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Acid Gelation Properties of Heated Skim Milk as a Result of Enzymatically Induced Changes in the Micelle/Serum Distribution of the Whey Protein/κ-Casein Aggregates

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Changes in the acid gelation properties of skim milk as a result of variations in the micelle/serum distribution of the heat-induced whey protein/κ-casein aggregates, induced by the combination of heat treatment and limited renneting, were investigated. No dramatic change in the zeta potential or the isoelectric point of the casein micelles was suggested, whether the aggregates were all attached to the casein micelle or not. Fluorescence intensity measurement using 8-anilino-1-naphthalenesulfonic acid (ANS) showed that the heat-induced aggregates were highly hydrophobic. Dynamic oscillation viscosimetry showed that acid gelation using glucono-δ-lactone (GDL) started at a higher pH value in prerenneted milk. However, no change in the gelation profile of skim milk could be related to the proportion of aggregates bound to the surface of the casein micelles. The results support the idea of an early interaction between the serum aggregates and the casein micelles on acidification.

KEYWORDS: Milk; heat treatment; chymosin; whey protein; κ-casein; acid gel

INTRODUCTION

Heating skim milk at 85–95 °C for 5–15 min induces denaturation of the whey proteins and formation of heat-induced complexes of whey proteins and κ-casein that are located on the surface of the casein micelle and in the serum phase of milk (*1–5*). The occurrence of aggregates in heated milk has been linked to acid gels having an earlier onset of gelation, higher firmness, and lower syneresis than gels of unheated milk (*6–8*). The increased pH of gelation has been accounted for by changes in the apparent isoelectric pH of the protein system (*8, 9*), by the increased hydrophobicity of the particles present in the milk (*10, 11*), or by the reduced hydration (*12*) or steric (*13*) barrier between micelles. The improved texture could be attributed to the high water-binding capacity of the denatured whey proteins (*6, 14, 15*), an increased number of acid-sensitive particles in milk (*14*) yielding a more connected and denser network (*12, 15, 16*), and the involvement of covalent disulfide bonds in the gel structure (*16*) that might even form throughout acidification of milk (*17*). It is also suspected that the respective proportions of the micelle-bound and serum types of aggregates affect the onset and connectivity of the acid gel. Within a range of pH values between 6.3 and 8.1, it has been reported that the proportion of serum aggregates increased as the pH on heating increased (*1–3, 18–22*). This change in the distribution of aggregates in the milk was linked with higher gelation pH values, faster development, and higher final firmness of the acid gels (*3, 23, 24*). However, no such clear relationship could be found when the proportions of micelle-bound, serum aggregates, and/or heat-particulated whey proteins were varied in skim milk systems at pH 6.7 (*14, 16, 25–29*). A factor other than the micelle/serum distribution of aggregates may therefore be suspected in pH-dependent studies. In a recent study, it was reported that the combination of heat treatment at 90 °C for 10 min and 0–67% hydrolysis of the κ-casein could yield stable milk systems with different distributions of the heat-induced whey protein/κ-casein aggregates between the micelle and the serum phases of skim milk at its natural pH (*30*). When κ-casein hydrolysis by chymosin preceded heat treatment, only micelle-bound aggregates were formed to the extent that the average size of the casein micelles significantly increased, whereas the reverse sequence led to systems comparable to heat-treated milk. It was suggested that when renneting was performed first, the whey proteins preferably bound to nonrepulsive para-κ-casein on heating, yielding hydrophobic whey protein/para-κ-casein aggregates that were unable to dissociate and to form serum aggregates. In the present study, this opportunity to control the micelle/serum distribution of the heat-induced aggregates was

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used to investigate the respective role of the two types of aggregates on acid gel formation.

MATERIALS AND METHODS

Reconstituted Skim Milk. Milk was reconstituted as 100 g L^{-1} ultralow-heat skim milk powder (whey protein nitrogen index = 9.5) (*31*) and 0.5 g L^{-1} sodium azide in stirred deionized water at 40 °C. The milk was stirred for at least 1 h following complete dissolution and then left overnight at 5 °C to complete equilibrium.

Milk Ultrafiltration Permeate (MUF). The serum phase of heated (90 °C for 10 min) or unheated skim milk was separated from the casein micelles using ultracentrifugation and was ultrafiltered at room temperature using a 10 kDa cutoff stirred cell system (Pall Life Science, St Germain en Laye, France). To measure the zeta potential of isolated milk protein materials as a function of pH, milk permeates were also prepared using unheated skim milk samples previously adjusted at pH values ranging from 2 to 8.

Isolated Milk Protein Materials. Micellar casein was separated from skim milk using microfiltration (0.1 μm cutoff, SCT Ceramics, Tarbes, France) and diafiltration against deionized water (*32*) and was kept frozen until used. Heat-induced whey protein/κ-casein aggregates were isolated from the ultracentrifugal supernatant of heated (90 °C/ 10 min) skim milk using size exclusion chromatography (SEC) on Sephacryl S-500 with an aqueous mobile phase at pH 7, extensive dialysis, and freeze-drying as described in Jean et al. (*11*). The aggregates were reconstituted in a 5 g L^{-1} suspension in MUF and equilibrated overnight under continuous stirring.

