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Limited Enzymatic Treatment of Skim Milk Using Chymosin Affects the Micelle/Serum Distribution of the Heat-Induced Whey Protein/κ-Casein Aggregates

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The effects of heat treatment and limited κ-casein hydrolysis on the micelle/serum distribution of the heat-induced whey protein/κ-casein aggregates were investigated as a possible explanation for the gelation properties of combined rennet and acid gels. Reconstituted skim milk was submitted to combinations of 0−67% hydrolysis of the κ-casein at 5 °C and heat treatment at 90 °C for 10 min. The protein composition of the ultracentrifugal fractions was obtained by reverse-phase high-performance liquid chromatography (RP-HPLC). The aggregates contained in each phase were isolated by size-exclusion chromatography and analyzed by RP-HPLC and sodium dodecyl sulfate−polyacrylamide gel electrophoresis. Upon heating only, 20−30% of the total κ-casein dissociated, while 20−30% of the total whey protein attached to the micelles. When heated milk was renneted, little changes were observed in the distribution and composition of the aggregates. Conversely, the heat treatment of partially renneted milk induced the formation of essentially micelle-bound aggregates. The results were discussed in terms of the preferred interaction between hydrophobic *para*-κ-casein and denatured whey proteins.

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

INTRODUCTION

Formation of acid dairy gels, e.g., in the manufacture of yoghurt, usually involves extensive heat treatment of the milk at 85−95 °C for 5−15 min. This treatment induces denaturation of the whey proteins, which has been correlated with the early onset of gelation, higher firmness, and lower syneresis of acid gels, all desirable qualities for yoghurt (*1*−*3*). During heating, the denatured whey proteins interact with each other and with κ-casein essentially through thiol/disulfide exchanges and hydrophobic interactions to form heat-induced whey protein/κ-casein aggregates located on the surface of the casein micelle and in the serum phase of milk (*4*−*8*). The dissociation of κ-casein from the casein micelle upon heating has been linked with the formation of the serum type of aggregates, but whether the amount of serum aggregates is a cause or a consequence of the κ-casein dissociation is unclear (*5*, *9*−*12*). However, it is suspected that the respective properties of the two types of aggregates are affecting the course of acid gel formation (*6*, *13*). Various studies indicated that the occurrence of whey protein/κ-casein aggregates in the serum phase was important for the formation of early, elastic acid gels (*14*−*16*). Other authors conversely proposed that it was the micelle-bound aggregates (*17*−*19*) or the interaction between serum aggregates and the surface of the casein micelles upon acidification or heat treatment that were important (*14*, *20*). Better knowledge of the route by which the respective proportions of serum and micelle-bound aggregates are established is therefore necessary to understand the formation and quality of acid dairy gels.

Interestingly, the combination of heat treatment and κ-casein hydrolysis has also been shown to increase the pH of gelation and the final elastic modulus G′ of acid gels in both conditions of prehydrolysis of the heated milk by chymosin prior to acidification (*21*) or simultaneous hydrolysis and acidification (*21*−*24*). In such milks, little is known of the actual structure and properties of the casein micelles and heat-induced aggregates or of the possible interactions between them. Heat treatment and renneting may also affect pH or mineral equilibrium in milk, which in turn may affect milk gelation. Dependent upon conditions, the acid and rennet coagulations seem to proceed somewhat synergistically, allowing larger G′ values to be reached (*21*−*23*). However, it has been shown that κ-casein as involved in the heat-induced aggregates may be hydrolyzed by chymosin (*25*, *26*). It would therefore be interesting to know whether the hydrolysis of κ-casein affects the distribution of the heat-induced whey protein/κ-casein

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aggregates between the micelle and the serum phases of milk, as another explanation to the improved acid gelation properties of combined gels. In the present study, controlled hydrolysis of the κ-casein and heat treatment of skim milk were therefore combined in various sequences and their effect on the distribution of the heat-induced aggregates was investigated.

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; *27*) 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. The serum phase of heated (90 °C for 10 min) or unheated reconstituted skim milk was separated from the casein micelles using ultracentrifugation (as described below) and was ultrafiltered at room temperature using a 10 kDa cutoff stirred cell system (Pall Life Science, St. Germain en Laye, France).

Heat Treatments. When heat treatment was performed prior to controlled renneting, 2 L of reconstituted skim milk was heat-treated at 90 °C for 10 min (+6 min of heating-up time) in a recirculating tubular heat exchanger. The flow rate was 135 L h^{-1}, and the flow was turbulent to ensure proper heat transfer (Re ~ 3200). After heat treatment, the milk was rapidly cooled to room temperature in ice water.

When heat treatment was performed on partially renneted milk in 250 mL bottles, inactivation of the enzyme was performed by immersing and hand-shaking the bottles continuously in a thermostated water bath for the required length of time. In series, where no 90 °C/10 min treatment was performed (samples 0−7 h) or when heated milk was renneted (samples HT_0h−HT_7h), inactivation was performed at 70 °C for 1 min (heating-up time of ~5 min). In series, where heat treatment followed hydrolysis by chymosin (samples 0h_HT−7h_HT), heat treatment and inactivation were performed together by heating the milk at 90 °C for 10 min (heating-up time of ~8 min). The milk was then rapidly cooled to room temperature in ice water.

Controlled κ-Casein Hydrolysis. A volume of 200 μL L^{-1} of recombinant chymosin freshly diluted to 10% (v/v) in deionized water (Maxiren 180 batch AG1520, 900 mg L^{-1} enzyme, 180 IMCU, DSM, Seclin, France) was added to agitated milk at 5 °C. After 3 min of stirring, the milk was divided into 200 mL fractions in identical 250 mL Schott bottles and incubated at 5 °C in a thermostated water bath for up to 7 h. After 0, 3, 5, or 7 h of incubation, one sample of the series was withdrawn and heat-treated at 70 °C for 1 min or 90 °C for 10 min to inactivate the enzyme.

