH-dependent (*18*); instead, the amount of released caseinomacropeptide, CMP, was measured.

Rennet and GDL were added to milk at 30 °C within 5 s of each other. The mixture was stirred for 15 s. Acidification was quantified by recording the pH every 10 s over the gelation period. Plots of the measured pH against time were analyzed by curve-fitting using Sigmaplot 10.0 (SPSS, Chicago, IL, USA) so that it was subsequently possible to interpolate the pH for all of the experimentally measured points for each of the acidification experiments.

The release of CMP was monitored using established methods (*19*, *20*). Renneted milk (4 mL) was pipetted to several test tubes in a water bath maintained at 30 °C. The rennet reaction was stopped at specific time intervals by the addition of 4 mL of 4% trichloracetic acid with subsequent vortexing. After overnight refrigerated storage, the supernatant was collected after centrifugation at 4500*g* (Beckman Coulter Canada Inc., Mississauga, ON, Canada) for 15 min, filtered through a 0.45 μm filter (Millipore Corporation, Bedford, MA, USA), and analyzed by reversed phase HPLC, using a system with a degasser, pump, autosampler, and UV detector set to 210 nm (ThermoFinnigan, Burlington, ON, Canada). The sample injection volume was 100 μL, and the oven temperature was 40 °C. The column was Pharmacia Biotech μRPC C2/C18 ST 4.6/100 (Piscataway, NJ, USA). The mobile phase consisted of a nonlinear gradient of 0.1% TFA in water and 0.1% TFA in 90% acetonitrile. The total peak area between about 7 and 23 min on the chromatograms was integrated using ChromQuest software v. 4.1 (ThermoFinnigan, Burlington, ON, Canada) and compared to the maximum peak area for unheated milk with 0.0351 IMCU/mL rennet. Two replicates were performed for each test.

Confocal laser scanning microscopy was carried out to characterize the gel microstructure. Fluorescein isothiocyanate (FITC, 0.5% w/v in water) was added to heated milk in a proportion of 4 μL/mL milk. Immediately after the addition of the gelling agents, a small volume was applied to a concave slide and covered with a coverslip which was sealed to prevent evaporation. The slides were placed in a water bath at 30 °C and viewed after 4.5 h, corresponding to ~pH 4.6, using a Leica upright CLSM (TCS SP2, Leica Microsystems, Heidelberg, Germany) with a 63× oil immersion objective lens. The gel structure was visualized in both fluorescence and reflectance modes. Images of 1024 × 1024 formats were collected with software (LCS, version 2.61, Leica Microsystems, Heidelberg, Germany) as the average of two consecutive scans. The speed of scanning was 400 Hz, and the pinhole was 122 μm. Several images were collected for each sample, and representative images are presented. Images presented in this article are from reflectance mode.

The development of the gel was followed using a controlled stress rheometer (AR-1000, TA Instruments, New Castle, DE, USA). Milk samples (20 mL) were loaded into the concentric cylinder geometry within 5 min of addition of the gelling agents. The temperature was maintained at 30 °C by means of an external water bath. A time sweep at 0.01 strain and 1 Hz frequency was performed for 4.5 h. At the end of the time sweep, the pH of the sample was ~4.6. The gelation pH was determined as the point when the viscous and elastic components were equal. This point usually coincides with a rapid increase in the storage modulus (G'). The value of G' 4.5 h after the addition of gelling agents was compared to determine differences in gel stiffness.

The gelation process was also monitored by transmission DWS as previously described (*6*, *21*, *22*). Samples of heated milk mixed with rennet and acid were transferred to an optical glass cuvette with 5 mm path length (Hellma Canada Limited, Concord, ON, Canada). The cuvette was immersed in a 1 L tank of water maintained at 30 °C by a circulating water bath. Correlation functions and the intensity of transmitted scattered light were measured for 2 min (118 s collection, 2 s break) continuously for 2−4 h, depending on the length of time taken by the milk system to gel.

Standard latex spheres of diameter 269 nm (Portland Duke Scientific, Palo Alto, CA, USA) were used to calibrate the laser intensity. The laser

intensity and the average intensity of transmitted light were subsequently used to calculate the $1/I^*$ of the milk system for each interval. The characteristic decay time of the fitted correlation function was used to calculate the diffusion coefficient, which was transformed into the apparent particle radius via the Stokes−Einstein equation. The details of this procedure are described elsewhere (*21*). Data processing was carried out using specialized software (Mediavention, Guelph, ON, Canada).