Skim Milk Systems. Skim milk systems having different micelle/serum distributions of the heat-induced whey protein/κ-casein aggregates were prepared using different combinations of heat treatment and renneting, as described in Renan et al. (*30*). Only heat-treated or only renneted skim milk samples were also prepared as reference samples. Briefly, 200 μL L^{-1} of freshly diluted (10% v/v in deionized water) recombinant chymosin (Maxiren 180 batch AG1520, 900 mg L^{-1} enzyme, 180 IMCU, DSM, Seclin, France) was added to previously heat-treated [90 °C/10 min with extensive denaturation of the whey proteins (*6, 7*)] or unheated skim milk and incubated for 0, 3, 5, or 7 h at 5 °C to make the "heated then renneted" (HT_0 h−HT_7 h) and the "renneted only" (0−7 h) milk samples, respectively. Inactivation of the chymosin was obtained by heat-treating the samples at 70 °C for 1 min, when heat-induced changes on the whey proteins are negligible compared to those obtained at 90 °C/10 min. "Renneted then heated" milk samples (0h_HT−7h_HT) were obtained by incubating unheated skim milk with chymosin at 5 °C for 0–7 h and by combining inactivation of the enzyme and heat treatment using incubation at 90 °C for 10 min. The low renneting temperature slowed the reaction and allowed heat treatments to be performed after every sampling. Renan et al. (*30*) reported that the rate of conversion of the κ-casein into para-κ-casein and caseinomacropeptide increased from 0 to ~65% in the only renneted (h) and the renneted then heated (h_HT) samples, and from 0 to 50% in the heated then renneted (HT_h) samples as the incubation time increased. No residual enzymatic activity could be found in any of the samples. Despite the fact that both the protein hydrolysis and heat treatment may slightly lower the pH of milk, no significant pH change was measured as a result of these treatments. In HT_h milk samples, heat-induced whey protein/κ-casein aggregates were found in both the serum and the micelle phases of milk, and their distribution between the two phases was about the same as that of only heat-treated milk HT_0 h or 0h_HT. In h_HT milk samples, however, the increase in particle size with renneting (+5−20% size increase as conversion increased from 36 to 67%) and the protein composition of the serum and micelle phases of the milk samples showed that almost all of the whey protein/κ-casein aggregates were bound to the casein micelles, possibly as a result of a preferred interaction between the denatured whey proteins and hydrophobic, hence immobilized, para-κ-casein.

It is known that calcium phosphate is displaced toward the colloidal phase on heating, thus lowering the ionic calcium concentration and contributing to adversely affect rennet action (*33–35*). This partly explained that lower conversion rates of κ-casein were obtained in HT_h compared to h or h_HT milk samples (*30*). However, as both h_HT and HT_h systems eventually received similar heat loads, it was not believed that the calcium phosphate equilibrium varied across these two systems on the time acid gelation was studied.

Ultracentrifugation. Separation of the serum and colloidal phases of the milk samples was performed on 15 mL aliquots of milk using ultracentrifugation on a Sorvall Discovery 90 SE centrifuge (Kendro Laboratory Products, Courtaboeuf, France) equipped with a 50.2 Ti rotor (Beckman Coulter, Fullerton, CA). The samples were spun at 19400 rpm (~33000 average *g*) for 65 min at 20 °C. The supernatant was collected without further draining or washing of the pellets and was designated the serum phase of milk.

Zeta Potential ξ Zeta potential in the different milk samples was measured within 24 h after preparation on a Zetasizer Malvern 3000 HS (Malvern Instruments, Orsay, France). The laser was He−Ne, with a 633 nm wavelength. The samples equilibrated at 25 °C were diluted in the appropriate milk ultrafiltration permeate at 25 °C to meet the Zetasizer operating range and left at 25 °C for 15 min to ensure proper mineral equilibrium of the diluted system. The HT_h and h_HT samples were diluted in permeate of heated milk, whereas h samples were diluted in permeate of unheated milk. The isolated micellar casein and heat-induced aggregates were diluted in a range of permeates of unheated skim milk adjusted at various pH values. The dynamic shear viscosity of permeates was 0.99 mPa·s at 25 °C. The dielectric constant ε was taken as that of water (79 at 25 °C), and voltage was 125 V. The zeta potential is calculated from the measurement of the electrophoretic mobility of particles, assuming that they are spherical (*11, 19*) with a radius largely exceeding the thickness of their ionic double layer [taking the same Debye length as that of the casein micelles, i.e., ~1 nm (*36*) and a hydrodynamic radius of ~35 nm (*11*)].

