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Role of the Soluble and Micelle-Bound Heat-Induced Protein Aggregates on Network Formation in Acid Skim Milk Gels

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Gel formation was monitored by low amplitude rheometry during acidification at 40 °C with 1.5% glucono-δ-lactone in combined milk systems containing soluble and/or micelle-bound heat-induced (95 °C/10 min) aggregates of denatured whey proteins and κ-casein and in heated dairy mixes with varying micellar casein/whey protein ratio (CN/WP). Both soluble and micelle-bound aggregates increased gelation pH and gel strength. Micelle-bound aggregates seemed to modify the micelle surface so that micelles were destabilized at a pH of 5.1 (instead of 4.7), while soluble aggregates precipitated at their calculated pI of ∼5.3, and initiated an early gelation by interacting with the micelles. Decreasing the CN/WP ratio produced larger aggregates with higher whey protein: κ-casein ratio, which gave more elastic gels. The specific effects of the micellar and soluble aggregates on gel strength are discussed with respect to their relative proportions in the heated milk.

KEYWORDS: Protein aggregation; heating; elastic modulus G′; acid gel; texture

INTRODUCTION

In many commercial dairy processes, thermal treatment of milk and dairy mixes is an obligatory operation to ensure the safety and shelf life of the final product and to improve the functional properties of the milk proteins. In yogurt manufacture, heating at 90−95 °C for 5−10 min considerably enhances gel firmness and homogeneity and reduces syneresis, in parallel with the extent of denaturation of the whey proteins (*1*−*4*).

Pearse et al. (*5*), Smits and Van Brouwershaven (*6*), Jang and Swaisgood (*7*), and others have shown that aggregates are formed between κ-casein and denatured whey proteins during heating, but little is known of the actual formation, size, composition, and other properties of the aggregates, or of their contribution to direct gel texture during acidification of the milk. Recently, Anema and Li (*8*) have shown that the composition and the amount of the whey protein/κ-casein complexes in the serum are strongly dependent on the conditions of heating, namely temperature and pH. We have also recently shown (*9*) that both soluble and micelle-bound forms of whey protein/κ-casein complexes could be isolated. Primary aggregates of β-lactoglobulin (β-lg) and α-lactalbumin (α-la) in a 3 to 1 ratio were probably involved in the aggregation between denatured whey protein and κ-casein. The final aggregates were 3.5−5.5 million Da in weight and probably over 10 nm diameter in size. By contrast, casein micelles are between 5×10^7 and 2×10^9 Da in weight (*10*) and have diameters of between 50 and 500 nm. Larger amounts of whey proteins in the milk or less available κ-casein (i.e., higher whey protein/casein ratio in the milk) gave larger soluble aggregates, with higher whey protein/casein ratios in the aggregates.

Early studies (*11*−*14*) using electron microscopy have shown the formation of linear aggregates of denatured whey proteins (and κ-casein) in heated milk, forming protrusions attached to the micelles or loose particles in the serum phase. These aggregates were associated with the higher gelation pH, stronger gel strength, and finer microstructure shown by acid gels made with heated milks. Application of low amplitude viscosimetry has allowed detailed and dynamic investigation of the acid-gelation of skimmed milk. Lucey et al. (*15*) have proposed that denatured whey proteins initiate early gelation at high pH values (related to the isoelectric pH of β-lg), after which the gelation was dominated by casein−casein interactions as acidification proceeds. O'Kennedy and Kelly (*16*) further suggested that increased gel strength and gelation pH were caused by the early interaction between denatured whey proteins and casein, while casein aggregation dominated gelation at lower pH. Compared to loose soluble heat-induced whey protein/κ-casein aggregates, the micelle-bound aggregates have often been found to exert a major effect on gel formation (*12*, *15*, *17*). There are, however, some conflicting reports. O'Kennedy and Kelly (*16*) found a larger impact of soluble denatured whey proteins mixed with unheated micelles than of the heated mix of native whey proteins and micelles. In addition, micrographs of Kaláb et al (*12*) did not show large differences between gels formed from heated

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whey and unheated micelles, and gels formed from heated micelles and unheated whey, even after further heating of the mixture. The different studies raise the question of the importance of the type of whey protein used (isolated serum or reconstituted WPI) and of the effect of the presence of casein on the preparation of the aggregates. We have shown (*9*) that these conditions will define the size and composition of the aggregates in the serum, especially with respect to the amount of κ-casein incorporated in them. The change in composition may alter their ability to interact with the micelles on acidification and also modify their function in the gel network (active or passive fillers, or structure breakers).

The goals of the research described in this paper were (1) to provide further understanding of the acid-induced gelation of heated skim milk by using endogenous material and (2) to extend this understanding to dairy mixes close to those used in yogurt manufacture (4.7% total protein content, varying protein ratios) and their effect on the formation and final quality of acid milk gels.