Statistical Analysis. Unless otherwise specified, three replicates were performed for each test. Statistical significance was determined through ANOVA (general linear model procedure) and pairwise least-squares means comparisons with $\alpha = 0.95$, calculated using SAS, version 9.1 (SAS, Cary, NC, USA).

RESULTS AND DISCUSSION

The pH of the unheated milk was 6.62 ± 0.13, and there was no significant change ($p < 0.05$) in pH as a result of heating. The partition of whey protein complexes between the soluble phase and those attached to the casein micelles depends on the pH of heating; at pH values of about 6.7, as in the current work, the complexes are expected to be predominantly located on the micelle surface (*23*).

The size exclusion chromatography elution profiles of the sera of unheated and heated milks are shown in **Figure 1**. The treatments 90 °C/20 s and 80 °C/300 s had similar elution profiles (data not shown) and corresponded to those reported in the literature (*16*, *23*, *24*), indicating that the effect of heat treatment on whey protein denaturation and soluble complex formation depended on the combination of heating temperature and length of heating time. Furthermore, it has been shown before (*25*, *26*) that 90 °C/20 s and 80 °C/300 s treated milks yield about the same percentage of denaturation of β-lactoglobulin and that both systems have similar mechanical properties in yoghurts, hence, probably, the same amount of complexes. The first peak present in all treatments (at 55 min) has been shown to contain residual fat globules (*23*). A peak eluting between 70 and 95 min was present only in the serum of milk heated to the greatest extent (85 °C/300 s, ▲), and a smaller peak starting at about 80 min was shown for all the other treatments, indicating that it was composed of particles of smaller size and, in the case of unheated and mildly heated milk, probably composed of small, κ-casein-rich micelles and κ-casein polymers. Increasing the extent of heating significantly increased the size of particles as well as the quantity of particles in this peak (inset of **Figure 1**), as evidenced by the earlier values of elution time and greater values of absorbance ($p < 0.05$), respectively. The peak eluting between 100 and 120 min, containing unaggregated whey proteins, decreased with increased extent of heating. The two peaks eluting after 120 min are not of interest in the current research, as they contain small molecular weight solutes (*16*).

The peak eluting between 80 and 95 min has previously been reported to appear also in unheated milk when prepared from skim milk powder (*23*), but the present results clearly demonstrate that soluble proteins and small soluble aggregates eluting before the monomeric whey proteins are always present in fresh unheated milk.

The fractions collected from the aggregate peaks separated by chromatography were analyzed by SDS−PAGE to determine possible changes in the composition of the aggregates with heating treatment. **Figure 2** summarizes the results for analyses conducted under reducing and nonreducing conditions. While the peak eluting between 80 and 95 min (see **Figure 1**) in unheated milk contained mainly caseins, increasing the extent of heating increased the amount of β-lactoglobulin present in the complexes within this peak, as well as the ratio of β-lactoglobulin/caseins. The complexes were held together by disulfide covalent bonds and, in the samples heated at 85 °C/300 s, also by hydrophobic

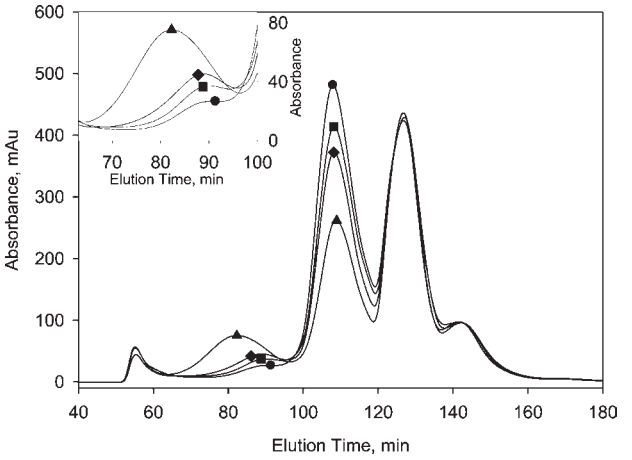

Figure 1. Elution profiles of the centrifugal sera of milk heated 74 °C/20 s (●), 85 °C/20 s (■), 80 °C/300 s (♦), and 85 °C/300 s (▲). The inset shows an expanded view of the aggregates' peaks. Curves show profiles for representative samples.