Surface Hydrophobicity. Surface hydrophobicity in the milk samples and corresponding supernatants was estimated using the ANS-binding fluorometric assay. A concentration of 1.5 mM of fresh 8-anilino-1-naphthalenesulfonic acid (ANS) was dispersed in deionized water, left overnight at room temperature under constant stirring to allow solubilization, and then filtered through 1 μm pore size before use. Fifteen milligrams of each milk or 100 mg of each serum sample (having comparable amounts of total protein) was completed to a total weight of 3 g by addition of first the appropriate amount of MUF and then of up to 600 mg of ANS solution (i.e., up to 0.3 mM final concentration of ANS). The mixture was shaken to ensure homogeneity, and then the fluorescence of the sample was immediately measured on an LS 50B spectrophotometer (Perkin-Elmer, Saint Quentin-en-Yvelines, France). The range of ANS concentrations (from 0 to 0.3 mM) obtained in the mixtures corresponded to a range of the ANS: protein ratio from 0 to ~10^4 mol/mol for both milk and serum samples. It also corresponded to an estimated range of ANS: particle ratios of 0−10^8 mol/mol for the milk samples and 0−10^6 mol/mol for the serums of heated milk [taking molecular masses of 10^6 and 5 × 10^3 kDa for hydrated micelles and aggregates, respectively (*5, 37*)]. The excitation and emission wavelengths were 390 and 480 nm, respectively. The emission and excitation slits were both set at 2.5 nm bandwith. Only the maximum fluorescence intensity was considered.

Viscoelastic Properties of Acid Milk Gels. Formation of the acid gels was monitored by measuring the elastic (G') and viscous (G'') moduli of the system under acidification at 38 °C using an AR1000 rheometer (TA Instruments, Saint Quentin-en-Yvelines, France) equipped with a coaxial DIN geometry (lateral gap, 1.95 mm; bottom gap, 4 mm). After equilibration of a volume of 60 mL of milk at 38 °C, 11 g kg^{-1} of glucono-δ-lactone (GDL) was added and dispersed for 1 min under stirring prior to transferring the milk into the rheometer. The geometry was installed, and then a thin Inlab 423 pH probe (Mettler-Toledo, Viroflay, France) was put in place so that the pH could be measured directly in the rheometer and recorded (pHM220 Meterlab, Radiometer Analytical SAS, Villeurbanne, France). The applied deformation was 0.1%, frequency was 1 Hz, and gel formation was followed for at least 6 h. The milk sample was covered with a thin layer of mineral oil to prevent evaporation.

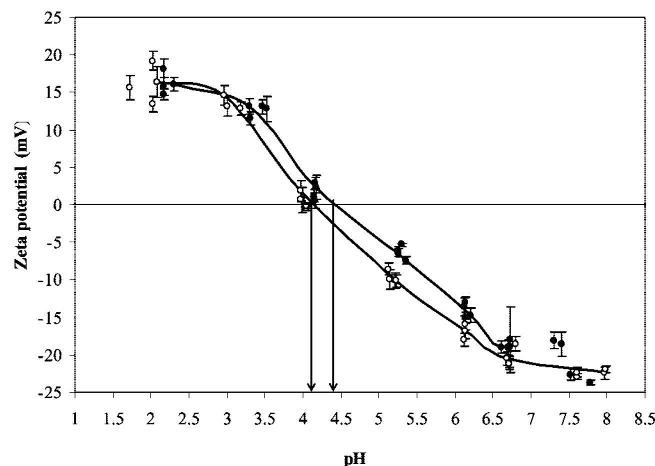

Figure 1. Zeta potential of heat-induced whey protein/κ-casein aggregates (●) and of micellar casein (○) dispersed in milk ultrafiltrates from a range of pH-adjusted skim milk samples. The arrows indicate the approximative isoelectric point of each type of protein particle.

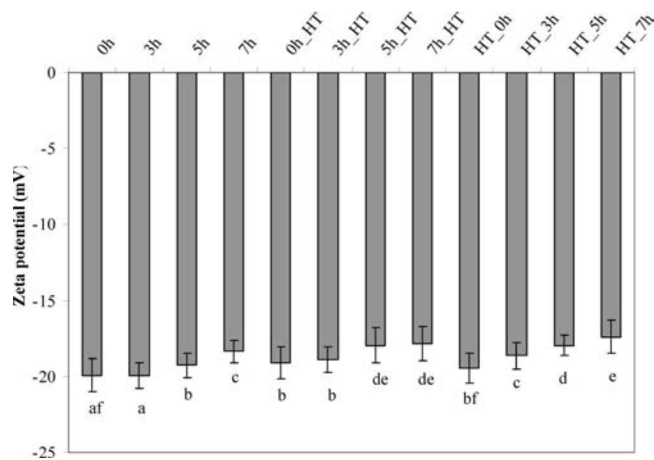

Figure 2. Zeta potential measured in milk samples at 25 °C after submission of unheated milk to chymosin for 0−7 h at 5 °C followed by heat treatment at 70 °C for 1 min (0−7 h) or at 90 °C for 10 min (0h_HT−7h_HT), or after submission of heat-treated (90 °C/10 min) skim milk to chymosin for 0−7 h at 5 °C followed by inactivation at 70 °C for 1 min (HT_0 h−HT_7 h). Different letters a−f indicate significant difference ($P_0 < 0.05$).

