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ARTICLE

Role of the Heat-Induced Whey Protein/κ-Casein Complexes in the Formation of Acid Milk Gels: A Kinetic Study Using Rheology and Confocal Microscopy

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The effect of heat treatment of milk on the formation of acid gel was examined using confocal scanning laser microscopy and low-amplitude dynamic oscillation throughout acidification. Milk samples were reconstituted by mixing colloidal phase from unheated or preheated skim milk, labeled with rhodamine B isothiocyanate, with the aqueous phase from unheated or preheated milk, labeled with fluorescein isothiocyanate. Gels were made by acidification with glucono-δ-lactone. The presence of material from preheated milk, that is, either the colloidal or the aqueous phase or both, led to an increase in the gelation pH and in the final elastic modulus and to a more branched network with larger pores. During acidification, the heat-induced serum complexes and the casein micelles did not appear to form separated gels with time or in space. Moreover, the colocalization in the final network of serum heat-induced complexes and casein micelles is particularly well observed in the presence of an aqueous phase obtained from preheated milk. Finally, because the rheological and microstructural properties of acid gels containing either micelle-bound or serum heat-induced complexes were similar, it was suggested that the serum heat-induced complexes interacted with the casein micelles early in the course of acidification and that formation of the network did not differ significantly whether the heat-induced complexes were initially found in the aqueous phase of milk or bound to casein micelles.

KEYWORDS: Acid milk gels; heat-induced complexes; confocal scanning laser microscopy

INTRODUCTION

In the manufacture of acid milk gels such as yogurt, the extensive heat-treatment of milk at 80−95 °C for several minutes has been shown to dramatically increase the pH of gelation and final elasticity of the set gel (*1*−*5*), to enhance connectivity of the protein network (*6*), and to reduce syneresis (*7*, *8*). These changes correlate well with denaturation of the whey proteins (*2*, *3*, *9*, *10*) and formation of heat-induced whey protein/κ-casein complexes (*4*, *5*, *11*). In heated skim milk at its natural pH (∼6.6), the whey protein/κ-casein complexes are parted between the colloidal and serum phases (*4*, *5*, *12*). Since the complexes precipitate on acidification (*13*) and are, in large part, retained within the acid curd on drainage (*14*, *15*), it is expected that they physically take part in network formation in acid milk gels. This was clearly demonstrated by Vasbinder et al. (*16*) who used confocal laser scanning microscopy (CLSM) on acidified milk systems of separately labeled casein micelles and whey protein complexes. They reported that both micelle-bound and serum complexes were involved in the final protein network, where they appeared to be colocated with the casein micelles. However, the actual contribution of either type of complex to the building of the gel in the course of acidification is not known. Lucey et al. (*3*) first suggested that heated milk started to gel at pH ∼5.3 as a result of the important role of β-lactoglobulin (isoelectric pH (p*I*) ≈ 5.3) in the formation and properties of heat-induced whey protein/κ-casein complexes, when casein micelles exhibit a lower p*I* of ∼4.8. This proposition was supported by Alting et al. (*17*), who showed that modification of the p*I* of β-lactoglobulin affected the acid gelation point of heated whey protein isolate, and was extended to skim milk by Vasbinder et al. (*18*). Because gel formation of heated skim milk exhibits a two-step process and the apparent p*I* of casein micelles was thought to increase from 4.8 to 5.6 as 0−100% of their surface was covered by denatured whey proteins (*18*), it was further suggested that the serum complexes first gelled at their apparent p*I* of ∼5.3, followed by partly whey protein-coated casein micelles with an apparent p*I* of ∼5.1 (*12*). However, this hypothesis is at variance with studies where milk systems in which β-lactoglobulin was substituted with ovalbumin (p*I* ≈ 4.8) or soy protein (p*I* ≈ 4.0) started to gel at pH 5.9−6.0 (*19*, *20*); neither is it supported by the finding that the

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whey protein/κ-casein serum complexes isolated from milk have an apparent p*I* of ~4.5 (*21*, *22*). Alternatively, it was proposed that the increased hydrophobicity of particles as a result of heat denaturation of the whey proteins accounted for the higher gelation pH of heated milk. However, whether or not the heat-induced complexes destabilize separately or concomitantly to the casein micelles is yet not known. Dubert-Ferrandon et al. (*23*) took CSLM images of the forming network throughout acidification from pH 6.5 to 4.2 and did not report separate gelation of the complexes and casein micelles with time or in space. However, as the labeled whey proteins were added to skim milk after heat-treatment, the authors mentioned that their observations may not have reflected the behavior of the heat-induced complexes. The purpose of the present study was therefore to investigate the specific contributions of the heat-induced whey protein/κ-casein complexes and the casein micelles to the formation of acid milk gels, using a modified labeling procedure.