MATERIALS AND METHODS

Reconstituted Milk and Milk Ultra-Filtrate. Skim milk powder, reconstituted skim milk, and skim milk ultra-filtrate (MUF) were made as described by Guyomarc'h et al. (*9*, *18*). The skim milk powder was prepared with very mild drying conditions to minimize lactosylation of the milk protein. The milk was reconstituted as 10% w/v in warm water + 0.05% sodium azide and was left overnight at 4 °C to complete reequilibration. The MUF was the permeate obtained by the filtration of reconstituted skim milk through a 10 kDa-cutoff membrane.

Other Materials. Sodium azide (NaN_3) was purchased from Sigma (St Louis, MO). Glucono-δ-lactone (GDL) was purchased from Fisher Scientific (Loughborough, UK). All chemicals were of analytical grade.

Heat Treatment 95 °C/10 min. The reconstituted skim milk systems were heated at 95 °C for 10 min using the small-scale tubular heat exchanger described in Guyomarc'h et al. (*9*). This heat-exchanger allowed almost instantaneous come-up time, and therefore a good control of the actual temperature of heating. This heat-treatment has been proved sufficient to denature >90% of the β-lg and α-la in skim milk (*19*) and exceeds what is generally applied in yogurt manufacture.

Preparation of milk Systems Containing 3.3% Crude Protein Combining Micelles of Heated or Control Milk with Serum of Heated or Control Milk, or Heated Serum. Heated (95 °C/10 min) and control (unheated) reconstituted skim milk were spun at 19 000 rpm (33 025*g* average) for 60 min on a Sorvall RC28S centrifuge equipped with a SS-34 fixed angle rotor (Kendro Laboratory Products Ltd, Hertfordshire, UK). Temperature was 20 °C ± 1 °C. The supernatant gently poured out of the tubes was defined as the serum; the remaining pellet was defined as the micelles. Draining and rinsing of the pellet were omitted to prevent protein loss. Micelles of control and heated reconstituted skim milk were designated as M0 and M1, respectively, and sera of the milks were designated as S0 and S1. Some of the serum of control reconstituted skim milk was heated at 95 °C for 10 min and was designated as S2.

The crude protein contents of M0, M1, S0, S1, and S2 were determined by macro-Kjeldahl analysis (*20*), using $N = 6.38$ as conversion factor. Six mixed systems were then produced by resuspending the appropriate amount of micellar pellet in 300 g of serum, so that the final crude protein content of the mix was 3.3%. These mixtures of heated and unheated micelles and heated and unheated sera were made as shown in **Table 1**.

Micelle suspension was carried out overnight at 4 °C using gentle stirring. Final dispersion was then achieved by homogenizing each mixture for 90 s with an Ultra-Turrax drive T25 (Janke and Kunkel GmbH, IKA Labortechnik, Staufen, Germany), set at its lowest speed. This treatment was shown not to alter the average hydrodynamic diameter of skim milk micelles as measured by photon correlation spectroscopy in a preliminary experiment (not shown). The six systems were then stirred gently overnight at 4 °C to complete the process.

Table 1. Compositions of the Six Milk Systems at 3.3% CP with Either Heated or Control Milk Parts

	M0S0	M1S1	M0S1	M1S0	M0S2	M1S2
micelles cont milk M0	X		X		X	
micelles heated milk M1		X		X		X
serum cont milk S0	X			X		
serumheated milk S1		X	X			
heated serum cont milk S2					X	X

Kjeldahl analysis of the crude protein content of the six systems confirmed that they all contained 3.3 ± 0.1% CP.

Fine protein composition of the 6 systems was also analyzed using the same RP-HPLC method used in the first part of this work (*9*). In the absence of internal standards for quantitative comparison, total area of the profiles were standardized on the basis of their consistent protein content. The results are shown in **Table 2**.

As expected, control heated (M1S1) and unheated skim milks (M0S0) showed similar overall compositions. Slightly lower contents in κ-casein and sometimes whey proteins were found in the heated milk, which was consistent over repetitions of the experiment. This could be attributed to some fouling during heat treatment of the milk. The M0S1 system was 25% richer in κ-casein and 13% poorer in whey proteins, because of the κ-casein that was solubilized into S1 during the heating of the milk, and the loss of some whey proteins from S1 as they were bound to casein micelles by the heat treatment. Similarly, the M1S0 system was 42% poorer in κ-casein and 7% richer in whey proteins. Here too, fouling could partly account for the unbalanced reciprocal transfers between the micelles and serum phases, but there may also be other experimental differences between unheated and heated skim milk that have remained unidentified. When the serum was heated separately (S2), the transfers between serum and micelles could not occur, and the composition of M0S2 did not differ from the control milks. M1S2 contained 33% less κ-casein than the controls but the same amount of whey proteins.

Differences in the casein/whey protein ratio (CN/WP) between some systems could also be accounted for by the heat-induced transfer of some whey proteins to the colloidal phase: at consistent CP content, more casein pellet M0 was needed to adjust S1 in M0S1, resulting in a higher CN/WP ratio; while the casein pellet M1 used to adjust S0 in M1S0 brought attached whey proteins and decreased the CN/WP ratio. Because of the nature of the complexes formed, it is difficult to work at a fixed ratio of proteins after the heating of the milk.