Stability and Viscosity of the Milks at 38 °C. Visible stability of the milks was evaluated by incubating a ~20 mL aliquot of each sample at 38 °C for at least 6 h. Further evaluation was provided by the measurement of the dynamic shear viscosity at 38 °C using a thermostated Low Shear 30 viscosimeter (Contraves, Zurich, Switzerland) equipped with coaxial cylinders of 11 and 12 mm in diameter. Deionized water and 200 g kg^{-1} ethanol in deionized water, having respective viscosities of 0.6814 and 1.2288 mPa s at 38 °C, were used as calibration standards. The viscosity of each sample equilibrated at 38 °C was measured at 5.96, 14.98, and 20.40 s^{-1} to check for Newtonian behavior. In the absence of a significant dependency of the viscosities on the shear rate ($R^2 < 0.66$), the values collected for each sample were averaged.

Analysis of the Released Caseinomacropeptide (CMP). Aliquots of 9 g of milk were precipitated at pH 4.6 using 5 M HCl (~100 μL) under constant agitation, and the weight of the aliquot was completed to 10 g with deionized water. The precipitated milks were transferred to 15 mL Corning centrifugation tubes and spun at 3000*g* for 15 min at 20 °C in a Biofuge Primo Heraeus centrifuge (DJB Labcare, Newport Pagnell, Buckinghamshire, U.K.). The supernatants were diluted to $^1/_2$ (v/v) with 2 mL L^{-1} trifluoroacetic acid (TFA) in deionized water immediately prior to reverse-phase high-performance liquid chromatography (RP-HPLC) analysis as described below, except that the loop size was 100 μL, the temperature was 40 °C, and the proportion of buffer B increased from 25 to 100% in 41 min. The rate of κ-casein hydrolysis was calculated as a percentage of the total CMP released in each sample. The total CMP was assayed using unheated skim milk incubated at room temperature for 24 h.

Particle-Size Analysis. The particle size in the different milk samples was measured within 24 h after preparation using dynamic light scattering (DLS) at a set angle of 90° on a Zetasizer Malvern 3000 HS (Malvern Instruments, Orsay, France). The laser was He−Ne, and wavelength was 633 nm. The samples equilibrated at 25 °C were diluted in the appropriate milk ultrafiltration permeate at 25 °C (from either heated or unheated milk) and left at 25 °C for 15 min to ensure proper equilibrium of the diluted system. The dilutions were then transferred to 2 mL disposable cuvettes and allowed to stand for 5 min prior to measurement. The refractive index and dynamic shear viscosity of both permeates were 1.3416 and 0.99 mPa s at 25 °C, respectively. The data were computerized into a particle-size distribution using a CONTIN modeling routine.

Separation of the Serum and Colloidal Phases of Skim Milk. The separation of the serum and colloidal phases of milk was performed on 15 mL aliquots 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 19 400 rpm (~33 000 average *g*) for 65 min at 20 °C. The supernatants were designated as the serum phase of milk. The pellets were resuspended at 5 °C for up to 48 h in the same weight of ultrafiltration permeate of unheated milk as that of the removed serum fraction, under constant agitation. This fraction was designated as the micellar phase of milk (*8*). The sodium dodecyl sulfate/ethylenediaminetetraacetic acid (SDS/EDTA) mobile phase was used instead of permeate to dissociate the pellets prior to the separation of the micellar heat-induced aggregates using size-exclusion chromatography (SEC) within 48 h.

Analysis of the Milk Fractions Using Size-Exclusion Chromatography (SEC). Size-exclusion chromatography analysis of the serum and micellar phases of the milks was performed on a Sephacryl S-500 Hi-Prep 16/90 column (Amersham Biosciences, Orsay, France). The samples were filtered through 1.2 μm filters (Pall Life Science, St. Germain en Laye, France). Separation of the serums was performed at room temperature in native and isocratic conditions using 0.1 M Tris, 0.5 M NaCl, and 10 mM NaN_3 at pH 7 as the mobile phase. The separation of the micellar phases was performed at room temperature in dissociating and isocratic conditions using 25 g L^{-1} SDS, 7.63 mM Tris, 0.76 mM EDTA, and 10 mM NaN_3 at pH 8.2 as the mobile phase. The loop size was 0.5 mL; the flow rate was 0.5 mL min^{-1}; and the detection was at 280 nm. The eluted peaks were collected, extensively dialyzed against deionized water, and concentrated by freeze-drying. The powder was then resuspended in deionized water and immediately analyzed by RP-HPLC. When micellar-phase samples were separated, residual SDS in the freeze-dried protein prevented the use of RP-HPLC. These samples were alternatively analyzed by SDS−PAGE.

Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC). The protein composition of the serum and micellar phases of milk or of the peak fractions collected during SEC was obtained by RP-HPLC. The samples were diluted 5 times with denaturing buffer (7 M urea and 20 mM Bis-Tris Propane at pH 7.5 plus 5 μL mL^{-1} of fresh β-mercaptoethanol) and incubated for 1 h at room temperature. The column was an Apex wide-pore C18 column of 25 cm length, 0.46 cm inner diameter, and 7 μm bead diameter (Jones Chromatography, Hengoed, U.K.). Buffer A was 0.106% (v/v) TFA in Milli-Q water (Waters, Molsheim, France). Buffer B was 0.1% (v/v) TFA in 80% (v/v) acetonitrile in Milli-Q water. The temperature was 46 °C, and buffer B increased in steps from 43 to 100% (v/v) in 23 min. The loop size was 30 μL; the flow rate was 1 mL min^{-1}; and detection was at 214 nm.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). Protein composition of the peak fractions collected by SEC in SDS/EDTA conditions was determined by SDS−PAGE on a Mini-Protean III Bio-Rad Electrophoresis unit (Bio-Rad, Hercules, CA). The concentrated eluates (~3 g L^{-1} protein in deionized water) and the low-molecular-weight standard protein kit (Amersham Biosciences, Orsay, France) were diluted 2 times with Laemmli sample buffer (1.2 M Tris-HCl, 40 g L^{-1} SDS, 200 g L^{-1} glycerol, 0.2 g L^{-1} bromophenol blue at pH 6.8, and 30 mL L^{-1} β-mercaptoethanol) and heated in boiling