MATERIALS AND METHODS

Reconstituted Skim Milk. Milk was reconstituted at 140 g kg^{-1} dry matter (DM) using ultralow heat skim milk powder (*24*) and 0.5 g kg^{-1} sodium azide in stirred deionized water at 40 °C. The milk was stirred for a further ~2 h after reconstitution then left overnight at 5 °C to complete dissolution. Repeated fractionation of the reconstituted milk into its micelle and serum phases to prepare combined milk systems induced protein losses. The 140 g kg^{-1} DM content of the reconstituted milk was chosen as to allow adjustment of the final total protein content of the combined systems at skim milk's usual value of ~35 g kg^{-1}.

Milk Ultrafiltrate. Milk ultrafiltrate (MUF) was prepared from reconstituted skim milk on a 10 kg mol^{-1} molecular weight cutoff organic Centramate membrane (Pall France, St Germain en Laye, France) and stored at 5 °C after addition of 0.5 g kg^{-1} sodium azide. MUF was designated as S0 in reconstituted milk systems.

Effect of pH on Absorption and Emission of the Fluorescent Probes. The absorption and emission spectra of fluorescein isothiocyanate (FITC) or rhodamine B isothiocyanate (RITC; Sigma-Aldrich, St Quentin Fallavier, France) were measured in citric acid/disodium phosphate solutions in order to range from pH 4.39 to 6.65. The absorption and emission spectra were collected in 1 cm path-length cuvettes over the UV−visible wavelength range using a double-beam Uvikon 922 spectrophotometer (Kontron Instruments, Trappes, France) and a LS 50B spectrofluorimeter (Perkin-Elmer, Saint Quentin-en-Yvelines, France), respectively. To measure emission, FITC and RITC were excited at 488 and 543 nm, respectively.

Protein Labeling and Heat Treatment. One milliliter of freshly prepared dye solution (4 g L^{-1} FITC or RITC in dimethylsulfoxide (DMSO), Sigma-Aldrich) was added to 200 g of agitated, unheated reconstituted milk; then the cross-linking reaction was left to occur over 1 h at room temperature under gentle stirring. To obtain labeled heated milk, the labeled unheated skim milk was then distributed in 25-g fractions into glass tubes then heat-treated at 90 °C for 10 min in a thermostatted water bath (come-up time ~3 min). After heating, the milk was cooled in ice water then left to equilibrate at room temperature for ~2 h prior to ultracentrifugation.

Labeling of proteins by the isothiocyanate derivatives of fluorescein or of rhodamine occurs through formation of thiourea covalent bonds between the isothiocyanate group and unprotonated primary amines of the protein (*25*). Reactivity will strongly depend on the class and dissociation constant, p*K*, of amines; but typically, conjugation is optimal at pH 8.5−9.5 and ~3 orders of magnitude less likely at the pH of milk if only considering the effect of protonation on activity of the amines. However, globular proteins like egg white lysozyme or bovine serum albumin were reported to bind FITC at pH values lower than 7 (*26*), and an example exists of binding of FITC to cell wall proteins at pH 5 (*27*). Because alkaline conditions strongly affect the structure of casein micelles (*28*), albeit somewhat reversibly (*29*, *30*), labeling of the milk proteins was therefore preferably performed at pH 6.65 despite the unfavorable conditions.

Separation of the Serum and Colloidal Phases of Milk. Separation of the serum and the colloidal phases of unheated or heat-treated labeled milk was performed on 15 mL aliquots using ultracentrifugation on a Sorvall Discovery 90 SE centrifuge (Kendro Laboratory Product, Courtaboeuf, France) equipped with a 50.2 Ti rotor (Beckman Coulter, Fullerton, CA). The samples were spun at 34 207 average *g* for 65 min at 20 °C. The supernatant and pellet were defined as the serum (S) and colloidal (C) phases, respectively, of milk. The serum of unheated or heat-treated milk was designated as S1 or S2, respectively, and their colloidal phases were designated as C1 or C2, respectively. Unless otherwise stated, S1 and S2 were prepared from milk labeled with FITC, while C1 and C2 were prepared from milk labeled with RITC.

Removal of the Unreacted Fluorescent Probes. In order to avoid cross-labeling of the unreacted FITC and RITC on reconstitution of the experimental milk systems, the ultracentrifugal fractions of the labeled milks were carefully washed. Unreacted FITC was removed from S1 and S2 using extensive dialysis (6−8 kg mol^{-1} MWCO, Medicell International, London, U.K.) against commercial UHT milk (Orlait, Compiègne, France) at 5 °C. Unreacted RITC was removed from C1 and C2 using repeated redispersion in fresh milk ultrafiltrate using blending at 9500 rotations min^{-1} for 3.5 min (Ultra-Turrax T25, Janke & Kunkel GmbH, Staufen, Germany), resuspension at 5 °C for 48 h under gentle stirring, and recollection using a further centrifugation stage. The dialysis treatment of S1 and S2 was shown to reduce the emission signal of FITC down to a minimum plateau value equal to ~5% and 11% of the initial fluorescence intensity, respectively (not shown), and to increase the weight of S1 and S2 by ~5%, due to water transfer from the aqueous phase of the UHT milk to that of the reconstituted milk. The washing procedure of C1 or C2 was shown to reduce the emission signal of RITC down to a minimum plateau value of ~5% and 15% of its initial value, respectively (not shown).