Skim Milk Powder Enriched in Casein Micelles or in Whey Proteins. Reconstituted skim milk was centrifuged at 12 000 rpm (14 750*g* average) and at 20 ± 1 °C for 2 h on the same Sorvall centrifuge as above, equipped with a GSA rotor. The final material showed a solid white pellet topped with a thick layer of white interphase. RP-HPLC of the protein in the initial reconstituted skim milk and in the final supernatant showed that the two white fractions together contained about 87% of the casein (i.e., most of the casein micelles of the milk). About two-thirds of the clear supernatant was then pumped out of the centrifuge bottles and stored at 4 °C. The casein-containing material was then homogenized as for the dispersion of the micelles in serum as described above, to give casein micelle-enriched skim milk.

Two volumes of the collected serum were mixed with one volume of reconstituted skim milk, then the protein: (minerals + lactose) ratio was brought slightly above milk's value by ultrafiltration of the mix to a concentration factor of 3. Both the retentate (whey protein-enriched skim milk) and MUF, as well as the casein micelle-enriched milk were then freeze-dried. The dried materials were crushed into powders and stored at −20 °C in doubled sealed bags.

Table 3 shows the composition of the three powders. The casein/whey protein ratio of the casein-enriched milk powder was 9.2, and those of the whey protein-enriched milk powder was 2.1.

Dairy Mixes at 4.7% TP with Varying Casein/Whey Protein Ratio. The appropriate compositions of 5 dairy mixes containing 4.7% total protein and varying casein/whey protein ratio were determined through matrix calculation of $|X| = |A|^{-1} \times |B|$; where $|A|$ is the matrix of data including the composition of the materials (skim milk powder,

Table 2. Protein Composition (g/L) of the Six Milk Systems as Obtained by RP-HPLC analysis

system	κ-casein	αs2-casein	αs1-casein	β-casein	total CN[a]	WP[b]	CN/WP
M0S0	2.6 ± 0.2	4.3 ± 0.3	9.5 ± 0.0	11.2 ± 1.1	27.7 ± 1.2	6.8 [c]	4.1
M1S1	2.2 ± 0.0	4.0 ± 0.1	10.0 ± 0.1	12.1 ± 0.1	28.4 ± 0.2	7.0 ± 0.2	4.1
M0S1	3.0 ± 0.1	4.6 ± 0.1	10.0 ± 0.0	11.8 ± 0.3	29.3 ± 0.4	6.0 ± 0.3	4.9
M1S0	1.4 ± 0.3	4.3 ± 0.4	9.9 ± 0.1	12.4 ± 0.2	28.0 ± 0.2	7.4 ± 0.2	3.8
M0S2	2.2 ± 0.1	4.6 ± 0.1	9.5 ± 0.4	12.4 ± 0.5	28.7 ± 0.3	6.7 ± 0.2	4.3
M1S2	1.6 ± 0.2	4.0 ± 0.1	9.9 ± 0.0	13.3 ± 0.4	28.7 ± 0.1	6.8 ± 0.1	4.2

[a] CN = casein [b] WP = whey proteins. [c] Values are the average of 2 aliquots prepared per system, except where only one value was valid.

Table 3. Composition of the Starting Skim Milk Powder and of the Casein-Enriched Skim Milk Powder, the Whey Protein Enriched Skim Milk Powder and the MUF Permeate Prepared from It[a]

	skim milk powder (%)	casein-rich powder (%)	WP-rich powder (%)	MUF permeate (%)
dry matter[b]	97	89.34	94.43	94.39
total protein[c]	33.93	45.32	42.46	0.27
casein[d]	28.11	40.89	28.91	0.13
whey protein[d]	5.82	4.43	13.55	0.14
lactose[e]	48.80	31.05	39.96	80.55

[a] The results are in g/100 g. [b] BS 1743 (*20*). [c] BS 1741 (*21*). [d] Precipitation of the casein with tungstic acid and Kjeldahl analysis of the soluble fraction (NCN). Precipitation of all the milk proteins with TCA and Kjeldahl analysis of the soluble fraction (NPN). Casein = TP − NCN. Whey protein = NCN − NPN. [e] Calibrated polarimetric measurement.

casein micelle-enriched milk powder, whey protein-enriched milk powder, dried MUF, and distilled water) in total solids, total protein, whey protein and lactose; |B| the matrix of target concentrations; i.e., TP 4.7%, lactose 5.3%, protein from skim milk powder 2.1%, and casein whey protein ratio x of 3.0, 3.9, 4.8, 5.7, or 6.6 w/w (defined as the proportion of whey protein in the total protein fraction, i.e., 4.7/(1+x)%); and |X| the matrix of the unknowns (i.e., the proportion of each ingredient). Because the materials involved were made from the same skim milk powder, it was assumed that the adjustment of lactose with dried MUF allowed adjustment of all other plasma elements (minerals and salts). Only one combination existed for each casein/whey protein ratio. The mixes were reconstituted as described above with the appropriate blend of the powders. All the mixes had pH values of 6.70 ± 0.02 and 6.67 ± 0.02 before and after heating, respectively.