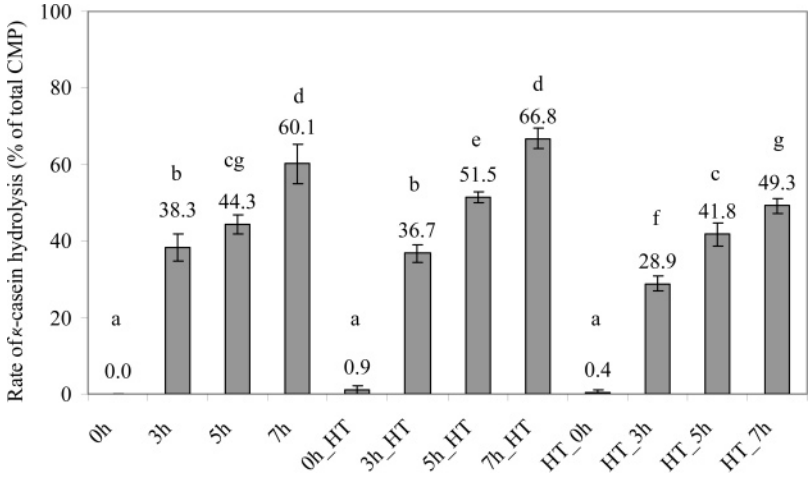

Figure 1. Rate of hydrolysis of the κ-casein, measured as a percentage of total CMP released, after submission of unheated milk to chymosin for 0–7 h at 5 °C followed by heat treatment at 70 °C for 1 min (0–7 h) or at 90 °C for 10 min (0h_HT–7h_HT) or after submission of heat-treated (90 °C/10 min) skim milk to chymosin for 0–7 h at 5 °C followed by inactivation at 70 °C for 1 min (HT_0h–HT_7h). Different letters a–g indicate significant difference ($\alpha = 0.05$).

water for 5 min. The resolving gel contained 14% (v/v) acrylamide [0.5% (v/v) bis-acrylamide] in 0.4 M Tris-HCl at 1 g L^{-1} SDS at pH 8.8; polymerization was catalyzed by 0.5 g L^{-1} ammonium persulfate and 1.5 mL L^{-1} *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (Temed). The stacking gel contained 4% (v/v) acrylamide in 0.125 M Tris-HCl and 1 g L^{-1} SDS at pH 6.8, catalyzed by 0.5 g L^{-1} ammonium persulfate and 1 mL L^{-1} Temed. The electrophoresis buffer was 25 mM Tris-HCl, 14.4 g L^{-1} glycine, and 1 g L^{-1} SDS at pH 8.5. Aliquots of 25 μL were loaded onto the gels. The separation was performed at 100 V and 50 mA for 2 h. The gels were stained with Coomassie blue and scanned with an Image II scanner (Amersham Bioscience).

Significance. The presented results were obtained from 2 to 4 preparations of the samples, analyzed at least twice each. *T* tests and linear regressions were performed using Excel (Microsoft, Courtaboeuf, France).

RESULTS

Rate of Hydrolysis. The extent of hydrolysis of κ-casein by chymosin, measured as a percentage of the total CMP released after incubation of the various milk samples for up to 7 h at 5 °C followed by inactivation of the enzyme, is shown in **Figure 1**.

In all cases, the results showed that the extent of κ-casein hydrolysis increased with the incubation time from less than 1% in samples without chymosin (0h, 0h_HT, and HT_0h) up to 60−67% in skim milk samples that were unheated at the time of renneting (7h and 7h_HT) and to ∼50% in heated skim milk (HT_7h). A comparison between renneted (h) and renneted and then heated milk (h_HT) showed that heat treatment did not affect the amount of CMP released ($P_0 > 0.05$), except at 5 h for an unknown reason. At each time, the extent of hydrolysis in heated milk (HT_h) was significantly lower ($P_0 < 0.05$, except at 5 h) than that of the two other series. No significant changes in the pH of milk were measured as a result of renneting and/or heat treatment (not shown). Significant reductions of the initial velocity and/or extent of the primary phase of the rennet coagulation process as a result of heating have been reported previously (*28*−*31*) and explained by a decreased accessibility of the Phe_{105}−Met_{106} bond of κ-casein as a result of the interaction of the casein with denatured whey proteins or because of changes in the mineral equilibrium of milk as a minor factor (*31*). Extensive heating induces the precipitation of calcium phosphate, hence a decrease in soluble calcium that may adversely affect renneting. Overnight storage in the cold, as performed in the present study prior to chymosin addition, usually restores soluble calcium (*29*) but at the expense of the colloidal calcium phosphate rather than the heat-precipitated form, which is also a cause of decreased renneting properties of heated milk (*28*). κ-casein involved in heat-induced whey protein/κ-casein aggregates may be hydrolyzed by chymosin (*25*, *26*), which indicates that the presence of attached whey proteins does not totally inhibit hydrolysis. However, it is likely that the kinetics and possibly the actual mechanism of the reaction actually differ from that occurring in unheated milk.

Stability of the Milk Samples. No visible destabilization of any of the milk sample was observed after incubation at 38 °C for 6 h. Measurement of the dynamic shear viscosity of the various milks at 38 °C and at different shear rates further showed that all samples were Newtonian. The averaged values are shown in **Figure 2**.

In only renneted milk, ∼38% hydrolysis of the κ-casein (sample 3 h) yielded a slight but significant decrease in viscosity ($P_0 < 0.05$); no further decrease was observed between 3 and 7 h of renneting. This early effect of renneting upon the viscosity of milk has long been attributed to the loss of CMP as result of up to ∼80% hydrolysis of the κ-casein and subsequent reduction in the volume fraction of the casein micelles (*28*, *32*−*35*). However, no significant change in viscosity was observed in renneted and then heated (h_HT) or in heated and then renneted milk samples (HT_h) as a function of the incubation time, i.e., of the extent of κ-casein hydrolysis ($P_0 > 0.05$). Altogether, heated milk samples rather showed slightly higher viscosities than unheated skim milks. This effect of heat treatment could be explained by the formation of water-binding heat-induced whey protein/κ-casein aggregates (*16*) and/or their attachment onto the surface of the casein micelles, thereby increasing their volume fraction (*4*). This will be discussed further below. Renneted and then heat-treated milk samples showed slightly higher viscosities than the heat-treated and then renneted ones, including at zero time of hydrolysis, probably because the former were heated in incubated bottles with less shear stress and longer heating-up time than those applied to the latter, where a recirculating pump was used.