Despite this washing, preliminary experiments have shown that low amounts of the free probes were still present in our milk systems and that cross-labeling of the unreacted FITC and RITC must therefore be considered. However, other experiments where only one fraction of the experimental milk systems was labeled showed that emission signals corresponding to possible cross-binding were within the background noise of that of the designated probe, when present (not shown).

Estimation of the Total Protein Contents in the Milk Fractions. The total protein contents of S1, S2, C1, and C2 were estimated using measurement of their absorbance at 280 nm on the Uvikon 922 spectrophotometer in 1 cm path-length cuvettes after 1/100 v/v dissociation in 10 mmol L^{-1} ethylenediaminetetraacetate (EDTA), 1.6 g kg^{-1} dithiothreitol (DTT), pH 11, followed by incubation at 37 °C for 1 h, to avoid light scattering. Calibration was made on 1/5 to 1/200 v/v diluted unheated reconstituted skim milk at 140 g kg^{-1} to determine the linear range in which to convert absorbance at 280 nm into milk total protein, without distinction between casein and whey proteins (not shown).

Reconstitution of the Experimental Milk Systems. Four milk systems (C1S1, C2S1, C1S2, and C2S2) were prepared by resuspending the appropriate mass of colloidal phase C1 or C2 in the appropriate mass of serum phase S1 or S2 and of milk ultrafiltration permeate S0 to reach a total protein content of 35 g kg^{-1} with a mass ratio of serum phase to colloidal phase of 4. Two casein micelle systems were prepared by resuspending C1 or C2 into S0 to reach a total protein content of 28 g kg^{-1}. The systems were homogenized as described above then left to disperse and equilibrate at 5 °C for 48 h under gentle stirring. All the systems had final pH values of 6.65 ± 0.05 at room temperature. Prior to acid gelation, the systems were equilibrated at room temperature then gently centrifuged at 1000*g* for 15 min (Kri4 centrifuge, Jouan, Saint Herblain, France) to remove residual insoluble particles. Because ultracentifuged casein particles are sometimes difficult to fully redisperse in the milk serum, all four systems tested, including the unheated and heated milk controls, were prepared from reassembled fractions of milk so that the drawback of residual insoluble particles was consistent across all comparisons.

Acid Gelation. Reconstituted systems equilibrated at 35 °C for 30 min were acidified using addition of 19.5 g L^{-1} glucono-δ-lactone (GDL, Sigma-Aldrich) and agitation for 2 min. The pH was recorded in a milk aliquot at 35 °C (pHM220 Meterlab, Radiometer Analytical SAS, Villeurbanne, France) and reached a final value of 4.5 within 4 h.

Confocal Scanning Laser Microscopy. Formation of the acid gels in acidified reconstituted systems was observed with time on an inverted

TE2000-E microscope equipped with a Nikon C1Si laser scanning imaging system (Nikon, Champigny-sur-Marne, France) with argon ion and helium−neon lasers emitting at 488 and 543 nm, respectively. Immediately after dispersion of the GDL, 1 mL of the sample was transferred in a 1.5 cm diameter cuvette mounted on a glass slide, covered with a thin layer of paraffin oil, and maintained at 35 °C using a Peltier thermostatic plate. A Nikon 60× oil-immersion objective with 1.40 numerical aperture was used. The FITC and RITC were excited at 488 and 543 nm, respectively, and the emitted light was recorded at 515 ± 15 and 590 ± 25 nm, respectively. The software used for image acquisition was EZ-C1, version 3.40 (Nikon). Digital images were recorded at 30 μm depth into the sample (i.e., above deposition of residual insoluble material) in 512 pixels × 512 pixels resolution and at 10.56 μs pixel dwell. Pinhole diameter and all other settings were also kept constant. Image brightness was auto-adjusted using the software EZ-C1 FreeViewer, version 3.20 (Nikon).

Rheology. Formation of the acid gels was monitored by measuring the elastic modulus (G') and loss tangent (tan δ, defined as the ratio of the viscous to the elastic modulus of a system, G''/G') of the reconstituted milk systems under acidification at 35 °C using an AR2000 rheometer (TA Instruments, Guyancourt, France) and a coaxial geometry. Immediately after dispersion of the GDL, ~12 g of sample was introduced into the geometry, and measurements were recorded every 4 min. The milk sample was covered with a thin layer of paraffin oil to prevent evaporation during analysis. The applied deformation was 0.1%, frequency was 1 Hz, and gel formation was followed for at least 5 h. The onset of gelation was defined as the time when $G' > 1$ Pa. In agreement with Vasbinder et al. (*16*), preliminary results showed that neither the presence nor the nature of the chosen fluorescent probes (RITC or FITC on either the serum or micelle phase) had significant influence on formation of the acid gel as measured by rheology (not shown). If the probes had some effect on the formation of the heat-induced complexes, variations would have been expected depending on the probe used.