Syneresis of Acid Skim Milk Gels. The method was adapted from Dannenberg and Kessler (*1*). Set gels were obtained by addition of 4% GDL in 25 mL of each skim milk system (**Table 1**) placed in standard closed plastic containers, with aluminum foil between lid and container. Samples were shaken for 1 min and incubated upside down at room temperature for 2 h, during which time gelation occurred. The lid was then removed and the container placed on a disk of Whatman paper no. 41 supported on a metal grid above a funnel and measuring cylinder. The foil was slid sideways to allow full contact of the gel with the filter paper, with minimum stress of the gel's structure. A pinhole was pierced into the container's bottom to prevent depression. Syneresis was defined as the volume of serum exuded from the gel and collected in the cylinder over a period of 18 h at +10 °C.

Viscoelastic Properties of Acid Skim Milk Gels. The elastic modulus G′ and loss tangent tan δ of the gels made from the six reconstituted systems by addition of 1.5% GDL and incubation at 40 °C were followed during gel formation by low amplitude dynamic oscillation viscosimetry using a Bohlin VOR rheometer 19:02 (Bohlin Instruments, Cirencester, UK) equipped with a C25 cup-and-bob measuring geometry. After addition of GDL, the milk was stirred for 1 min, then 13 mL was transferred to the rheometer and the geometry's gap was covered by an adapted O-ring to prevent evaporation. Measurements were made at a constant strain of 1%, at a frequency of 0.1 Hz, and the stress generated by the resistance of the gel was sensed with a calibrated torsion bar (nominally 1 g · cm). Measurements were taken every 30 s over a period of 9 h. In parallel, pH was measured at regular intervals on the remainder of the sample (maintained at 40 °C in a thermostated water-bath) with a Mettler-Toledo 345 pH-meter equipped with a Russell electrode (Russell pH, Fife, UK).

RESULTS

Specific Effects of Soluble and Micellar Aggregates on Acid-Gelation of Skim Milk. The development of viscoelastic properties in gels made by acidification with GDL of dairy systems combining micelles of control or heated reconstituted skim milk (M0 and M1, respectively) with serum of control or heated reconstituted skim milk (S0 and S1, respectively) or heated serum of reconstituted skim milk (S2), were measured. **Figure 1** shows the increase in elastic modulus G' during acidification in the M1S0 and M0S1 systems (i.e., containing only micellar or soluble heat-induced aggregates, respectively) compared with control milk M0S0 and heated milk M1S1. The systems M0S2 and M1S2 are not shown in **Figure 1** for reasons of clarity.

The control skim milk M0S0 began to gel at pH 4.7 (i.e., the isoelectric pH of casein micelles). G' increased slowly, following a sigmoidal curve versus time, and the final G' did not exceed 15 Pa at the end of the 9-hr run. In contrast, heated skim milk M1S1 had an earlier gelation at about pH 5.3 and higher final values of G' around 205 Pa. In this milk, the value of G' followed a double sigmoid time curve, with a plateau in the G' versus t plot that also showed in the G' versus pH plot between pH 5.1 and pH 5.0 (**Figure 1**). In the control milk M0S0, tan δ decreased throughout the run, following a decreasing hyperbolic time-curve tending toward a final value of 0.3. In heated milk M1S1, the values of tan δ tended toward a final value of 0.15, but had a marked maximum of ∼0.5 at pH 5.0 (not shown). These profiles were similar to those obtained with the starting unheated and heated skim milks used to make the corresponding M0S0 and M1S1 systems, respectively; only gel strength was slightly lower (not shown). These patterns were in accordance with other reports (*3*, *4*, *15*, *22*−*24*) and demonstrated that a heated milk produced stronger and more elastic gel structure. The profile of M0S2 (not shown) superimposed on that of M1S1. The profile of M1S2 started at a slightly earlier gelation pH of ∼5.4 had about only 5 Pa-higher G' values than M1S1 during the exponential growth of G' but plateaued toward the same final G' value (not shown). It was evident that all the systems containing serum aggregates (i.e., M0S1, M1S2, and M0S2) behaved like heated milk M1S1 during acidification with respect to the pH and time courses of both G' and tan δ. The M1S0 system, containing micellar aggregates and undenatured serum protein, showed an intermediate profile. It gelled at pH 5.1, followed a single sigmoid time curve and reached ∼85 Pa after 9 h. This pattern was also found by Kelly and O'Kennedy (*25*) for phosphocasein. Similarly to M0S0, the values of tan δ for M1S0 did not show any intermediate maximum, but tended toward 0.2, a value closer to that of heated milk and the mixtures containing aggregate particles in the serum. It therefore seemed that micellar