Size of the Casein Micelles. Despite the likely formation of heat-induced 25−70 nm diameter aggregates in the serum of heated milks (*6*, *36*, *37*), the intensity of the DLS signal is sensitive to the diameter of particles to the power of 6. It is

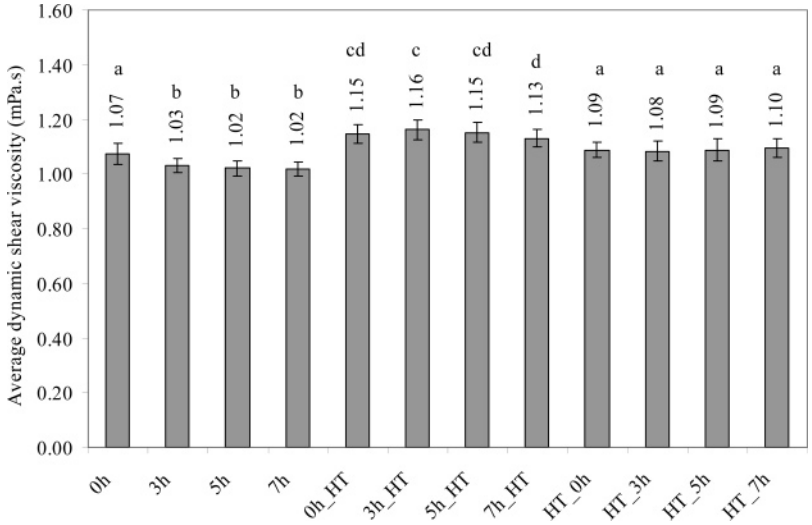

Figure 2. Dynamic shear viscosity of the milk samples at 38 °C (average of the measurements taken at 5.96, 14.98, and 20.40 s^{-1}) after submission of unheated milk to chymosin for 0–7 h at 5 °C followed by heat treatment at 70 °C for 1 min (0–7 h) or at 90 °C for 10 min (0h_HT–7h_HT) or after submission 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_0h–HT_7h). Different letters a–d indicate significant difference ($\alpha = 0.05$).

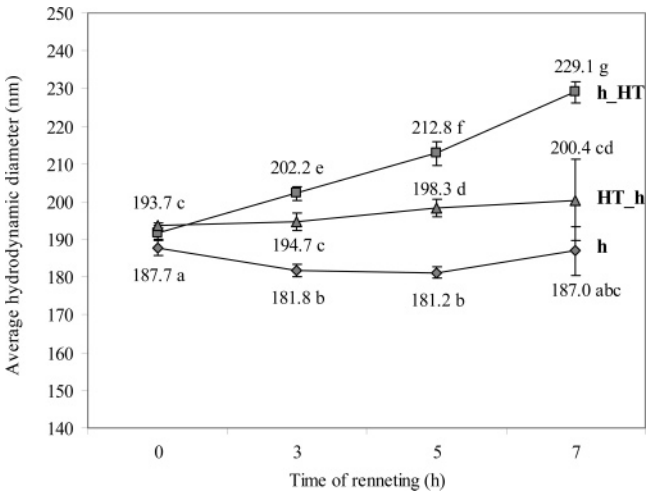

Figure 3. Average particle hydrodynamic diameter in the milk samples after submission of unheated milk to chymosin for 0–7 h at 5 °C followed heat treatment at 70 °C for 1 min (0–7 h; ◆) or at 90 °C for 10 min (0h_HT–7h_HT; ■) or after submission of heat-treated (90 °C/10 min) skim milk to chymosin for 0–7 h at 5 °C followed by inactivation at 70 °C for 1 min (HT_0h–HT_7h; ▲). Different letters a–g indicate significant difference ($\alpha = 0.05$).

therefore believed that the results shown in **Figure 3** were essentially a description of changes in the casein micelle size.

In unheated milk samples, partial hydrolysis of the κ-casein by chymosin induced a significant reduction of the average diameter of the casein micelles by about 6−7 nm after up to 5 h of incubation ($P_0 < 0.05$), in agreement with previous studies (*33*, *38*). In only heated milk samples 0h_HT and HT_0h, the average particle size was significantly higher by ∼5 nm than that measured in unheated milk ($P_0 < 0.05$). This value was however lower than the 20−25 nm increase reported by Anema and Li (*4*) after heating skim milk at pH 6.55 at 90 °C for 10 min. In heated and then renneted samples (HT_h), the particle size slightly increased by 6−13 nm as the rate of κ-casein hydrolysis increased up to ∼50%. This increase was barely significant. However, in renneted and then heated samples (h_HT), the particle size increased by 4−40 nm as the rate of hydrolysis increased up to ∼67% ($P_0 < 0.001$). Because the rheological properties of these samples were similar to those of original milk, chymosin-induced aggregation of the casein micelles was unlikely. Because some κ-casein left the serum phase upon hydrolysis (**Figure 4**), it is possible that it associated with the casein micelles and slightly increased the particle size in the 7h, HT_5h, or HT_7h samples. However, this would only partly explain the increase in particle size in the h_HT samples.

Distribution of the Heat-Induced Aggregates between the Serum and Micellar Phases of Skim Milk. Upon heating, it is believed that some denatured whey proteins attach to κ-casein on the surface of the casein micelle to form micelle-bound aggregates (*39*, *40*) and that some κ-casein is concomitantly dissociated from the casein micelle as a result of the formation of whey protein/κ-casein aggregates in the serum phase of milk (*9*, *12*). **Figure 4** shows the changes in the distribution of κ-casein and the whey proteins between the serum and micellar phases of skim milk after heat treatment and/or controlled κ-casein hydrolysis.