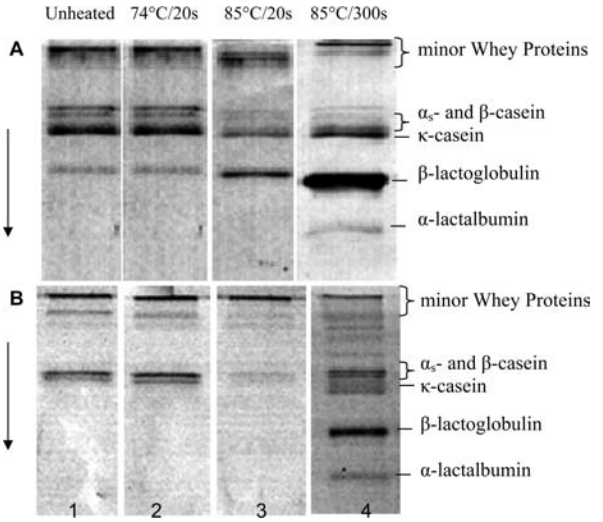

Figure 2. SDS−PAGE profiles of different milk treatments for samples collected from size exclusion chromatography. The peaks analyzed are those eluting in the aggregate fraction (i.e., between 70 and 95 min). Analyses were carried out under reducing (**A**) and nonreducing conditions (**B**). Arrows indicate migration direction.

interactions, as evidenced by the presence of proteins in the nonreducing gel (**Figure 2**B, lane 4). The electrophoretic pattern of mildly heated milk (74 °C/20 s) was similar to that of unheated milk. **Figure 2** clearly shows that while β-lactoglobulin was largely present in the soluble whey protein complexes, less α-lactalbumin was involved in the aggregates. This is in disagreement with what has been reported before (*23*, *27*). Electrophoresis under reducing and nonreducing conditions of the centrifugal sera (results not shown) confirmed that as the extent of heating increased, more protein became involved in disulfide-linked complexes, and less soluble caseins were present. All of the serum proteins bands were present, and heating did not seem to change the ratio between the proteins when samples were analyzed under reducing conditions.

Few studies have quantified the release of CMP from casein micelles in milk during simultaneous acidification and renneting (*12*). As the rennet activity depends on pH (*18*) and milk processing history (*28*), the amount of CMP released during acidification was determined as a function of pH for the differently heated milks and for the two levels of rennet used (**Figure 3**).

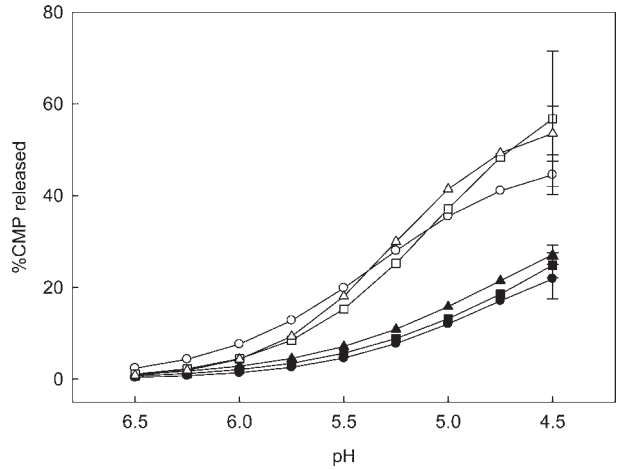

Figure 3. Percentage of CMP-released as a function of pH for milks heated 74 °C/20 s (●, ○), 85 °C/20 s (■, □), and 85 °C/300 s (▲, △) and gelled by combined acidification with low rennet (closed symbols) or high rennet (open symbols) treatments. Plotted values are the average of 2 replications, and standard deviation error bars are shown for the final point.

A greater amount ($p < 0.05$) of CMP was released from milks treated with the higher amount of rennet (53 ± 8%, at the final pH 4.6, open symbols) than milks treated with the lower amount (23 ± 3%, closed symbols). The enzymatic reaction did not achieve completion in either of the gelation treatments.

Heat treatment did not significantly affect ($p < 0.05$) the amount of CMP released. It has been previously reported that heating of milk impairs the rennet-induced gelation of milk, either by interfering with the enzymatic phase (*28*, *29*) or the aggregation phase (*10*, *30*) of the rennet reaction. The current work confirms previous results by Anema et al. (*10*) that time−temperature combinations did not affect the primary phase of the rennet reaction.

The sol−gel transition of differently heated milks acidified with the low and high amounts of rennet was followed using small deformation oscillatory rheology; the G' and tan δ behaviors are illustrated in **Figure 4**. A control sample of skim milk heated at 85 °C/300 s acidified with GDL but without rennet is also shown in **Figure 4** (cross symbol) which showed the typical pH-dependent development of G' and tan δ of acidified heated milk (*3*). For clarity, only three treatments are shown in the figure, and the results from all treatments are summarized in **Table 1**.