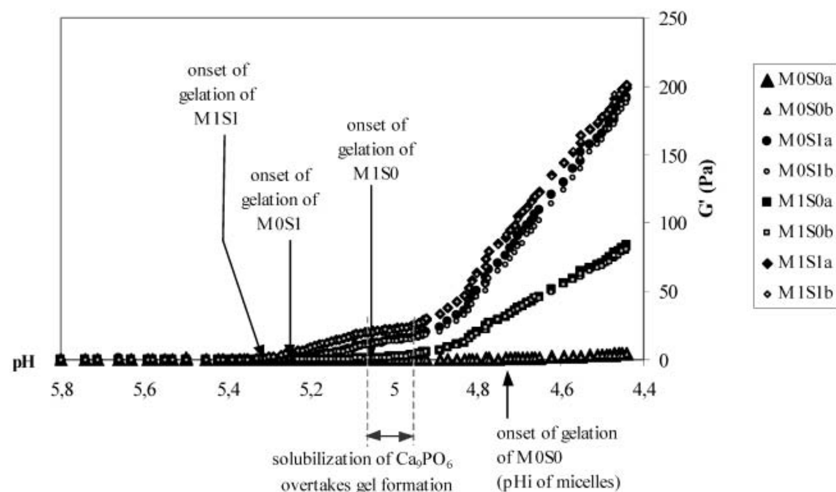

Figure 1. Effect of the occurrence of heat-induced (95 °C/10 min) soluble or micellar whey protein/κ-casein aggregates in milk on the profile of G' during acidification, plotted as a function of pH. The systems were made of micelles of heated (M1) or control milk (M0) resuspended in the serum of heated (S1) or control (S0) milk. There were two runs a and b for each type of system.

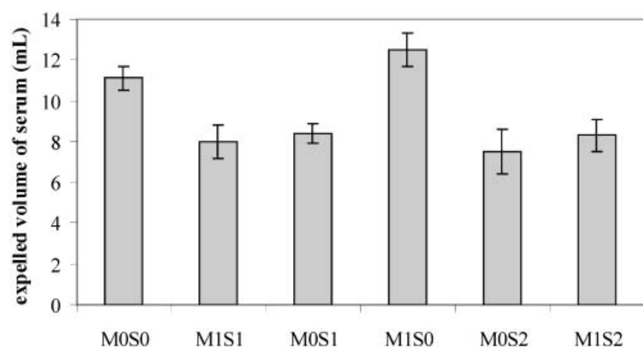

Figure 2. Volume of serum drained from standardized acid gels of milk systems containing heat-induced (95 °C/10 min) soluble or micellar whey protein/κ-casein aggregates, during 18h at +10 °C. The systems were made of micelles of heated (M1) or control milk (M0) resuspended in the serum of heated (S1) or control (S0) milk.

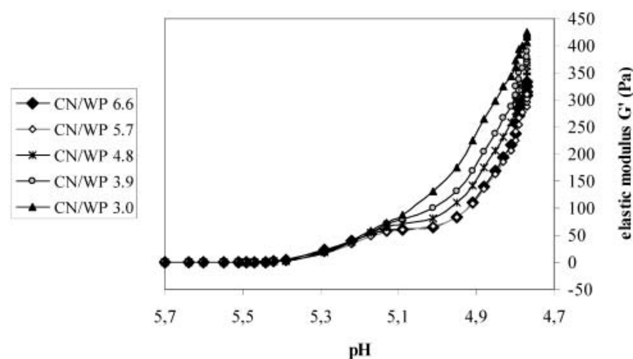

Figure 3. Effect of the casein/whey protein ratio (CN/WP) of dairy systems at 4.7% total protein on the profile of G' during acidification, plotted as a function of pH. Two runs were carried out for each CN/WP value (repetition not shown).

aggregates alone only allowed a moderate increase of gelation pH and final G', while the occurrence of aggregates in the serum gave larger increases in these responses, no matter whether micellar aggregates from heated milk were present (**Figure 1**).

The reason for the S2-containing systems M0S2 and M1S2 showing slightly higher G' values than the corresponding systems M0S1 and M1S1 may arise from the larger size and different composition of the aggregates formed in S2 (*9*), and their higher efficiency in bridging casein micelles on acidification and in giving a more reticulate and more resistant gel (*26*).

Syneresis of the Acid Skim Milk Gels. Standard acid skim milk gels were prepared from every system by addition of 4% GDL and incubation at room temperature. **Figure 2** shows the average amount of serum expelled by the gel overnight at +10 °C. Two significantly different groups were found ($p < 0.01$). Control skim milk M0S0 and skim milk containing only micellar aggregates M1S0 both exuded a large amount of serum (11−12.5 mL for a 25-mL gel), while all skim milk systems containing at least serum aggregates (i.e., M0S1, M1S1, M0S2, and M1S2) exuded only about 8 mL of serum.