Significance. The presented results were obtained from two repetitions of complete sample preparation and analysis. Significance was evaluated using t test ($\alpha = 0.05$).

RESULTS

Absorption and Emission of RITC and FITC as a Function of pH. In citric/phosphate buffer at pH 6.65, the RITC showed maximum absorption and emission at ~550 and ~576 nm, respectively, while FITC showed maximum absorption and emission at ~492 and ~515 nm, respectively. These values were in reasonable agreement with reported data on either probe in alkaline aqueous conditions (*25*, *31*). Distinct maxima make FITC and RITC adapted for use together in one system where location of two components is needed (see, for example, Nigen et al. (*32*) and Sanchez et al. (*33*)). However, the absorption and fluorescence properties of probes, especially charged probes like FITC, may be dramatically affected by pH and polarity of the medium (*34*). In citric/phosphate buffer, the absorption maximum of FITC shifted from 492 nm at pH 6.65 to ~480 nm at pH 5.16 and to ~450 nm at lower pH values (not shown). In comparison, the absorption spectrum of RITC in the same conditions was barely affected by pH (not shown). In other words, the absorption spectrum of FITC shifted away from that of RITC on acidification, so that virtually no risk of overlapping existed throughout pH 6.65 to 4.39.

Conversely, the wavelengths of maximum fluorescence of either FITC or RITC did not significantly change as pH decreased from 6.65 to 4.39 (not shown), although the emission spectra themselves may change shape or area with decreasing pH. In particular, relative intensities of the primary and secondary fluorescence maxima somewhat changed with pH and slightly modified the spectrum of FITC (not shown). Furthermore, the quantum yield of RITC increased about 3-fold on decreasing pH from 6.65 to 4.39, while that of FITC dramatically decreased (about 8-fold, not shown), due to a decreased quantum yield of its protonated forms (*34*). In acidifying milk systems where the two probes are used, it should therefore be expected that emission of the RITC becomes more intense on acidification, while that of the FITC fades out, so overlay images show changing color where colocated. However, the results also show that FITC exhibited a much larger fluorescence intensity than RITC at pH values 6.65−5.16, down to comparable intensities at pH 4.75−4.39. Appropriate concentrations of the two probes should therefore allow both emission signals to be clearly visible at all times throughout acidification.

Contribution of the Heat-Induced Micelle-Bound Complexes to the Acid Gelation of Resuspended Casein Micelles. Figure 1 shows the acid gelation behavior of labeled unheated casein micelles C1 or casein micelles coated with whey protein/κ-casein complexes C2 dispersed in milk ultrafiltration permeate S0, compared with reconstituted unheated and heated reference milk systems C1S1 and C2S2.

The effect of heating on the acid gelation of skim milk is widely documented (e.g., see Lucey and Singh (*35*), Lucey (*36*, *37*) and Livney et al. (*38*)). As expected, heat-treatment at 90 °C for 10 min increased the pH of gelation from ~4.9 in unheated milk C1S1 system to ~5.3 in heated milk system C2S2. On acidification at 35 °C, gels developed slowly in C1S1 and yielded final elastic modulus values lower than 30 Pa at pH 4.5. In contrast, C2S2 exhibited a higher increase in elastic modulus G' and reached values of about 420 Pa at pH 4.5 ($P_0 < 0.05$). In contrast to C1S1, tan δ of C2S2 exhibited a local maximum at pH ~4.9, typical of acidifying milk systems that start to gel before dissociation of the colloidal calcium phosphate is completed (*36*). At pH 4.5, tan δ was ~0.27 in C2S2 vs ~0.34 in C1S1 ($P_0 < 0.05$), which confirmed that heat-treatment increased solid-like properties of the acid gels. Microstructure of C1S1 acid gels at pH 4.6 exhibited rather homogeneous clusters of aggregated protein, with small pores and poor connectivity between clusters. In contrast, C2S2 gels showed larger pores and denser protein clusters connected by thin linear strands. These structural features are in agreement with previous observations (*6*, *16*, *39*).

Figure 1 furthermore showed that the acid gelation behavior of unheated (C1S0) or heated (C2S0) casein micelles alone was very similar to that of the corresponding unheated or heated milk systems, only that gelation of C1S0 and C2S0 started at slightly lower pH values and reached somewhat lower final G' values than C1S1 and C2S2, respectively ($P_0 < 0.05$). Other features (changes in tan δ and final microstructure) were identical to that of the reference milks ($P_0 > 0.05$ for tan δ). Lower values of the final G' were accounted for the lower total protein content (28 g L^{-1}) of the systems C1S0 and C2S0 compared with C1S1 and C2S2 (35 g L^{-1}, (*40* and *41*)). The results therefore showed that micelle-bound complexes contribute to a large extent to the heat-induced changes observed in acid skim milk gels.