At each gelation pH, the sol−gel transition resulted in a sudden increase in G' and concurrent decrease in tan δ to values which reached a plateau at about 0.3. The gel stiffness, indicated by the G' value, continually increased as the acidification progressed. The rheological behavior depended on the amount of rennet added. Aggregation of casein micelles occurred at higher pH when more rennet was present (**Figure 4B**,**D** and **Table 1**) because of the more rapid decrease in the steric repulsion through cleavage of the hairy layer. Furthermore, the increased amount of hydrophobic *para*-κ-casein patches in the high rennet system allowed more contact points in the network as shown by the higher G'. These results are consistent with the notion that as the rate of κ-casein cleavage increases, the micelles become more prone to aggregation by the action of GDL. It is important to note, however, that the percentage of CMP released at the gelation pH values was low in both cases (11 ± 1% and 26 ± 5% for the low and high rennet treatments, respectively; see **Figure 3**) and that the aggregation of the micelles was, therefore, predominantly acid-induced. As such, the gelation pH and final gel stiffness were directly proportional to the severity of milk heating treatment (**Table 1**). However, the effect of heat treatment was noticeably different for milks gelled with different amounts of rennet, with the effects being more predominant in milk containing low (3.14×10^{-4} IMCU/mL) rennet (**Table 1**). The small difference in the extent of κ-casein hydrolysis was clearly significant in modifying the micelles, and the effect of heat treatment was reduced in the high rennet gels.

Milk with the high amount of rennet exhibited two stages in the development of G' (**Figure 4B**). There was an initial increase in G' at pH 5.3−5.4, corresponding to casein micelle coagulation; and at pH ∼5, there was a second change in slope in the G' curve. This pattern of G' development was not observed in the control treatment of milk (85 °C/300 s, cross symbol) gelled in the absence of rennet or in the gelation of milks with the low amount of rennet (**Figure 4A**). All of the milk samples with a gelation point above pH 5.0 exhibited a maximum in tan δ at a constant pH of 5.02 ± 0.04. The change in slope of G' and concurrent increase in tan δ toward tan δ_{max} has been previously attributed to the loosening of the viscoelastic structure and colloidal calcium solubilization (*12*, *13*, *31*).

Figure 5 illustrates (**A**) the radius and (**B**) $1/l^*$ parameters observed by DWS for two control samples acidified without rennet and heated mildly at 74 °C/20 s or extensively at 85 °C/300 s. The casein micelles had an initial apparent radius of approximately 120 nm and displayed hard-sphere interactions. The initial radius and $1/l^*$ were not affected ($p < 0.05$) by heat treatment. From the initial pH to pH of ∼5.3, the casein micelle radius decreased by 10−15 nm, consistent with the collapse of the hairy layer at decreased pH (*2*). With further acidification, the behavior of the micelles was dependent on milk heat treatment, confirming previous research (*21*).

For the mildly heated milk, the destabilized micelles began deviating from hard-sphere interactions at pH 5.3, as evidenced by the rapid increase in $1/l^*$ (**Figure 5B**). This increase in $1/l^*$ without a noticeable change in particle size (**Figure 5A**) indicates a change in the positional correlations of the micelles prior to aggregation. The dramatic increase in $1/l^*$ continued until a maximum value, which was followed by gelation, as indicated by the rapid increase in particle radius at pH ∼4.9. Near the end of the experiment, the $1/l^*$ values continued to increase, indicating continued particle interactions and network rearrangements, though the formation of the gel eliminated the ergodic nature of the signal. This development of DWS parameters for acidification of mildly heated (74 °C/20 s) milk was similar to that previously reported for acidification of unheated milk (*21*), demonstrating that the mild heat treatment was not severe enough to induce the behavior typical of heated milk.

The samples heated at 85 °C/300 s (open symbol) showed a very different gelling behavior. The initial decrease in radius to pH ∼5.3, (see inset) was followed by a substantial increase in the apparent radius at pH ∼5.1. This sudden increase in radius corresponds to the coagulation point of the milk and appeared earlier than that in the mildly heated samples, consistent with the higher gelation pH for heated milks during acidification as observed by rheology (**Table 1**). Shortly after the gelation point, the signal became nonergodic due to the formation of a gel, and the measurements were stopped. These results are consistent with previous reports on acidification of heated milk (*21*).