In accordance with the results found with viscoelastic measurements, it seemed that the presence of aggregates in the serum enhanced the serum binding capacity of the gel and/or reduced gel contraction, probably through the increase in density of gelling protein particles (soluble aggregates + micelles) and the consequently decreased permeability of the gel, the high water-binding capacity of particles involving denatured whey proteins (*1*), and lower brittle character and higher flexibility of the protein network, as concluded by Puvanenthiran et al. (*26*). However, most of the published studies observed syneresis in co-heated systems (i.e., M1S1 or M1S2). Schorsch et al. (*17*) used preheated WPC in casein dispersions and found that their system resembling our M0S2 exuded more serum than it did after further heating (i.e., ∼M1S2), both systems being far more prone to syneresis than the co-heated and even the unheated blend of low-heat WPC and casein. Because the gel network appears different when the whey proteins and the casein are heated separately or co-heated, the source of whey proteins seems also important. In particular, preheated WPC as used by Schorsch et al (*17*) would form very large aggregates containing no κ-casein (unpublished results), that might interact differently with the casein micelles on gelation, compared with "endogenous" heat-induced aggregates.

Effect of the CN/WP Ratio. Figure 3 shows the G' profiles of the five heated dairy mixes at 4.7% TP and with different casein/whey protein ratios, during acidification with GDL. **Figure 4** shows the corresponding time-curve of G' (**Figure 4a**) and of tan δ (**Figure 4b**). The profiles matched those found in reconstituted skim milk (**Figure 1**), except that the final pH tended toward 4.7 instead of 4.3, and the final G' also tended toward higher values, above 300 Pa. These changes were explained by the higher TP content of these systems, which

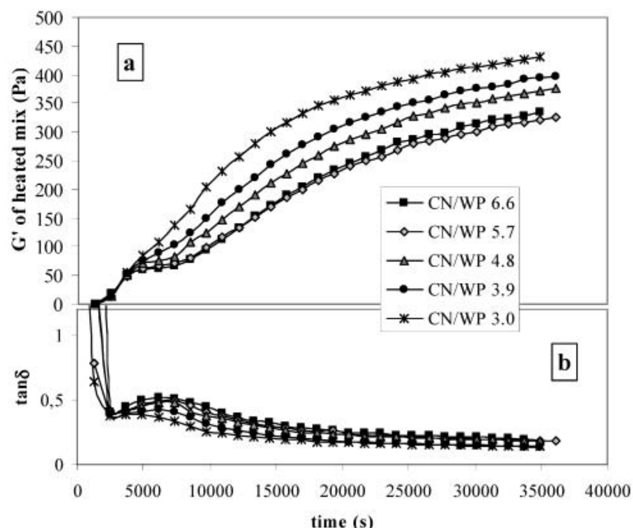

Figure 4. Effect of the casein/whey protein ratio (CN/WP) of dairy systems at 4.7% total protein on the profile of G' (a) and of tan δ (b) during acidification, plotted as a function of time. Two runs with identical results were performed for each CN/WP value. (Repetition not shown).

provided higher buffering capacity toward a fixed amount of GDL, and a greater amount of material for network formation.

Figures 3 and **4a** show that the higher proportion of whey proteins in the mix (i.e., the lower the casein/whey protein ratio), the higher were the G' values and the earlier was the resumption of G' growth after the plateau starting at pH 5.1. At the end of the run, G' ranged from 300 to 450 Pa as the casein/whey protein ratio decreased from 6.6 to 3.0. **Figure 4b** also shows that the maximum of tan δ shifted toward higher pH values as the casein/whey protein ratio decreased, following the pH shift of the resumption of G' growth. These observations confirmed that both the resumption of G' after the plateau and the maximum of tan δ related to the same event(s) then occurring in the system, which was (were) affected by the casein/whey protein ratio in the mix.

The increase in G' value at final pH in relation with lower casein/whey protein ratio could partly be explained by a shorter plateau around pH 5.1, that is, a shorter period of suspended G' growth over a standard 9-hr run. However, tan δ also tended toward lower values as the proportion of whey proteins increased in the mix, shifting from 0.3 to 0.1, thus showing that the gels were also positively stronger and more elastic when more whey proteins were present.

The study of both serum and micellar aggregates by SEC and RP-HPLC (*9*) has demonstrated a positive relationship between the whey protein/κ-casein ratio in the milk and the whey protein/κ-casein ratio in the aggregates, the size, and concentration of the aggregates. Combined results then suggested that the higher proportion of whey protein in the mix, the higher whey protein/κ-casein ratio in the aggregates, the larger and more numerous the aggregates (*9*), and the larger the increase in G' (i.e., the stronger the gel formed by the aggregates and casein micelles). This pattern was in agreement with the results of Kelly and O'Kennedy (*25*) using blends of heated WPI and phosphocasein in simulated milk ultrafiltrate with increasing proportion of WPI. Puvanenthiran et al. (*26*) also found that varying the casein/whey protein ratio of yogurts using skim milk and WPC led to higher gel strength and more elastic behavior on relaxation using an Instron texture analyzer, albeit the initial gradient (approximately equivalent to G') tended to decrease.

DISCUSSION

In agreement with previous authors, we identify the two major effects of the heat-induced whey protein/κ-casein aggregates on the properties of acid skim milk gels as (1) an increase in the pH of the onset of gelation and (2) an increase in gel strength as measured by the elastic modulus G'. Our results show that both responses seemed more affected by the presence of soluble aggregates than micellar ones, as shown by the closeness of the G' profiles of M0S1 and M1S1 and their difference from M1S0.