Acid Gelation Behavior of the Heat-Induced Serum Complexes. Labeled heat-induced serum complexes of denatured whey protein and κ-casein were prepared by ultracentrifugation of FITC-labeled heated skim milk and removal of the casein micelle pellet. The resulting serum phase S2 was estimated at ~12 g L^{-1} total protein. The behavior of the heat-induced serum complexes on acidification is presented in **Figure 2**.

The results showed that, even at a relatively low concentration, the heat-induced complexes were able to form a weak gel in the absence of casein micelles. Gelation started as pH reached ~5.2 and yielded G' and tan δ values at pH 4.5 of less than 10 Pa and ~0.28, respectively. Despite its low elastic modulus, the final gel therefore exhibited a final tan δ value that was similar to those found in acid milk gels C2S2 or C2S0 where heat-induced complexes were present (**Figure 1**). Similarly, the pH of gelation of acidified S2 was in the range of those found in C2S2 and C2S0.

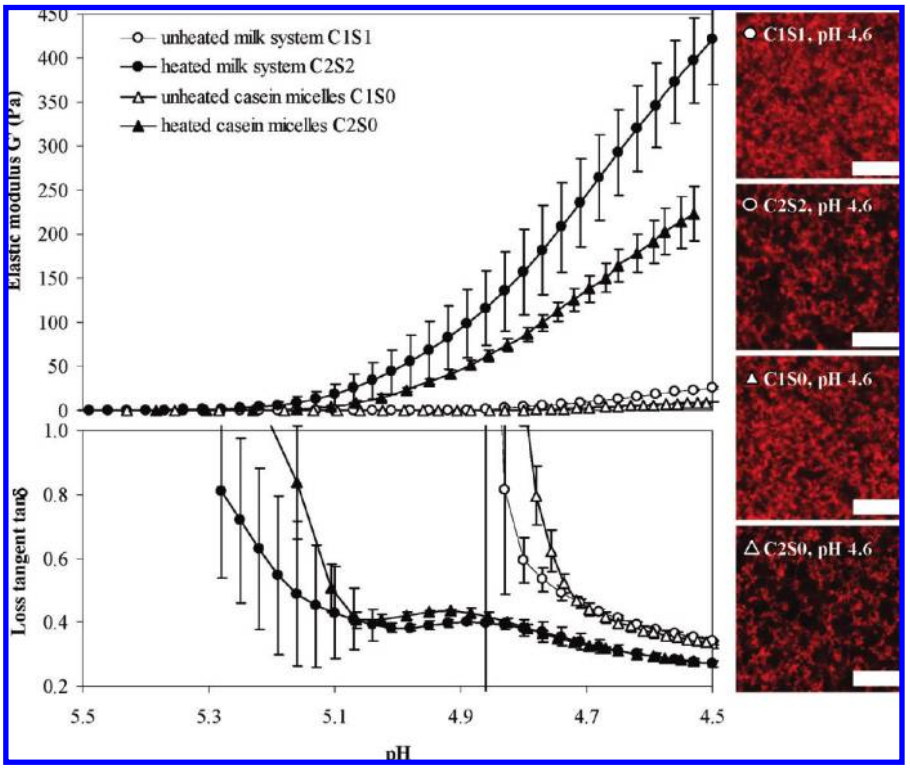

Figure 1. Development of the elastic modulus, G' (Pa), and loss tangent, tan δ, of reconstituted unheated skim milk system C1S1 and of heated skim milk systems C2S2 at ~35 g kg^{-1} total protein and of the ultracentrifugal pellet (i.e., casein micelles) of unheated (C1) or heated (C2) skim milk dispersed at ~28 g kg^{-1} total protein in milk ultrafiltration permeate S0, in the course of acidification by 19.5 g kg^{-1} GDL at 35 °C. Data are the average of two preparations. Right hand side images show the final microstructure of C1S1, C2S2, C1S0, and C2S0 at pH 4.6 (scale bar 10 μm). Labeling probe was RITC, excitation was at 543 nm, and emission was recorded at 590 ± 25 nm.