Significance. The presented results were obtained from two to four preparations of the samples, analyzed at least twice each. *t* tests and linear regressions were performed using Excel (Microsoft, Courtaboeuf, France).

RESULTS

Apparent Isoelectric pH of Particles. In heated milk systems, both the casein micelles and the heat-induced whey protein/κ-casein aggregates actively contribute to the formation of acid gels. **Figure 1** shows the changes in the zeta potential, ξ, of each of the protein particles at 25 °C and as a function of pH.

At the natural pH of milk (6.7), both the casein micelles and the heat-induced aggregates bear not significantly different negative charges of −20.3 ± 0.8 and −18.8 ± 1.7 mV, respectively ($P_0 < 0.05$). Comparable values were reported for casein micelles of unheated skim milk (*38–40*) and for the heat-induced aggregates (*11*) in milk ultrafiltrate at 25 or 30 °C. The zeta potential of both the casein micelles and the aggregates increased with comparable rates as the pH was decreased, except that the heat-induced whey protein/κ-casein aggregates consistently showed slightly higher values of ξ than the casein micelles. Although the method differs from isoelectrofocusing, which is the reference method for determination of the isoelectric point (p*I*), an "apparent" p*I* could be defined from the results as the pH value at which ξ is zero and changes sign. In the present conditions, the apparent p*I* values of the casein micelles and the heat-induced aggregates were ~4.2 and ~4.4, respectively. Although some conditions were assumed as being met for the calculation of an "effective" value of ξ (roughly spherical particles with radii >20 times larger than the Debye length), such calculation should be considered with care due to nonideal conditions such as softness and ion penetrability of milk protein particles, surface roughness, and possible chemical surface heterogeneity that will affect the position of the plane of shear (*41*). Of interest is also the fact that particles near their p*I* will move very slowly, making measurement of the electrophoretic velocity difficult. Casein micelles precipitated when pH was <5.3, and the aggregates precipitated at pH values ranging from ~3.5 to 5.3 (not shown). Reported data also using zeta potential calculation showed that the p*I* of casein micelles from unheated skim milk is indeed below 4.5 (*38*), whereas that of the heat-induced aggregates has been reported to be ~4.5 in similar conditions (*11*). The present results showed that the surface charge of the casein micelles and the heat-induced aggregates showed similar changes as a function of the pH of a milk medium, only that the values of ξ and therefore of the p*I* were slightly higher for the heat-induced aggregates throughout the pH 3−6.5 range in which variations are largest.

Zeta Potential of the Renneted and/or Heat-Treated Milk Samples. Changes in the overall zeta potential at 25 °C of the milk systems as a function of the extent of hydrolysis of κ-casein and of the sequence of renneting and/or heat-treatment are shown in **Figure 2**. Because the size of the casein micelles is large relative to that of the heat-induced whey protein/κ-casein aggregates occurring in heated systems, it is likely that the micelles are the essential origin of the changes in ξ described in **Figure 2**.

Comparison of the ξ values of unheated (h) and heat-treated milk (h_HT and HT_h) at each renneting time showed that heat treatment slightly decreased the zeta potential of the casein micelles by about 1 mV ($P_0 < 0.05$). Small heat-induced variations of the zeta potential of casein micelles were also reported by Anema and Klostermeyer (*38, 39*), although ξ increased on heating at pH 6.6 and decreased at pH 7.1. As expected, the absolute value of the zeta potential of the different milk systems significantly decreased as the incubation time and, hence, the extent of κ-casein hydrolysis increased. However, the amplitude of the decrease was <3 mV as up to 50–67% of the κ-casein was converted, depending on systems. This was less than the decreases of 6–8 or 7–8 mV previously reported just before (*38*) or after (*40*) completion of the enzymatic reaction, respectively. Aside from the above-described heat-induced decrease in ξ, no significant difference changes could be observed as a result of the applied treatments. Zeta potential decreased by 1.5–2 mV as the incubation time with chymosin increased from 0 to 7 h, whether the milk sample was unheated or heated prior to or after renneting. Considering the similarity between the zeta potentials of the casein micelles and of the heat-induced aggregates in pH 6.7 ultrafiltrate at 25 °C (**Figure 1**), this result is consistent with the fact that the occurrence of heat-induced aggregates either on the surface of the casein micelle (h_HT milks) or in both the serum and micelle phases of milk (HT_h milks) is unlikely to modify the surface charge of the casein micelles.