Figure 6 summarizes the changes in the DWS parameters for differently heated milks, treated with both GDL and rennet. Independent of heat treatment, the start of particle interactions occurred earlier in milk containing the high rennet concentration, as evidenced by the earlier onset of $1/l^*$ (**Figure 6C**,**D** and **Table 1**). In these samples, the changes in particle interactions were

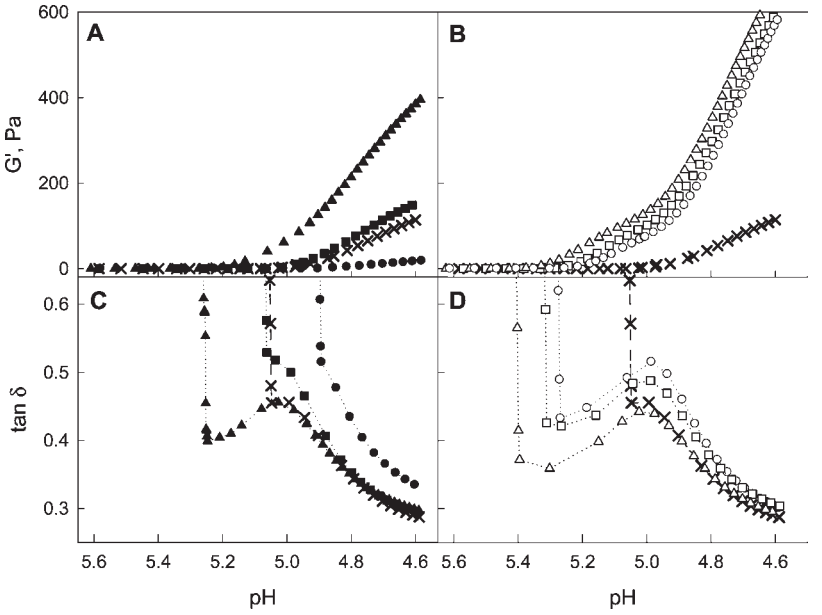

Figure 4. G' (**A** and **B**) and tan δ (**C** and **D**) of milks heated 74 °C/20 s (●, ○), 85 °C/20 s (■, □), and 85 °C/300 s (▲, △) and gelled by 1.5% GDL combined with low rennet (closed symbols) or high rennet (open symbols). The same parameters are shown for the control treatment of milk heated 85 °C/300 s and gelled with 1.5% GDL (**X**) but no rennet. Curves show representative experiments.

Table 1. Summary of Parameters Collected Using DWS and Rheology: pH Onset of 1/l* and Gelation pH Measured by DWS, Gelation pH Measured When tan δ Value = 1; Change in 1/l* from the Initial to pH 4.6, and G' Value at pH 4.6[a]

		gelation pH		at pH 4.6	
	pH onset of 1/l*	DWS	rheology	Δ1/l* (mm^{-1})	G' (Pa)
		No Rennet			
74 °C/20 s	5.25 a	4.91 a	4.82 a	1.61 a	17 a
85 °C/300 s	5.37 a	5.13 b	5.12 b	0.47 b	174 b
		Low Rennet			
74 °C/20 s	5.35 a	5.00 a	4.95 a	1.33 a	23 a
80 °C/20 s	5.36 ab	5.03 a	4.98 a	1.06 b	35 a
85 °C/20 s	5.39 abc	5.09 b	5.08 b	0.78 c	131 b
90 °C/20 s	5.42 bc	5.16 b	5.13 c	0.70 c	255 c
80 °C/300 s	5.42 bc	5.15 b	5.16 c	0.71 c	256 c
85 °C/300 s	5.44 c	5.23 c	5.27 d	0.36 d	415 d
		High Rennet			
74 °C/20 s	5.57 a	5.36 a	5.30 a	0.61 a	625 a
80 °C/20 s	5.57 a	5.32 a	5.32 ab	0.58 a	619 a
85 °C/20 s	5.54 a	5.29 a	5.34 ab	0.49 a	670 ab
90 °C/20 s	5.58 a	5.35 a	5.36 bc	0.49 a	680 b
80 °C/300 s	5.58 a	5.37 a	5.38 c	0.44 a	690 b
85 °C/300 s	5.61 a	5.40 a	5.42 d	0.41 a	707 b

[a] Values are the average of three replicates, and within the same block, different letters indicate statistical difference ($p < 0.05$). Samples contained 1.5% GDL or either low (3.14×10^{-4} IMCU/mL) or high (1.26×10^{-3} IMCU/mL) rennet.