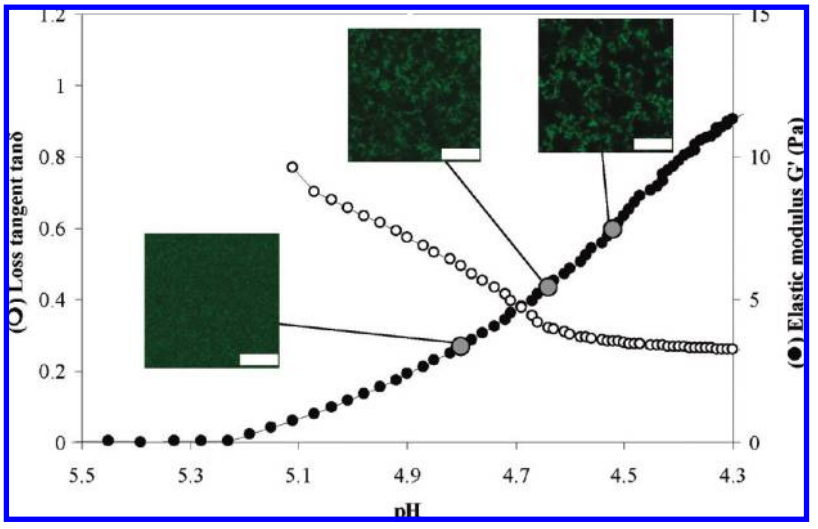

Figure 2. Development of the elastic modulus, G' (Pa), and loss tangent, tan δ, of the supernatant of heated skim milk S2 (i.e., heat-induced serum complexes) at ~12 g kg^{-1} total protein in the course of acidification by 19.5 g kg^{-1} GDL at 35 °C. Inserted images show the microstructure of S2 at pH 4.77, 4.64, and 4.52 (scale bar 10 μm).

Confocal images of S2 showed little organization at pH ~4.8, although already a gel ($G' > 1$ Pa). Microstructure of the S2 gel at pH ~4.6 showed barely connected protein clusters, which became denser and more branched as the pH further decreased to ~4.5 and as large pores opened in the network.

Contribution of the Heat-Induced Micelle-Bound or Serum Complexes to the Acid Gelation of Skim Milk. Skim milk systems with either only micelle-bound or only serum heat-induced complexes were prepared, and their response to acidification was compared to those of the unheated and heated skim milk systems C1S1 and C2S2. Formation of the corresponding acid gels as observed by rheology and final microstructure of the gels as observed by CSLM are shown in **Figure 3**.

The results clearly show that when either the micelle-bound or the serum fraction of the total heat-induced complexes was present, the milk system responded to acidification in a very similar manner to heated milk system C2S2. In systems C1S2 and C2S1, gelation indeed started at pH 5.2−5.3. Final tan δ values were 0.28−0.29 at pH 4.5 and were not significantly higher than those of C2S2. The G' of C1S2 and C2S1 reached respective final

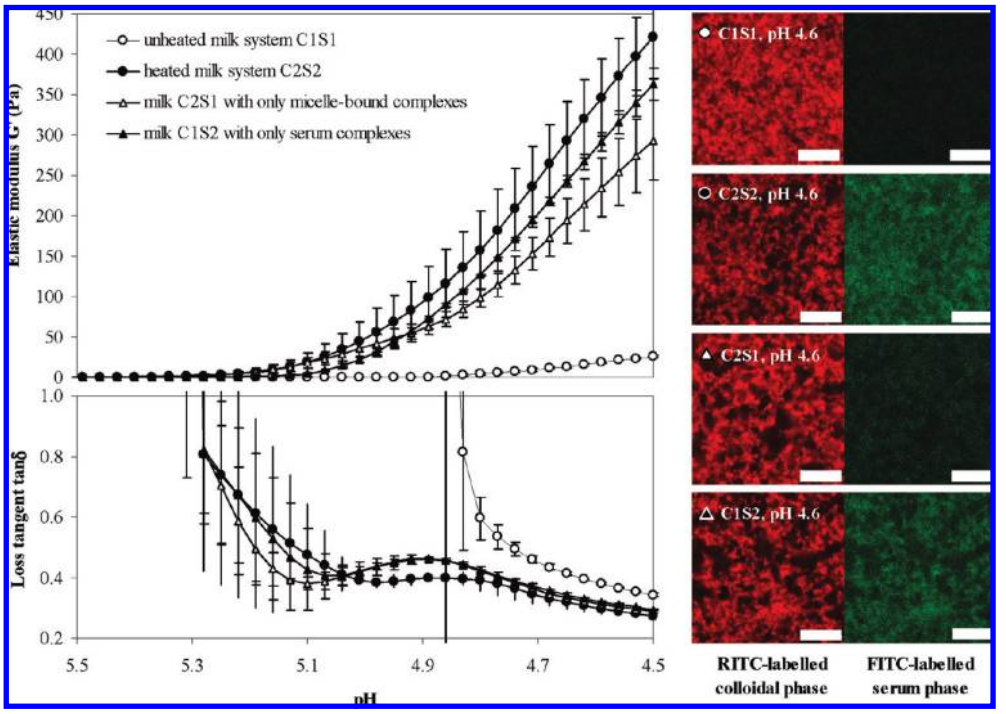

Figure 3. Development of the elastic modulus, G' (Pa), and loss tangent, tan δ, of reconstituted unheated skim milk system C1S1, of heated skim milk system C2S2, of combined skim milk system with only micelle-bound complexes C2S1, and of combined skim milk system with only serum complexes C1S2 at ~35 g kg^{-1} total protein in the course of acidification by 19.5 g kg^{-1} GDL at 35 °C. Data are the average of two preparations. Right hand side images show the final microstructure of systems C1S1, C2S2, C2S1, and C1S2 at pH 4.6 (scale bar 10 μm). Ultracentrifugal pellets C1 and C2 (casein micelles with or without micelle-bound complexes) were labeled with RITC (in red), while supernatants S1 (native whey proteins) and S2 (serum complexes) were labeled with FITC (in green).