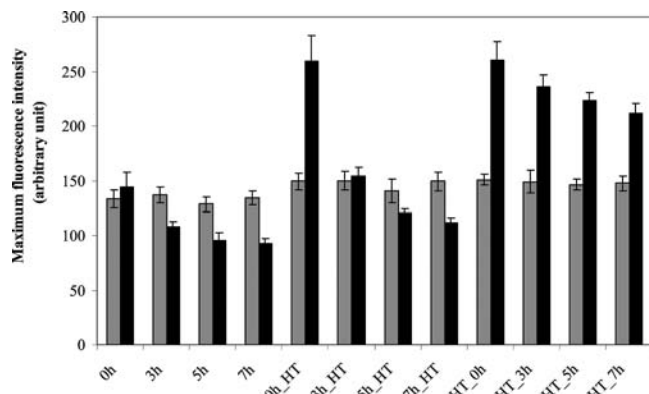

Figure 3. Maximum fluorescence intensity measured on addition of up to 0.3 mM ANS to milk (gray bars, 15 mg) or the corresponding serum (black bars, 100 mg) samples obtained after submission of unheated milk to chymosin for 0−7 h at 5 °C followed by heat treatment at 70 °C for 1 min (0−7 h) or at 90 °C for 10 min (0h_HT−7h_HT), or after submission of heat-treated (90 °C/10 min) skim milk to chymosin for 0−7 h at 5 °C followed by inactivation at 70 °C for 1 min (HT_0 h−HT_7 h). The serums were separated using ultracentrifugation.

Surface Hydrophobicity. The maximum fluorescence intensity obtained on binding ANS probes to either the casein micelles and/or the heat-induced aggregates present in the serum phase of the milk systems is shown in **Figure 3**.

In the milk samples, heat treatment significantly increased the surface hydrophobicity of the present particles (casein micelles and the heat-induced aggregates) by an average of 5% of its initial value across all incubation times (P_0 < 0.05). This was in agreement with previous studies in which denaturation of the whey proteins was held responsible for the increase in surface hydrophobicity of milk or milk fractions (*42, 43*), although the dependence of surface hydrophobicity on heat load exhibited a complex pattern due to the specific responses of the serum and micelle fractions. Conversely, no significant effect of the incubation with chymosin was found in any of the h, h_HT, or HT_h series. It is, however, expected that the surface hydrophobicity of casein micelles as measured using anionic probes increases as κ-casein is hydrolyzed by chymosin (*44, 45*). The lack of such variation in the present study, consistent with the small change in zeta potential during renneting (**Figure 2**), may be due to the methods used. It may be that either the amount of released caseinomacropeptide is overestimated, so that renneting is less advanced than expected, or that the concentration of protein in the milk series was too low for hydrophobicity changes to be detected, although the heat-induced changes could be seen.

In the serum samples, heat treatment, renneting treatment, and the sequence by which these treatments were performed all induced significant changes of the fluorescence intensity (P_0 < 0.05). Comparison of the serum of unheated (0 h) with that of heated milk (0h_HT and HT_0 h) showed that the occurrence of heat-induced aggregates in the serum phase dramatically increased the maximum fluorescence intensity from ~145 to ~260. Analysis of the protein composition of the milk samples and of their ultracentrifugal fractions using reverse-phase high-performance liquid chromatography (RP-HPLC) showed that the serum of unheated milk contained 10–11% of the total caseins and 98% of the total whey proteins, whereas that of heated milk contained the same proportion of total caseins (not shown) and 72% of the total whey proteins (*30*). Furthermore, in heated systems h_HT and HT_h where the whey proteins are denatured and aggregated, a positive correlation could be found between the amount of whey protein and the hydrophobicity measured in the serum (R^2 = 0.83). These results indicated that the heat-denatured whey proteins, as part of the heat-induced aggregates present in the serum phase of heated milk, are highly hydrophobic as compared to native whey proteins, in agreement with previous results (*11, 46*). The formation of hydrophobic whey protein/κ-casein aggregates also explained the increase in overall hydrophobicity in the heat-treated milk samples, as previously suggested by Iametti et al. (*43*). When heat treatment is performed prior to renneting (HT_h samples), the fluorescence intensity of the serums remained above 200 throughout κ-casein hydrolysis. The corresponding protein contents were slightly less than in heated milk, with ~8% of the total caseins (not shown) and ~70% of the total whey proteins across HT_3 h to HT_7 h samples (*30*). Conversely, the fluorescence intensity of the serum decreased to below 150 when the milk was renneted for 3–7 h prior to heat treatment (h_HT samples), which, as the fluorescence of milk did not change, indicated that surface hydrophobicity was larger in the micellar fraction of h_HT samples than in that of the corresponding HT_h milks, that is, independent of the only effect of renneting on casein micelles (*44, 45*). Furthermore, the protein contents in the serum of h_HT milks were lower than in the serum of heated milk. The proportions of total caseins and of total whey protein in the serum phase respectively decreased from 6.5 to 4.5% (not shown) and from 36.5 to 25.8% as κ-casein hydrolysis increased (*30*). These results were therefore in accordance with the essentially micelle-bound location of the heat-induced aggregates formed in h_HT milks, whereas those formed in heated milk or in HT_h milk showed a more even micelle/serum distribution (*30*). **Figure 3** eventually shows that the fluorescence intensity measured in the serum phase significantly decreased as renneting proceeded, no matter whether heat-induced aggregates were present (in HT_h milks) or not (in h and h_HT milks). Partial aggregation of the serum micellar material (*5*) or of the serum aggregates through hydrophobic interaction may lower the overall intensity of fluorescence (*45, 47*). However, no such aggregation could be detected from light scattering data or SEC analysis of these systems (*30*). As correlations existed between the content of the serums in κ-casein (R^2 = 0.94) or in denatured whey protein (see above) and the surface hydrophobicity measured in these serums, a likely explanation for the decrease in surface hydrophobicity of the serum phase of all three series rather is that a small proportion of its protein material (κ-casein-rich casein material or heat-induced aggregates), probably the most hydrophobic particles, associated with the casein micelles on renneting. This transfer of protein material from the serum to the micelle phase would explain the decrease in SEC area of the serum κ-casein-rich material (in h milks) or aggregate peak (in HT_h milks) on renneting (*30*). Interestingly, these results suggested that the surface hydrophobicity of the micelle fraction was slightly increasing in all of the series of samples as κ-casein hydrolysis proceeded, and that the aggregate-coated casein micelles of renneted and then heated skim milk (h_HT) are likely to bear a highly hydrophobic surface.