observed at pH 5.57 ± 0.03; this pH corresponded to ~15% κ-casein hydrolysis. However, in milk samples containing the low amount of rennet, these changes began at 5.40 ± 0.05, which corresponded to ~7% κ-casein hydrolysis (**Figure 3**). The occurrence of additional interactions at a higher pH corroborates the higher gelation pH for milks gelled with the higher amount of rennet, as observed by an earlier increase in radius (**Figure 6A**,**B**) and as previously described for rheological determinations (**Figure 4**). These results demonstrate that low levels of κ-casein

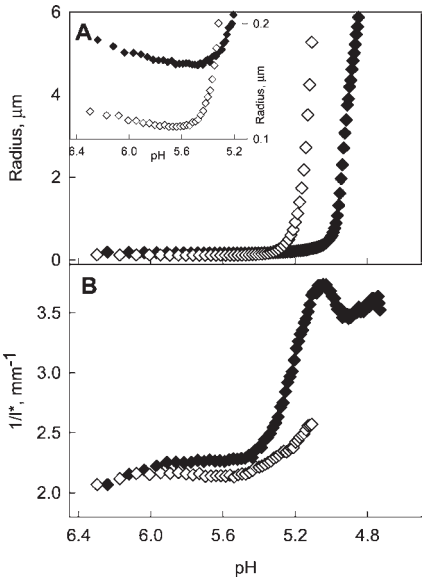

Figure 5. Apparent radius (**A**) and 1/l* (**B**) of control samples heated 74 °C/20 s (♦) and 85 °C/300 s (◇) and acidified with 1.5% GDL with no rennet. Curves show representative experiments.

hydrolysis significantly affect the casein interactions occurring in the stages preceding aggregation. The increased amount of κ-casein hydrolysis caused a greater reduction in the steric repulsion between micelles, allowing particle interactions and aggregation to occur at a higher pH. Interestingly, the onset of changes in particle interactions in milks gelled with low amounts of rennet was similar ($p < 0.05$) to those gelled by acidification alone, confirming that the interactions occurring in the milks gelled with the low amount of rennet are predominantly acid-induced. In contrast, milks gelled with the high amount of rennet all showed an earlier onset of particle interactions (**Table 1**) than the control samples with no rennet.

Heat treatment distinctly affected the interactions and aggregation of micelles gelled by combined acidification and rennet,

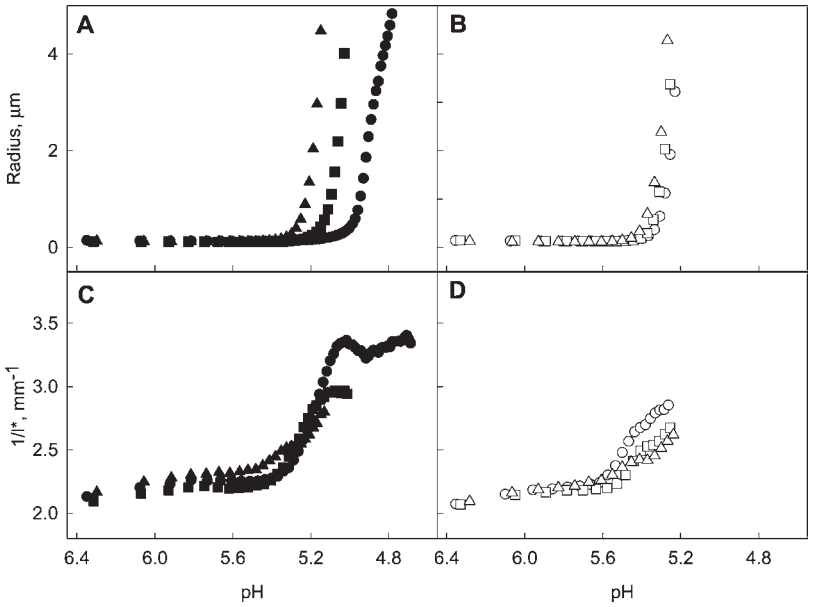

Figure 6. Apparent radius (**A** and **B**) and 1/*l** (**C** and **D**) of milks heated at 74 °C/20 s (●, ○), 85 °C/20 s (■, □), and 85 °C/300 s (▲, △) and gelled with 1.5% GDL and low rennet (closed symbols) and high rennet (open symbols). Curves show representative samples.

but only in milk with low amount of rennet. In these samples, the beginning of changes in particle interactions correlated directly with extent of heating ($p < 0.05$) as evidenced by the onset of $1/l^*$ at higher pH values (**Table 1**). This was not the case for milk samples containing higher rennet, as the effect was not statistically significant. The same behavior was noted for the increase in particle radius (**Figure 6A**,**B** and **Table 1**). These results are consistent with rheology (**Figure 4** and **Table 1**) in that heat treatment caused a greater effect on the gelation properties when a very low rennet activity is present. In this case, the interactions between the casein micelles and the κ-casein/WP complexes were prevalent over the limited hydrophobic intermicelle interactions due to the low hydrolysis caused by the rennet. This rendered the system acid-like and heavily dependent on the extent of heating. In milk with the high amount of rennet, however, the extensive hydrolysis of the κ-casein layer enabled the soluble complexes to associate with the micelles via rennet-induced hydrophobic patches at high pH before complex/casein interactions could take place, thereby eliminating the effect of heat treatment on gelation pH.