A slight overestimation of the whey protein area and underestimation of the κ-casein area, because of previously reported integration errors (*41*), induced mass balances slightly different from 100%. A negative relationship ($R^2 > 0.80$) was found in all series between the extent of hydrolysis and the RP-HPLC area of κ-casein; the generally lower balances obtained for this protein in the renneted milk samples could therefore be explained by the partial loss of CMP (∼36% of the total mass of nonglycosylated κ-casein; *42*). As expected, the hydrolysis of κ-casein in unheated milk did not induce any significant change in the distribution of the whey proteins between the serum and the micellar phases of milk ($P_0 > 0.1$). They were essentially soluble, with 10−15% of the total being found in the micellar phase most likely as part of the serum entrapped in the centrifugal pellet. Conversely, κ-casein was essentially found in the micellar phase of unheated milk, with ∼13% of the total in the soluble form. Upon renneting, κ-casein disappeared from the serum phase ($P_0 < 0.05$) and micellar κ-casein tended to increase ($P_0 > 0.1$). Because *para*-κ-casein is hydrophobic (*43*), it may have associated to the casein micelles upon hydrolysis. As expected, the heat treatment of milk induced significant changes ($P_0 < 0.05$) in the distribution of the two protein species. About 20−30% of the total whey protein was transferred to the micellar phase, while 20−30%

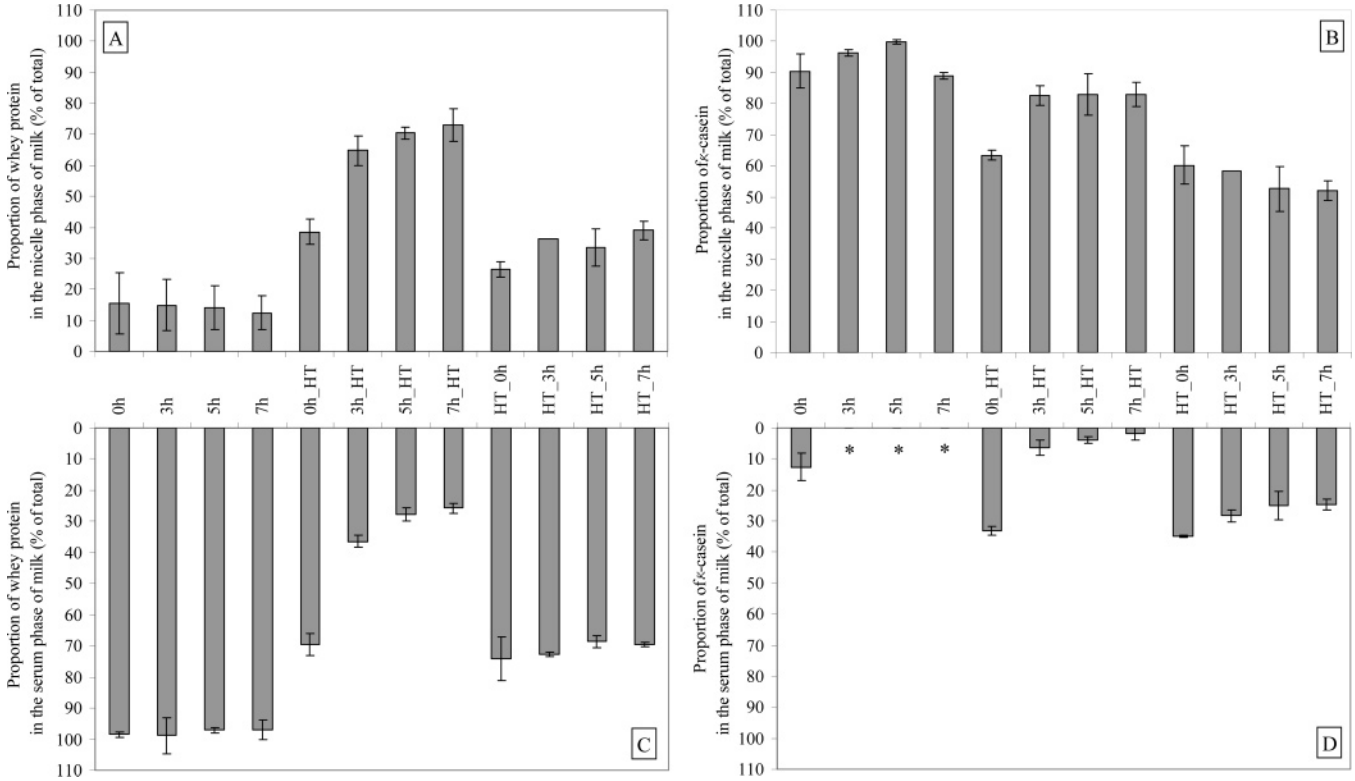

Figure 4. Distribution of the whey proteins (A and C) and κ-casein (B and D) between the ultracentrifugal pellet (A and B) and the supernatant (C and D) of skim milk samples obtained after submission of unheated milk to chymosin for 0–7 h at 5 °C followed by heat treatment at 70 °C for 1 min (0–7 h) 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_0h–HT_7h). The results are given in a percent of the total considered protein (total RP-HPLC area) found in the initial unheated skim milk. The asterisk indicates that trace amounts were too low for proper integration.

of the κ-casein was dissociated from the casein micelles upon heating, most likely as a consequence of the formation of both micelle-bound and serum heat-induced whey protein/κ-casein complexes. These figures were in accordance with earlier reports (*4*−*6*, *44*, *45*), where the heat-induced transfer of whey proteins to the micellar phase was ∼40% at pH 6.6 and ∼30% at pH 6.7−6.9, and with other data (*9*, *10*, *44*), where the proportion of nonsedimentable κ-casein increased from 15% in unheated milk to ∼20% at 60 °C and 25−30% at 90 °C for various heating times. Hydrolysis of the κ-casein in previously heated milk (HT_h) did not induce any significant change in the distribution of the protein between the micelle and the serum phases, although as in unheated milk, it seemed that slightly less κ-casein was found in the serum phase of milk upon renneting. Earlier reports (*7*, *25*, *26*) have discussed the fact that κ-casein involved in the heat-induced protein aggregates is as susceptible to hydrolysis by chymosin as whey protein-free κ-casein, although kinetics probably differ. The absence of destabilization of heated milk up to ∼50% hydrolysis of the κ-casein also indicated that limited proteolysis in milk conditions did not precipitate the aggregates (*25*) more readily than the casein micelles. In renneted and then heated milk however, the presence of *para*-κ-casein, rather than κ-casein, upon heating dramatically modified the heat-induced distributions of the whey protein and κ-casein. The proportion of whey proteins to be transferred from the serum to the micellar phase upon heating increased from 20−30% in the absence of hydrolysis (0h_HT) to 50−60% when κ-casein was partially hydrolyzed (3−7h_HT; $P_0 < 0.05$). Conversely, the proportion of κ-casein to be transferred from the micelle to the serum phase upon heating decreased from 20−30% at 0% hydrolysis to about 10% when 36−67% of the κ-casein was hydrolyzed ($P_0 < 0.05$). At the end of the renneting and heating sequence, the two protein species were therefore essentially colocated in the micellar phase of milk. Colocation of κ-casein and the whey proteins as a result of heating is usually taken as an indication of the formation of whey protein/κ-casein aggregates. It is therefore suspected that, in the presence of significant amounts of *para*-κ-casein, the formation of serum aggregates is prevented to the almost exclusive benefit of sedimentable, possibly micelle-bound ones. Surprisingly, the degree of hydrolysis of the κ-casein was not found to have a significant effect on either the whey protein or κ-casein mass transfers ($P_0 > 0.05$).