It is possible to interpret the results showing the increase in the pH of gelation by assuming that aggregates mostly formed of β-lg ($pI = 5.3$), κ-casein ($pI = 5.4$) and α-la ($pI = 4.8$) (*27*) will have an overall isoelectric pH of $\sim$5.3. The binding of these aggregates to micelles is very likely to modify the surface properties of the micelles. Depending on the quantity and size of the aggregates attached to the micelles, the apparent overall pI of micelles of 4.7 in normal milk will therefore increase toward the higher value of 5.1, as suggested by the gelation pH of the M1S0 system (**Figure 1**). Soluble aggregates, not being involved as part of micelle particles, would precipitate or perhaps form a weak gel at their actual pI of $\sim$5.3, as shown by M0S1 and M1S1 in **Figure 1**. This aggregation appears to initiate the destabilization of the micelles and bring about further gelation of the milk, presumably through the interaction between the soluble aggregates and the micelle surfaces as the former precipitate. Hydrophobic interaction between the aggregates and partly neutralized micelles is possible, because the repulsive charge interactions will be decreased, not only by the titration of negatively charged groups but also because of the increased ionic strength in the serum phase caused by the dissociation of colloidal calcium phosphate. It is also possible that the soluble aggregates first interact with the readily accessible soluble casein material found by SEC and RP-HPLC (*9*), possibly promoting or even allowing further interaction with the micelles. However, this soluble casein material cannot be taken to be responsible for the observed changes in G', as its peak area did not vary with the protein ratio and, rather, decreased on heating (*9*).

Vasbinder et al. (*28*) have shown that the pH of gelation of heated skim milk (70 to 90 °C for 10 min) was related to the amount of denatured whey protein in the milk, especially with denatured β-lg. The authors attempted to relate this relationship with the extent of coverage of the micelle surface with denatured β-lg (i.e., with bound whey protein), but further work by the same group (*29*) rather suggested a different effect of either the soluble or micelle-bound heat-induced whey protein aggregates on the pH of gelation. By plotting the gelation pH of heated milk versus the percentage of total denatured β-lg, the denatured β-lg in soluble aggregates and the denatured β-lg in micellar aggregates, they found three linear relationships, with the highest slope (and therefore, the highest impact) being those of the soluble aggregates. This result tends to confirm our hypothesis that the soluble aggregates are responsible for a larger increase of the gelation pH in heated skim milk compared to micelle-bound ones. The authors did not, however, draw any conclusions, because the ratio between soluble and micellar aggregates was constant across the samples and could not permit a specific observation of the effect of each type of aggregate on the relationship between the amount of denatured β-lg and the gelation pH.

Other changes in the properties of the micelle surface caused by the aggregates could account for early micelle destabilization. Aggregates of 10−60 nm in size (*9*, *29*) would, for instance, hinder the stabilizing 6−12-nm thick hairy layer of κ-casein,

as suggested by Horne & Davidson (*30*), although it is possible that this has collapsed during the decrease of pH (*31*, *32*). Protrusion of the aggregates as well as enlargement of the micelle would help coagulation (*33*). Anema and Klostermeyer (*34*) have also suggested that the modified micelles are more sensitive to calcium, and there could also be changes in their surface hydrophobicity.

The impact of the aggregates on gel elasticity could first be accounted for by the considerable increase in the number of gelling particles in skim milk after heating, several aggregates being possibly formed for each micelle (*9*). This would therefore increase the complexity, and decrease the porosity, of the acid gel. Disulfide bridges involved in the aggregates also introduce rigid covalent bonds in the gel network, whereas a casein acid gel is mostly based on hydrophobic interactions. The aggregates play a very active, specific role in connecting the micelles together on acidification, as is suggested by the dramatic effect of no more than 20% of the total protein content, or even less, on the gel elasticity (**Figure 1**). Serum aggregates could also enhance the continuity of the gel network and help trapping of the serum phase, as suggested by **Figure 2**. A wider pH range for gel formation, as when gelation is induced at high pH, would also give the gel more time to organize during a standardized acidification process. This could find an application in pH-monitored processes such as yogurt-making, where fermentation is stopped when pH reaches 4.6, no matter at what pH the onset of gelation occurs.