Previous studies have indicated that in heated, acid-induced gels, DWS detects the coagulation point of the micelles (rapid increase of radius) at a higher pH than rheology ($G' = G''$) (*31*). The current work showed no significant differences between the pH of gelation measured by DWS and rheology within the same treatment. The combined effect of acid and rennet-induced destabilization of the caseins, the soluble complexes, or both increased the opportunities for interactions between the species. When the system was heavily heat-treated, the presence of high amounts of heat-induced complexes increased the strength of the intermediate network and was able to be detected macroscopically by rheology at the same time as it was detected microscopically by DWS (*32*). In the case of low heat treatment (heat loads up to 80 °C/300 s), there were relatively few κ-casein/WP complexes formed. However, the onset of attraction between the micelles was significantly moved to higher pH even at low rennet amounts (pH onset of $1/l^*$, **Table 1**). This could be interpreted as a fragile state of equilibrium resulting in an abrupt change to aggregation as soon as acidification progressed. This would also result in similar pH of coagulation measured by rheology and DWS.

In the low rennet treatment, increasing the extent of heating caused a significant ($p < 0.05$) decrease in the change in $1/l^*$ ($\Delta 1/l^*$) from the initial value to the value at the aggregation point; the same trend existed but was not significant ($p < 0.05$) in the high rennet treatment (**Figure 6C**,**D** and **Table 1**). This value describes the changes in spatial correlations of particles and suggests that the final distribution of casein micelles in low-rennet acid gels was dependent on the extent of heating. In a low-heat milk, a network can only be achieved when the micelles come together, whereas in extensively heated milk, the micelles can become part of the network via links created by the soluble complexes and the micelles, creating a co-gel. This enables the scatterers of the system to remain, on average, relatively apart and similar to their liquid state. In a high rennet system, the early stickiness of the micelles makes them prone to immediate aggregation irrespective of whether an intermediate connecting link is available.

Figure 7 illustrates the gel microstructure of combination gels prepared from milk heated at 74 °C/20 s, 85 °C/20 s, and 85 °C/300 s. The microstructure of the milk samples heated at 74 and 85 °C for 20 s with the low amount of rennet (**Figure 7A**,**C**) contained roughly uniform protein flocs spanning the micrograph area, whereas the gel produced with the high amount of rennet (**Figure 7B**,**D**) exhibited larger protein aggregates and greater porosity. As mentioned above, the low-heat samples contained very few soluble aggregates; therefore, aggregation and gelation can only proceed via micelle−micelle contacts. At low-rennet conditions, the micelles still contained most of their stabilizing layer intact (albeit modified enough to affect the aggregation point), which therefore only allowed the casein micelles to aggregate in a mostly acid-induced manner. At high rennet levels, before the pH changes in the medium could influence micelle aggregation, the rennet action was severe enough to form important hydrophobic patches in the casein micelles, thereby creating a greater number of contact points between destabilized micelles. As acidification progressed, these larger aggregates came together further and resulted in a porous network with large protein aggregates.

Gels produced from the extensively heated milk (85 °C/300 s) differed from the mildly heated milks (74 and 85 °C for 20s) in that they had increased network connectivity and smaller protein