Acid Gelation. The changes in elastic modulus G' and in tan δ of the various skim milk systems as pH was decreased to 4.6 using GDL are shown in **Figure 4**. The pH at which gelation starts is defined by tan $\delta = G''/G' = 1$; that is, the milk shows a transition from a mainly viscous to a mainly solid system. The average pH value at which gelation started and the average final G' value reached by each type of sample are given in **Table 1**.

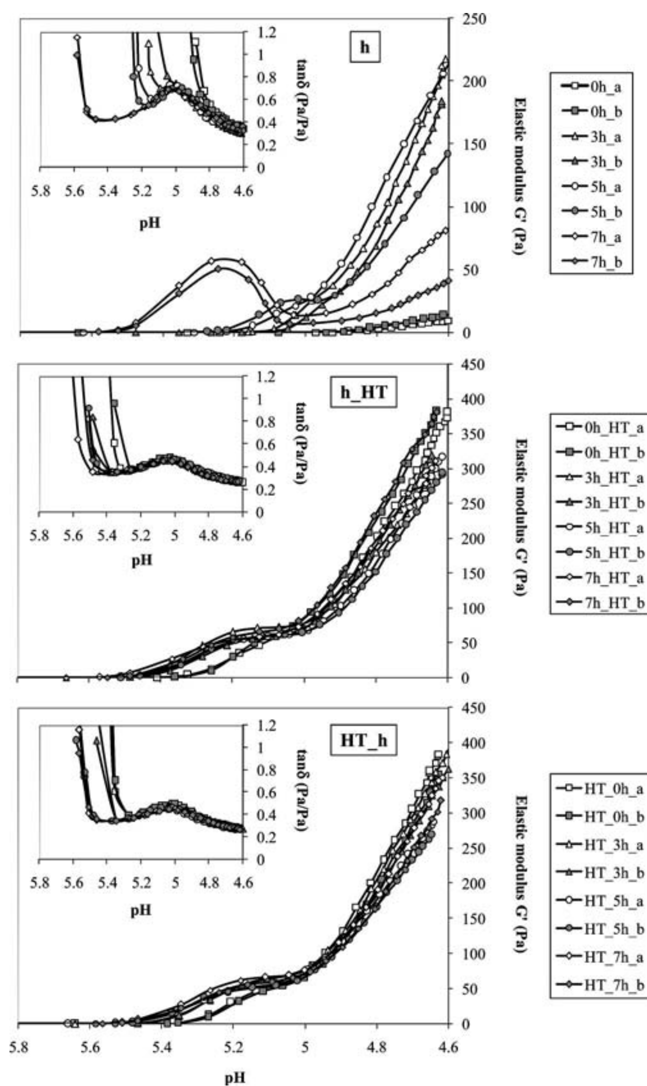

Figure 4. Typical acid gelation profiles of skim milk samples obtained after submission of unheated milk to chymosin for 0−7 h at 5 °C followed by heat treatment at 70 °C for 1 min (h) or at 90 °C for 10 min (h_HT) or after submission of heat-treated (90 °C/10 min) skim milk to chymosin for 0−7 h at 5 °C followed by inactivation at 70 °C for 1 min (HT_h); the samples were acidified to pH 4.6 at 38 °C by addition of 11 g L^{-1} GDL, and gelation was measured using low-amplitude oscillation rheometry. Letters a and b refer to repetitions. The insets show the corresponding changes in tan δ (G''/G') as a function of pH.