To confirm these results, the presence of aggregates was investigated using SEC on both the serum and micellar phases of milk. **Figure 5** shows the profiles obtained with the serum samples and the protein composition of peaks of interest. The other peaks were identified as previously reported (*8*, *46*). For clarity, only the samples obtained after 0 or 7 h of renneting are shown. Profiles obtained after 3 or 5 h of renneting were similar to those obtained at 7 h.

In the serum of unheated milk, large amounts of native whey proteins were found at large retention times (290−300 min) essentially corresponding to mono- and oligomers. Small aggregated material containing κ- and α_{s1}-casein was also found at ∼240 min, in agreement with previous reports (*8*, *46*). Upon renneting, the latter material was removed from the serum phase of milk (7 h trace), in accordance with **Figure 4D**. Upon heating, the amount of native whey protein decreased noticeably, while a new peak eluted at short elution times (160−260 min in HT_0h), indicating large material. Analysis of the corresponding eluate fraction (**Figure 5B**) confirmed that it essentially contained whey proteins and κ-casein in aggregated form with a whey protein/κ-casein area ratio of ∼2 (*8*, *12*, *46*). Previous

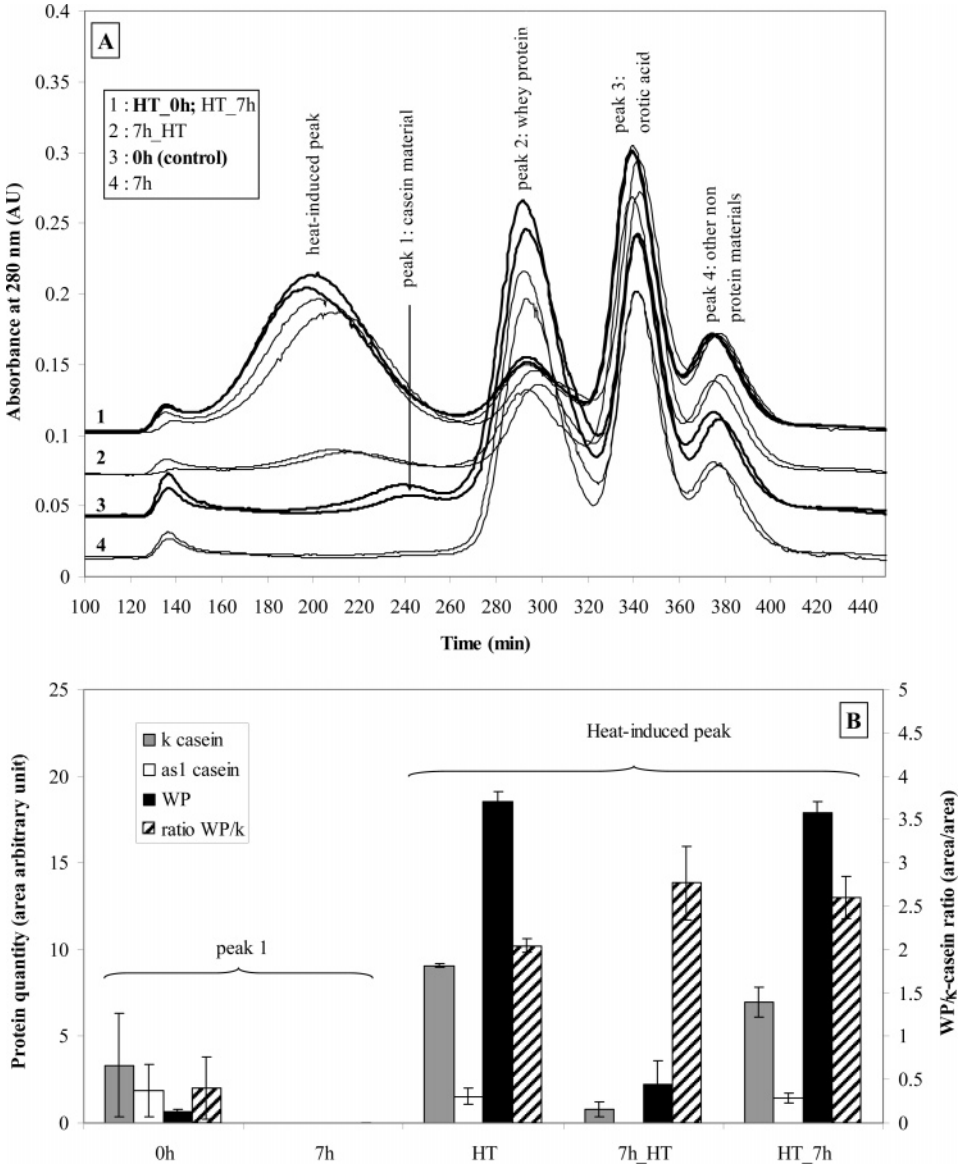

Figure 5. SEC elution profiles (A) and protein composition of the indicated peaks as analyzed by RP-HPLC (B) of the ultracentrifugal supernatants of skim milk samples obtained after submission of unheated milk to chymosin for 0–7 h at 5 °C followed by heat treatment at 70 °C for 1 min (0–7 h) or at 90 °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_0h–HT_7h). The profiles obtained from two different sample preparations are shown. For clarity, only the results obtained at 0 and 7 h are shown. As expected, the profiles of the heated milks HT_0h and 0h_HT superimposed and only the results for HT_0h are shown (bold trace). A shift of 0.03 AU was introduced between the various types of samples.

studies reported aggregate sizes of 30−90 nm in similar SEC fractions (*37*, *47*). When heated milk was renneted (HT_7h), no significant change in the SEC profile (**Figure 5A**) and composition of the aggregates was observed (**Figure 5B**). Only slight changes in the retention time and area were observed, as already reported (*25*). Conversely, major changes were observed in the SEC profiles when the milk had been submitted to chymosin prior to heat treatment (7h_HT). Despite the usual loss of native whey protein upon heating, little heat-induced protein material was collected between 160 and 260 min (parts **A** and **B** of **Figure 5**). However, the composition of the collected material was similar to that of the other heat-induced aggregates, i.e., whey protein and κ-casein with a ratio of 2:3. As a complement, **Figure 6** shows the SEC profiles obtained with the dissociated pellet samples and the protein composition of peaks of interest.