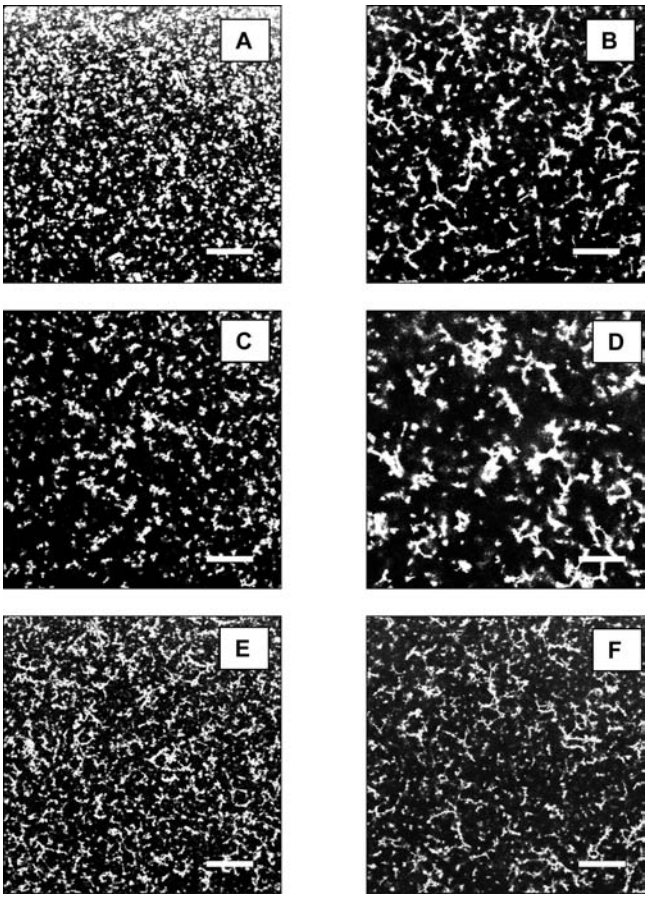

Figure 7. Confocal laser scanning micrographs of combination gels prepared using 1.5% GDL and low rennet (**A**,**C**,**E**) or high rennet (**B**,**D**, **F**) for milk heated at 74 °C/20 s (**A**,**B**), 85 °C/20 s (**C**,**D**), 85 °C/300 s (**E**,**F**). The scale bar is 20 μm. Images show representative replicates.

aggregates. The microstructure of combination gels from the extensively heated milk was similar to that observed for acid-gels of heated milk (*33*) with a high level of branching and diffuse protein structures. In this case, high levels of heating produced high numbers of soluble aggregates, both attached to the surface of the micelles and free in solution. As either rennet action or acidification took place, interactions between the soluble aggregates and the WP-coated micelles (either by hydrophobic patches caused by the action of rennet or the lower pH caused by the GDL) enabled the formation of a network without necessary direct micelle−micelle contact. This produced a much more open structure, with smaller aggregates. In the samples prepared with extensively heated milk, the level of rennet caused little difference in the gel microstructure. This can be interpreted as the product of two competing mechanisms. The action of rennet on the system is to create hydrophobic patches on both the micelles and the soluble complexes, as it is known that the primary phase of rennet action in milk is not impaired by heat (*10*). This drives the system to aggregation via hydrophobic interactions. However, the casein micelles are heavily coated by WP complexes on the surface of the micelle which in turn create a sterically stabilizing layer. Even though aggregation did indeed take pace through the hydrophobic action, this layer prevented the close approach of the micelles and impeded the creation of large aggregates. Both low and high levels of rennet would create the same effect. The similarities in the microstructure of gels formed with the low and high rennet for milk heated at 85 °C for 300 s confirm the reduced differences in the aggregation behavior as observed by rheology and DWS.

In conclusion, heating of milk using a pilot scale system with various heating regimes showed that the extent of soluble complex formation can be adjusted and that the relative proportion of casein to whey protein in the heat-induced aggregates changes with the extent of heating.

The levels of CMP released in this study would be insufficient to independently cause micellar aggregation. Increasing the level of rennet caused a more rapid reduction in steric stability and resulted in micellar interaction and aggregation occurring at higher pH. Furthermore, the combination gels with increased level of rennet exhibited increased stiffness due to the microscopically observed larger protein aggregates with increased connectivity.

With low rennet, the gels were characterized as predominantly acid-gels, with increasing milk heat treatment resulting in earlier gelation and the formation of stiffer gels. In combination gels with high amount of rennet, the slightly greater amount of κ-casein hydrolysis (about 10% higher) altered the micelles so that the effect of heat treatment on gel properties was less significant. The microstructure of combined gels was similar for the gelation of extensively heated milk (85 °C/300 s and 90 °C/20 s) with both levels of rennet, again indicating the reduced effect of rennet in extensively heated milks.

The current work was the first attempt at combining a detailed study of the soluble complexes formed in heated milk with the observation of the stages preceding combined gelation using DWS. It can be concluded that the microstructure and stiffness of the network can be modified by changes in the nature and extent of interactions present between the different states of the species in the system. These states can be modified by heating regimes as well as by small changes in the amount of rennet used, as the small difference in CMP released at the gelation point clearly caused significant changes in the microstructure and gelling behavior.

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