Unheated skim milk (0 h) started to gel at pH ∼4.9; G' increased continuously and reached low values of <20 Pa (**Figure 4**, top; **Table 1**). Heated skim milk (HT_0 h or 0h_HT) started to gel at pH 5.3–5.4, developed a two-stage gelation process marked by a transient loosening of the network at pH 5.0–5.1 (local maximum of tan δ) attributed to the adverse effect of ongoing calcium phosphate solubilization (*48*), and reached G' values ranging between 350 and 400 Pa (**Figure 4**, middle and bottom; **Table 1**). These effects of the heat-induced denaturation of whey proteins on the acid gelation of skim milk are widely documented (*8, 10, 14, 16, 17, 23*). In the three series h, h_HT, and HT_h, prehydrolysis of the κ-casein using chymosin increased the pH of gelation of the milks ($R^2 > 0.8$ in each series). Increasing the rate of conversion of κ-casein into para-κ-casein from 0 to ∼65% caused the gelation pH to shift from 4.9 to 5.5 in unheated milk and from 5.4 to 5.6 when heat treatment and renneting were combined (**Table 1**). This effect was also reported when unheated (*49–51*) or heated (*51*) skim milk had been renneted to similar extents prior to acidification. In these studies, it is proposed that prerenneting of the casein micelles allowed hydrophobic attraction to overcome electrostatic repulsion at higher pH values than in untreated milk.

Table 1. Average Values and Standard Deviations of the pH of Gelation and of the Final G' of Acid Gels Obtained by Acidification of the h, h_HT, and HT_h Systems Using Addition of 11 g kg^{-1} GDL[a]

sample	gelation pH (upH)	final G' (Pa)
0 h	4.87 ± 0.03	13 ± 4
3 h	5.09 ± 0.07	200 ± 23
5 h	5.23 ± 0.02	176 ± 48
7 h	5.54 ± 0.06	64 ± 33
0h_HT	5.36 ± 0.04	381 ± 1
3h_HT	5.48 ± 0.04	308 ± 16
5h_HT	5.50 ± 0.03	312 ± 26
7h_HT	5.55 ± 0.01	345 ± 49
HT_0 h	5.26 ± 0.12	370 ± 20
HT_3 h	5.46 ± 0.02	379 ± 20
HT_5 h	5.52 ± 0.01	288 ± 26
HT_7 h	5.55 ± 0.01	335 ± 21

[a] The corresponding gelation profiles are shown in **Figure 4**.

The effect of prerenneting skim milk on the final G' values of the acid gels depended on the type of sample. In unheated skim milk, final G' dramatically increased from 13 to ∼200 Pa as the rate of κ-casein hydrolysis increased from 0 to ∼40% (**Table 1**; $P_0 < 0.05$). At higher conversion rates, gelation occurred as a two-step process during which building of the network started early but poorly recovered from loosening at pH 5.0–5.1 (**Figure 4**, top). The samples also showed advanced syneresis (not shown). Similar gelation profiles have been reported when skim milk was prerenneted at high extents (≥60% of total κ-casein) prior to acidification (*51*) or when unheated skim milk was submitted to combined gelation using fast renneting rates [e.g., using high levels of rennet (*51−55*)]. In these cases, the decoupling between fast, early rennet gelation and late and/or slow acid gelation is believed to enhance partial loosening of the forming gel at pH ∼5.1, introducing large rearrangements, including syneresis and pore formation, concomitantly to solubilization of the calcium phosphate. In heated samples, the same gelation profiles were observed whether renneting preceded or followed heat treatment (**Figure 4**, middle and bottom, respectively). Gelation of the h_HT or HT_h samples occurred in a two-step process and reached higher final G' values (280–380 Pa) than for unheated milk samples h (**Table 1**; $P_0 < 0.01$). No visible syneresis could be observed in any of the h_HT or HT_h samples. In these samples, no significant change in the final G' value of the gels was found as the rate of κ-casein hydrolysis was increased, due to large standard deviations (**Table 1**; $P_0 > 0.05$) and despite the fact that almost all rennet-treated samples reached up to 100 Pa lower final G' values than nonrenneted milk (**Figure 4**, middle and bottom; **Table 1**). As a result of the increased gelation pH, the first stage of gelation shifted toward higher pH values without dramatic changes in the rate at which G' increased with decreasing pH. Durations of the transient increase in tan δ and of the corresponding retardation of G' growth increased from ∼0.2 to ∼0.4 upH with renneting, so that the second stage of gel formation consistently started at pH 5.0 across all samples. As previously reported by Schulz et al. (*55*) or Li and Dalgleish (*51*), the prehydrolysis of κ-casein essentially affected the first, rennet-type stage of gelation of the milks and hardly affected

the pH value at which the local maximum in tan δ occurs nor that at which the second, acid-dominant stage starts.