Because the pellets were resuspended in SDS/EDTA buffer, the dialysable nonprotein material present in **Figure 5A** was almost removed from the profiles of **Figure 6A**. Peak 2, containing the dissociated caseins and low amounts of whey proteins (**Figure 6C**) eluted ~20 min earlier than that of the native whey proteins in **Figure 5A**. This decrease in retention time was attributed to the binding of SDS molecules to the protein material (*8*). In unheated milk samples (0 and 7 h), low amounts of protein material were eluted ahead of peak 2 (as peak 1); this fraction essentially contained κ-casein and was identified as the polymeric form of the casein (*48*, *49*). No change was observed in the SEC profile as a result of κ-casein hydrolysis. In heated milk (HT_0h) and in heated and then renneted milk (HT_7h), peak 1 was removed from the profiles, while a new heat-induced peak appeared at short elution times (130−200 min). SDS−PAGE analysis of the heat-induced peak in reducing conditions showed that it was composed of whey proteins and κ-casein (**Figure 6B**), in agreement with Guyomarc'h et al. (*8*) for heated and unrenneted milk and with Mollé et al. (*25*) for heated and then renneted milk. Because of the binding

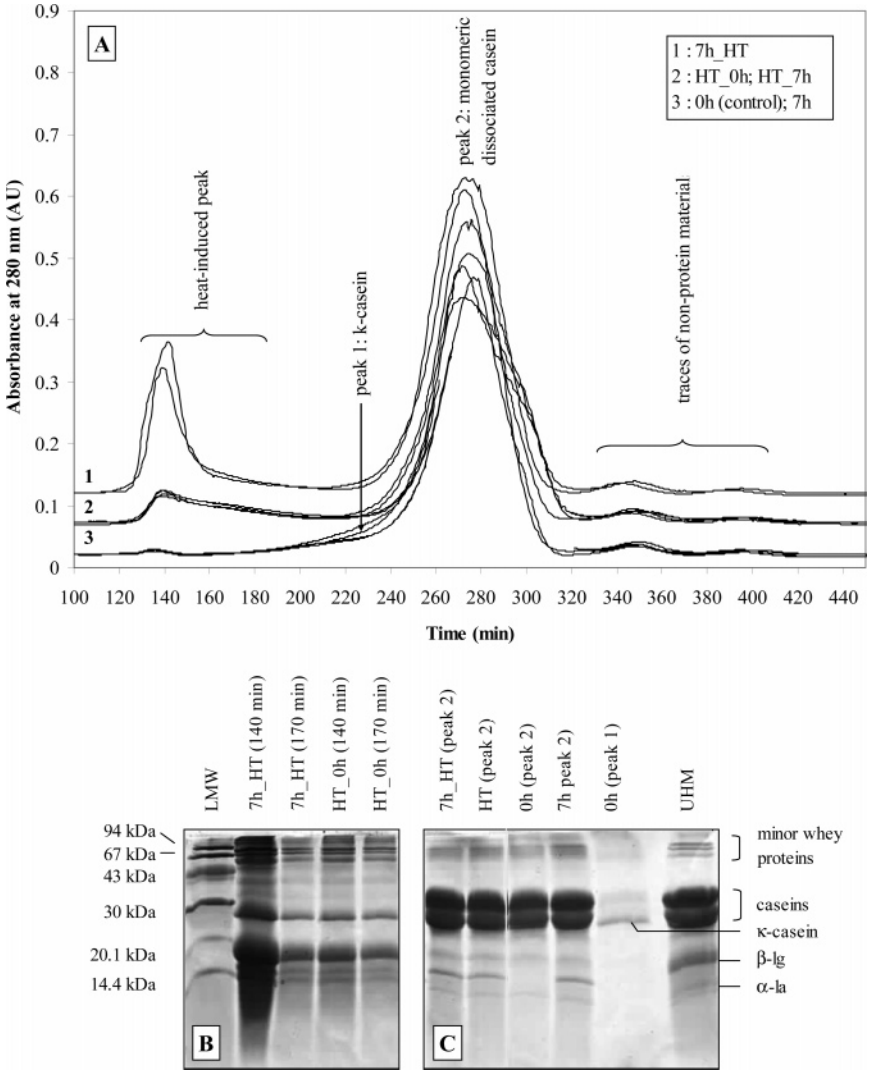

Figure 6. SEC elution profiles (A) and protein composition of the indicated peaks as analyzed by SDS–PAGE (B and C) of the ultracentrifugal pellets of skim milk samples obtained after submission of unheated milk to chymosin for 0–7 h at 5 °C followed by heat treatment at 70 °C for 1 min (0–7 h) or at 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_0h–HT_7h). The pellets were resuspended and dissociated in the SDS/EDTA mobile phase prior to injection. The profiles obtained from two different sample preparations are shown. For clarity, only the results obtained at 0 and 7 h are shown. As expected, the profiles of the heated milks HT_0h and 0h_HT superimposed and only the results for HT_0h are shown. UHM is the control unheated skim milk, and LMW is the low-molecular-weight marker kit. A shift of 0.05 AU was introduced between the various types of samples.

of SDS by the aggregates, the size of the sedimentable aggregates could not be compared to that of the serum ones (**Figure 5**). However, the separation of serums of heated milk samples using SDS/EDTA yielded similar retention time profiles than those of **Figure 6** (not shown), showing that the sizes of micellar and serum aggregates are probably close. In the absence of β-mercaptoethanol, no visible bands were seen on the gels (not shown). As expected, the protein material collected using dissociative SEC was missing the minor proportion of protein involved in the aggregates through hydrophobic interaction (*36*). These results showed that, in accordance with the results of **Figures 4** and **5**, no major quantitative difference was found between the proportions and compositions of the whey protein/κ-casein aggregates of heated milk prior to or after ~50% hydrolysis of the κ-casein. However, when κ-casein hydrolysis preceded heat treatment (7h_HT), parts **A** and **B** of **Figure 6** show that a much larger amount of heat-induced micelle-bound whey protein/κ-casein aggregates were present. In agreement with **Figures 4** and **5**, this result showed that as soon as hydrolyzed κ-casein was present in milk prior to heat treatment, the aggregates formed by the thiol/disulfide interaction between the denatured whey protein and κ-casein upon heating were almost exclusively found in the sedimentable phase of milk.