Figure 1 showed that M0S1, containing soluble whey protein/κ-casein aggregates, exhibited a higher gelation pH and final G' than M1S0, containing micelle-bound whey protein/κ-casein aggregates; indeed, M0S1 behaved similarly to heated skim milk M1S1. In addition, **Figure 1** showed that the slope of G' growth with time and with pH was lower in M1S0 than in M0S1 or M1S1, suggesting that soluble whey protein/κ-casein aggregates were more effective than micelle-bound ones in building the gel network. Our results are in agreement with those of O'Kennedy and Kelly (*16*) and Kelly and O'Kennedy (*25*), who found that serum aggregates alone enhanced the rate of formation and strength of acid skim milk gels. However, it is generally accepted that it is the interaction between denatured whey protein and micelle that enhances yogurt's quality. Lucey et al. (*15*) or Kaláb et al. (*12*) found that micellar aggregates alone rapidly generated elastic gels, while serum aggregates had a much smaller effect. However, all these results should be considered with respect to the amount of denatured whey protein present in the milk system, as suggested by Vasbinder et al. (*28*, *29*), because only denatured whey protein and casein can contribute to acid gelation and gel firmness (*4*). Lucey et al. (*15*), for instance, found that 80% of the β-lg in skim milk was bound to the micelles after heating, which agrees with a majority of reports (e.g., *6*, *29*, *35*, and *36*) where over 50% and up to 100% of β-lg was micelle-bound after heating. In our case, various results rather indicated that only a small amount (10−15%) of β-lg was attached to the micelle after heating:

- The RP-HPLC analysis of M1 and M0, re-suspended in MUF permeate and standardized to the same average casein content as control milks (M0S0 and M1S1), showed that M1 contained about 15% of the total whey proteins, excluding those being mechanically trapped in the pellet, as detected in M0.
- **Table 2** indicated that M0S1 had 13% less, and M1S0 7% more whey protein than the control milk, the difference being possibly due to fouling (i.e., not all whey protein that leaves the serum goes on the micelles) or other experimental source of error.
- Quantitative analysis of the milk proteins in the 4.7% TP milk system of CN/WP ratio of 4.8 (i.e. as in milk) showed that 9−12% of β-lg was lost by the serum phase during heating. Having corrected the loss due to fouling (the initial values were ~30%), this figure was the actual amount of β-lg attached to the micelles.

In comparison, the BDWP system of Lucey et al. (bound denatured whey protein, i.e., M1S0) (*15*), would be expected to contain 70−80% of the whey protein in a denatured form, while our corresponding system M1S0 would contain only ~10−15%. Also, **Table 2** indicated that this mixture had a slightly lower CN/WP ratio compared to the control. M0S1, on the other hand, would then contain up to 90% of the initial β-lg, in a denatured form, while its CN/WP ratio was slightly higher than that in the control. This probably largely accounts for the higher effect of the soluble aggregates in our study. In that case, the conflicting results of the present study and some of the literature might be explained only by the quantitative amount of aggregates present in the systems, independently of whether they are soluble or not.

Our lower rate of association of denatured whey protein to the micelle on heating can be explained by three causes. First, we took the fouling of some denatured whey protein into account in our calculation (i.e., whey protein that left the serum but did not bind the micelles) while most authors consider the amount of protein loss from the serum phase. Second, the low centrifugation speed that we used to separate the colloidal and the serum phase may have avoided the sedimentation of some of the soluble aggregates, as experienced by Lucey et al. (*15*), using 90 000g (up to 45% of soluble denatured β-lg was sedimented). Other centrifugation regimes used in the literature were ≥ 60 000g, and Anema and Li pointed out a relationship between the g force used and the amount of "micelle-bound" whey proteins (*8*). The use of rennet to separate the micelle and serum fraction (*29*, *35*) may induce the same bias, considering the size of the aggregates and the fact that they contain κ-casein. Finally, Anema and Li (*8*) reported a large effect of the pH at heating on the amount of whey proteins associated to casein micelles on heating. The instantaneous come-up time of our heating system may have considerably reduced the length of time the milk stayed at temperatures over 55 °C, which affects the heat-induced decrease in pH of the milk, and may have therefore limited the association of denatured whey proteins with the micelles compared to other studies.

A relationship between the heating of milk (i.e., the presence of whey protein/κ-casein aggregates) and the double-sigmoid feature on acidification at 40 °C has long been suggested (**Figure 2**) (*16*, *22*, *37*), but might not be straightforward. Horne (*38*) has indeed produced G' profiles with this double-sigmoid pattern from unheated milk acidified at 45 °C with high levels of GDL (≥4%), thus showing that the phenomenon must involve the casein micelles also. Several authors (*39*−*42*) have shown that the dissociation of colloidal calcium phosphate occurs between pH 6.7 and 5.1. According to Horne (*38*), the double-sigmoid pattern would therefore appear when gelation starts at pH higher than 5.1, where the dissociation of colloidal calcium phosphate is not yet complete. This is confirmed by the observation of a G' plateau in milk renneted at high pH and where acidification is also taking place (*43*, *44*). In this case, the weakening of the micellar structure would overlap and alter gel formation in the region slightly above pH 5.1.

Following the interpretation given above, the single sigmoid pattern of the unheated milk M0S0 or of M1S0 in **Figure 1**

can be explained by the pH of gelation values of these two milk systems of 5.1 or lower. Also, the earlier resumption of G' after the plateau in **Figure 3** could then be accounted for by the variation in the casein/whey protein ratio across the mixes. A decrease in this ratio indeed entails a decrease in the amount of colloidal calcium phosphate that has to be dissolved, which could lead to an earlier completion of the dissociation of colloidal calcium phosphate, and consequently shorten the G' plateau.

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