DISCUSSION

The results showed that casein micelles that bear almost the totality of the heat-induced whey protein/κ-casein aggregates formed during heat treatment of the milk at 90 °C for 10 min, as found when skim milk is renneted prior to heating (h_HT), are large in size (*30*) and have a high surface hydrophobicity but no difference in zeta potential compared to heat-treated and then renneted milk (h_HT), where the aggregates are more evenly distributed between the serum and micelle phases. On acidification, slightly lower zeta potential and higher isoelectric point of the aggregates relative to that of the casein micelles may introduce probably negligible charge differences between heavily and moderately coated micelles. However, neither the higher attractive surface hydrophobicity, the larger size, nor the possibly lower repulsive electrostatic attraction of h_HT micelles induced any change in the gelation pattern of skim milk compared to heated and then renneted milk or even to simply heated milk. Studies in which the physicochemical properties of natural or model heat-induced protein aggregates were described and varied clearly showed that the onset of gelation of dairy systems on acidification depended on the specific electrostatic/hydrophobic (repulsive/attractive) ratio of the aggregates (*9–11*). These findings should hold for the casein micelles, but no similar relationship was found in the present study. Only the decrease in zeta potential due to increasing κ-casein hydrolysis by chymosin seemed to relate to an increase of the pH of gelation of the milks, no matter the micelle/serum distribution of the aggregates. In addition, no change in the gelation profiles of the h_HT and HT_h milks could be related to the sequence by which renneting and heating were performed nor, therefore, to the proportion of heat-induced aggregates being attached to the casein micelles.

In the present study, no further increase of the firmness of the acid gel made with heated milk could be achieved using prerenneting. The large changes obtained using unheated milk ((*49–51, 55*); **Figure 4**) show that heat treatment is, in itself, very effective in increasing the final G' of acid milk gels. However, Li and Dalgleish (*51*) reported that final G' values increased as the rate of prehydrolysis of the κ-casein of heat-treated milk was increased to 81%. Heat treatment performed by the authors (80 °C/30 min) may have led to a smaller extent of aggregation of the whey proteins, yielding less dramatic heat-induced changes in the gelation profile of milk (gelation pH 5.1, final $G' = 200$ Pa) than that applied in this study (90 °C/10 min). With a possibly lower effect of heat treatment, that of prerenneting could then be seen, in agreement with Niki et al. (*50*), who used medium-heat skim milk powder and also observed rennet-induced changes in the gelation profile of acid gels. In extensively heated milk, it seems that increased G' values may rather be obtained when acid gels are made using simultaneous renneting and acidification, providing that the rate of κ-casein hydrolysis is finely tuned throughout acidification for a synergy to occur (*51–53, 56*).

The present results are therefore at variance with previous studies in which increasing the proportion of serum aggregates in skim milk using heat treatment at alkaline pH values increased both the pH of gelation and the final G' of acid gels (*3, 19, 23, 24*). It may be that the aggregates formed at pH 6.9 or 7.1 have properties different from those formed at the natural pH of milk, which would change their repulsive/attractive ratio. Aggregates formed at mildly alkaline pH values are, for instance, smaller, more numerous, and richer in κ-casein and possibly other caseins and they involve a higher proportion of intermolecular disulfide bonds than at pH 6.7 or lower (*20, 21, 57–59*). Studies undertaken at pH 6.7 in which the amounts of serum and micelle-bound aggregates were varied, however, indicated that the occurrence of whey protein/κ-casein aggregates in the serum phase was important for the formation of early and firm acid gels (*14, 25, 26*), albeit opposite results were also reported (*16, 27*). Changes in the protein compositions and/or heat treatment conditions in these studies, however, rendered identification of the respective roles of the serum and micelle-bound aggregates on acid gelation difficult. Schorsch et al. (*25*) proposed that it was the interaction between the serum aggregates and the surface of the casein micelles that was important and promoted it using heat treatment. Using dynamic wave scattering, Alexander and Dalgleish (*29*) and Donato et al. (*28*) have proposed that the serum aggregates interacted with the surface of the (heated or unheated) casein micelles early in the course of gelation, that is, within about pH 5.5–5.1. The absence of difference between the gelation behaviors of two skim milk systems having sensibly different micelle/serum distributions of the heat-induced whey protein/κ-casein aggregates, as found in the present study, either supports this view or indicates that said distribution is not a significant factor to account for the gelation process of skim milk, or both. In light of the results obtained by Donato et al. (*28*), the heat-induced aggregates found in the present milk systems probably become unstable at pH ~5.4 (**Figure 1**); the serum aggregates, if any, then associated with the casein micelles so that micelle/serum distribution differences no longer existed between the h_HT and HT_h systems at the time gelation started. Why serum aggregates would first associate with the casein micelles rather than with each other is, however, unclear; quite possibly, both interactions occur simultaneously, but it is the aggregation of the casein micelles, rather than that of the aggregates, that would more significantly affect the responses measured either by rheology or light scattering. In their study, Donato et al. (*28*) used ultrafiltration of the serum phase of heated milk to vary the concentration of heat-induced serum aggregates in skim milk systems. They reported that the pH of acid gelation of these systems increased as said concentration increased and attributed this effect to specific precipitation behavior of the serum aggregates. As no difference in the gelation pH of the HT_h and h_HT systems was found in the present study, it is possible that the varying total (micelle-bound + serum) concentration of heat-induced aggregates, rather than their micelle/serum distribution, is an important factor to control acid gelation. Further research is needed to improve our understanding of the specific roles of the casein micelles and of the heat-induced whey protein/κ-casein aggregates on the early stages of particle–particle interaction and gel formation.

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