DISCUSSION

The presented results showed that hydrolysis of the κ-casein by chymosin, even at as low of an extent as 30−40% of the total κ-casein, prevented the formation of serum heat-induced whey protein/κ-casein aggregates to the benefit of an increased amount of sedimentable ones. In accordance with the gradual increase in the particle size of h_HT milks (**Figure 3**), indications suggested that these new sedimentable aggregates were micelle-bound aggregates. Light scattering data showed that the polydispersity index consistently remained in the range of 0.1−0.16 across samples, which excluded aggregation as well as significant polydispersity changes induced by renneting and heating ($P_0 > 0.05$; not shown). No bimodal distribution was observed in any of the renneted and then heated milk samples, as could have been expected if they contained over 200 nm large serum aggregates and ~190 nm large casein micelles.

Previous reports (*9*−*12*) have established a clear relationship between the dissociation of κ-casein and the formation of the heat-induced whey protein/κ-casein complexes. Various studies have for instance reported that the proportions of serum aggregates and dissociated κ-casein increased with the pH of heat treatment (*4*−*6*, *37*, *41*, *46*, *50*, *51*). Although both heat treatment and renneting may decrease the pH of milk, no significant change in the pH value has been measured in the samples used in the present study as a result of either treatment (not shown). This factor was therefore not responsible for the observed changes in the micelle/serum distribution of the heat-induced aggregates. In accordance with the present results involving *para*-κ-casein, it therefore seems that, when κ-casein is made more hydrophobic and/or less repulsive to other proteins (e.g., in the *para*-κ-casein form or at pH values close to isoelectric pH), it becomes less likely to dissociate from the casein micelle in whatever form (aggregated or not) and more likely to attract denatured whey proteins on the micellar surface. However, whether the dissociation of κ-casein is a cause or a consequence of its interaction with denatured whey proteins upon the formation of the serum type of aggregates remains unclear. Interestingly, the fact that the renneted and then heated milks used in this study contained 33−63% of unhydrolyzed and therefore potentially heat-dissociable κ-casein and, despite this, that very little heat-induced serum aggregates were formed in these samples strongly suggested that the interaction of the denatured whey proteins with κ-casein on the surface of the casein micelle was the preferred route for the formation of serum aggregates. Of course, this result may be a consequence of a likely higher affinity of the denatured whey proteins for *para*-κ-casein rather than for κ-casein. Because κ-casein is probably not limiting (*8*), the denatured whey proteins may have all interacted with the only fraction of κ-casein that had been converted. However, previous studies using whey protein-free milk or β-lactoglobulin with a blocked thiol group also suggested that the interaction of the whey protein with κ-casein actively contributed to its dissociation from the casein micelle (*9*−*12*). Conversely, the addition of caseinate (*52*) or isolated κ-casein (*47*) to milk prior to heat treatment did not increase the amount of serum aggregates formed. These results all strongly suggest that the serum aggregates originate from the dissociation of micelle-bound ones and therefore that the serum and the micelle-bound aggregates are likely to show similar structures and properties.

The small changes in the properties of the serum aggregates formed in heated as a result of renneting (HT_h milks) as well as the absence of serum aggregates when renneted milk was heated (h_HT milks) showed that the conversion of κ-casein affected the heat-induced aggregates in both sequences of heat and chymosin treatments and therefore that *para*-κ-casein was somehow involved in the final form of the aggregates. However, a significant proportion of the whey protein/*para*-κ-casein aggregates formed in heated and then renneted milk (HT_h) were found in a stable dissociated form, while comparatively little dissociation of these aggregates occurred in renneted and then heated milk samples (h_HT). These results suggested that the sequence of the heat and chymosin treatments affected the properties of the whey protein/*para*-κ-casein aggregates. A current theory describes the action of chymosin on casein micelles as the random cleavage of κ-casein molecules as the enzyme collides with casein micelles through Brownian motion, leaving hydrophobic patches on the micelle surface (*38*, *53*). It is therefore possible that κ-casein involved in the aggregates of heated and then renneted milk samples was hydrolyzed to a similar extent as that measured in the overall milk (**Figure 1**), i.e., that the aggregates contained both κ-casein and *para*-κ-casein. The remaining κ-casein could help maintain the stability of the partially hydrolyzed aggregates in the serum phase of milk, as for partially renneted micelles. It has been reported that isolated serum aggregates resuspended in a buffer precipitated upon renneting, although the serums of heated milk samples did not (*25*). On the other hand, it is likely that the denatured whey proteins of renneted and then heated milk preferably interacted with the *para*-κ-casein patches previously formed on the surface of the casein micelles rather than with κ-casein, as a result of the decreased charge repulsion and increased hydrophobic attraction between *para*-κ-casein and denatured whey proteins. Such aggregates would therefore mainly contain *para*-κ-casein and be less stable in an aqueous environment and/or unlikely to dissociate. It is worth noting that the affinity of the denatured whey protein for *para*-κ-casein seems strong, because the almost exclusive formation of micelle-bound aggregates already took place at as low a rate of κ-casein conversion as ∼37%.

Further research is needed to better investigate how the changes induced by the coupling of chymosin and heat treatment affect the properties of skim milk. It could for instance be interesting to investigate how the likely changes in the surface properties of both the casein micelles and head-induced whey protein aggregates, e.g., surface charge and hydrophobicity, affect the combined coagulation behavior of skim milk (*21*), especially when the respective role of the serum and micelle-bound heat-induced aggregates on acid gelation of milk is still an open question (*14*−*16*, *19*, *20*). The heat treatment of prehydrolyzed milk could eventually open new perspectives for the complete recovery of milk proteins, e.g., in isolate preparations.

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