values of ~360 and ~300 Pa vs ~420 in C2S2. Considering the large standard deviations, the differences were not significant ($P_0 > 0.05$); however, the tendency of C1S2 and C2S1 systems to reach lower values of final G' than C2S2 could be accounted for by their lower content in total heat-induced complexes. In agreement with rheological measurements, observation of the acid gels of reconstituted milk systems by CSLM showed that C1S2 and C2S1 exhibited reticulated, porous microstructure similar to that of the heated control C2S2. When present, that is, in C1S2 and C2S2, FITC-labeled heat-induced serum complexes were clearly visible and showed the same pattern as the RITC-labeled colloidal phase, thus evidencing colocation in the protein gel. The above results showed that acid gels typical of heated skim milk could be formed whenever micelle-bound (C2) or serum (S2) heat-induced complexes were present, even in a fraction of the total amount found in C2S2. They also confirmed that the serum complexes were involved with the casein micelles in the final network, whereas native whey protein remained evenly distributed and did not aggregate into dense clusters, in agreement with Vasbinder et al. (*16*).

In order to identify a possible sequence of events in the gelation process of the reconstituted milk systems, CSLM fluorescence images of both RITC-labeled colloidal phase and FITC-labeled serum phase were recorded at various pH values throughout acidification. The resulting overlay images are shown in **Figure 4**.

From pH 6.65 to as late as pH 5.18, all systems showed blurred images as a result of yet highly mobile protein components. The dense 1−3 μm large particles visible on the images were identified as insoluble casein particles that failed to disperse on reconstitution of the systems. On acidification, the overlay images shifted to redder color (green shifted to orange or orange to red depending on systems) as a result of the decreased intensity of FITC fluorescence (in green) to that of RITC (in red) as a function of pH (see above).

At all pH values, including at pH 6.65 where no gelation is suspected, reconstituted systems that contained preheated serum complexes (C1S2 and C2S2), showed higher green relative intensity compared with systems that contained serum phase with native whey proteins (C1S1 and C2S1). This probably resulted from either a greater content in FITC probes in systems containing heat-induced complexes or to heat-induced changes in the local environment of the FITC probe within serum complexes. Indeed, heating increases the surface hydrophobicity of the complexes (*22*) and could lead to a further binding of free FITC to heat-treated whey proteins compared with native ones. Heating can also affect the local environment of the probe such as exposure of hydrophobic domains (*22*) or a modification of local charges, which may affect the quantum yield.

Systems C1S2, C2S1, and C2S2 that contained heat-induced complexes started to show arrested diffusion at ~pH 5.0 only, and network formation was visible in all systems at pH 4.81. Confocal microscopy therefore proved a less sensitive method to detect the onset of gelation in acid milk gels than rheology, as also observed by Dubert-Ferrandon et al. (*23*). Within this limitation of the method, CSLM in-time images did not evidence any particular sequence in the aggregation of either the casein micelles or the heat-induced serum complexes. In both C2S2 and C1S2, RITC-labeled casein micelles and FITC-labeled serum complexes aggregated simultaneously, and separation of the two fluorescence signals at pH 5.0 or 4.8 showed superimposable images, as shown in **Figure 3** at pH 4.6.

DISCUSSION

Observation of the acid gelation behavior of reconstituted milk systems with or without heat-induced serum or micelle-bound complexes (C1S1, C1S2, C2S1, and C2S2), of only casein micelles with or without micelle-bound complexes (C2S0 and C1S0), and

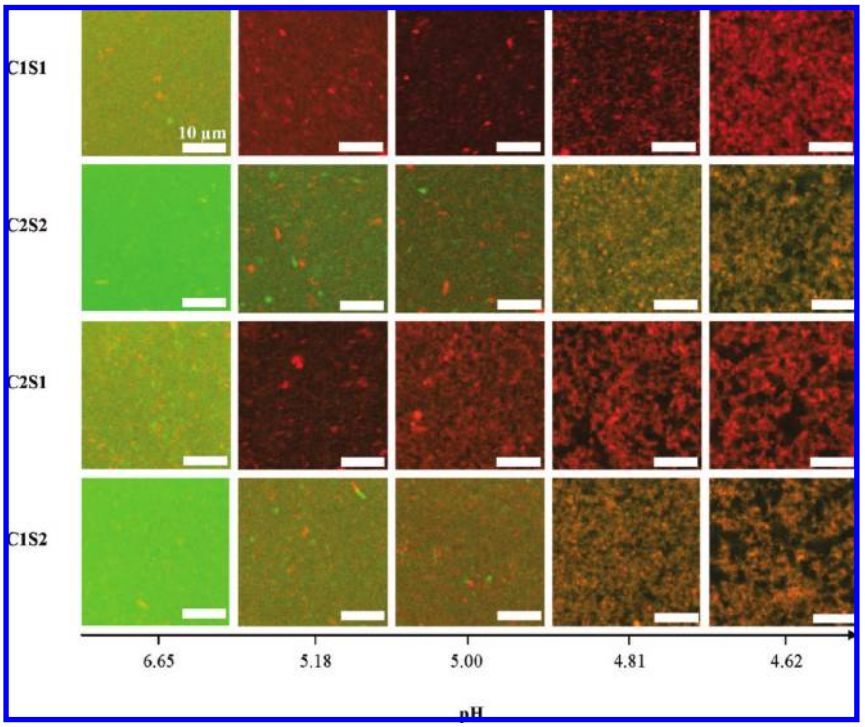

Figure 4. Change in the FITC/RITC overlay images of the reconstituted unheated skim milk system C1S1, of heated skim milk system C2S2, of combined skim milk system with only micelle-bound complexes C2S1, and of combined skim milk system with only serum complexes C1S2 at ~35 g kg^{-1} total protein, as observed by confocal scanning laser microscopy in the course of acidification by 19.5 g kg^{-1} GDL at 35 °C. The serum phase S1 or S2 of the milk systems was labeled with FITC and appeared green, while the colloidal phase C1 or C2 of the systems was labeled with RITC and appeared red on the confocal images. Overlay images (green + red) appeared yellow to red depending on systems.

of the serum phase of heated milk (S2) clearly showed that early and firm gels could only be formed in the presence of both the casein micelles and heat-induced complexes (micelle-bound or serum or both). In the absence of casein micelles (S2), gelation started at high pH but yielded low final G' (**Figure 2**). The absence of separated gelation of the serum complexes (S2) and of the casein micelles with time or in space in C1S2 and C2S2 (**Figures 3** and **4**), and similarity of the gelation behavior of C2S1 (only micelle-bound complexes) with that of C1S2 (only serum complexes) and C2S2 (both of them) strongly indicated that the heat-induced complexes were responsible for initiating destabilization of the whole milk protein system (i.e., including caseins) at pH ~5.3 and for enhanced viscoelastic moduli of the gel, probably through enhanced interactions between the casein micelles. Dubert-Ferrandon et al. (*23*) also reported that both the casein and whey protein fractions of heated milk were involved in the network from the point of gelation onward, although no distinction between serum and micelle-bound complexes was made. The absence of obvious differences in the rheological and microstructural changes of the forming acid gels as a function of the initial location of the heat-induced complexes in the serum or the colloidal phase of milk (**Figure 3**; Guyomarc'h et al. (*22*)), as well as colocation of the heat-induced complexes and casein micelles in the gels throughout acidification (**Figure 4**), further suggested that the heat-induced serum complexes interacted with the casein micelles at pH ~5.3 or earlier in the course of acidification. Therefore, systems C1S2, C2S1, and C2S2 were, in essence, probably similar in terms of micelle/serum distribution of the complexes at the time gelation started. This interpretation is in line with Alexander and Dalgleish (*42*) and Donato et al. (*43*) who proposed that the heat-induced serum complexes interacted with the surface of the casein micelles earlier (i.e., at higher pH) than the actual onset of gelation. Conversely, it rules out the gelation scheme proposed by Guyomarc'h et al. (*44*) where the serum complexes would first gel at pH 5.3, followed by the casein micelles with or without micelle-bound complexes at pH ~5.0.

From the same comparisons, it is also evident that the heat-induced complexes bear a large responsibility in the development of a solid, porous, reticulated acid gel microstructure at pH 4.5. As a complement, Famelart et al. (*19*), Graveland-Bikker and Anema (*45*) or Vasbinder et al. (*18*) showed that heated whey protein-depleted or whey protein-free milk exhibited acid gelation behavior close to that of unheated milk (i.e., low gelation pH and final G' values). Because S2 alone yielded very soft acid gels, it is suggested that the casein micelles are the building material for the milk gel while the heat-induced complexes that are attached to their surface (either as micelle-bound complexes or as an effect of acidification on serum complexes) provide the casein micelles with new gelation functionality, such as increased attraction between particles (possibly through increased surface hydrophobicity, Guyomarc'h et al. (*22*)), increased rigidity of the connections (possibly through formation of new disulfide bonds, Vasbinder et al. (*46*)), increased number of interactions (*44*, *47*, *48*), or increased "anisotropy" of the interactions, favorable to formation of linear strands and pores rather than evenly distributed clusters. Major efforts have been given to conceptualize gel formation in acidifying milk, using, for instance, adhesive spheres, percolation, or fractal models. Horne (*49*) however points out that none of these models accounts for heat-induced changes in the gelation kinetics of acidifying skim milk. Clearly, a better experimental description of the heat-induced structure of whey protein-coated casein micelles and of the early steps of their interaction on acidification is needed to aim at modeling the formation of typically porous, solid, and water-keeping acid gels of heated milk.

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