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Formation of Soluble and Micelle-Bound Protein Aggregates in Heated Milk

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The formation of heat-induced aggregates of κ-casein and denatured whey proteins was investigated in milk-based dairy mixtures containing casein micelles and serum proteins in different ratios. Both soluble and micelle-bound aggregates were isolated from the mixtures heated at 95 °C for 10 min, using size exclusion chromatography. Quantitative analysis of the protein composition of the aggregates by reverse phase high-performance liquid chromatography strongly suggested that primary aggregates of β-lactoglobulin and α-lactalbumin in a 3 to 1 ratio were involved as well as κ-casein, and α_{s2}-casein in micellar aggregates. The results gave evidence that heat-induced dissociation of micellar κ-casein was implicated in the formation of the soluble aggregates and indicated that a significant amount of κ-casein was left unreacted after heating. The average size of the aggregates was 3.5−5.5 million Da, depending on the available κ-casein or the casein:whey protein ratio in the mixtures. The size and density of these aggregates relative to those of casein micelles were discussed.

KEYWORDS: Milk proteins; aggregation; heating

INTRODUCTION

In most commercial dairy processes, thermal treatment of milk and dairy mixes is an essential operation aimed at both food safety and shelf life of the final product and improvement of the functional properties of the proteins. In yogurt manufacturing, heating at 90−95 °C for 5−10 min considerably enhances gel firmness and smoothness and reduces syneresis, in parallel with the denaturation extent of the whey proteins (WPs) (*1*−*4*). At temperatures above 65 °C, β-lactoglobulin, α-lactalbumin, bovine serum albumin (BSA), and the immunoglobulins unfold and interact with κ-casein (K-CN) to form heat-induced protein aggregates (*5*, *6*). These aggregates are acid precipitable and account for large increases in pH of gelation gel strength and serum binding capacity of acid gels formed with heated dairy media (*3*, *7*−*11*). There is evidence that the textures of acid milk gels depend on the protein composition of the milk (*12*−*14*), but no direct link has yet been drawn to relate the size and composition of the aggregates to the initial protein distribution in milk, nor to relate the gelation properties of heated milk to the characteristics of the aggregates. Also, little is known of the quantitative protein composition, size, shape, and physicochemical properties of these aggregates, although these characteristics are more likely to determine the liability of the dairy mixture to acid coagulation than simply the extent of WP denaturation. It is important to define a comprehensive view of the aggregation process, following the work of previous authors (*15*−*20*), to understand how compositional and processing factors can modulate characteristics of the aggregates and consequently the quality of acid milk gels. The aims of the present work were to describe in quantitative terms the heat-induced aggregates of WPs and κ-CN found in milk and to propose a pathway for their formation.

MATERIALS AND METHODS

Skim Milk Powder. Lactosylation of milk proteins occurring by Maillard reaction at the drying stage of the manufacturing of skim milk powder may interfere with β-lactoglobulin denaturation and aggregate formation (*21*−*23*). A skim milk powder with as low level of lactosylation as in pasteurized milk was therefore prepared according to Guyomarc'h et al. (*24*) and was used as the source of the reconstituted milk throughout this work.

Reconstitution of Milk. Milk was reconstituted from 10% w/v skim milk powder and 0.05% w/v sodium azide (NaN_3) in distilled water at 45 °C. The milk was stirred until completely dissolved and was left to stand overnight at 4 °C to ensure complete equilibration.

Milk Ultrafiltrate (MUF). MUF was prepared by filtration of reconstituted skim milk at 42 ± 1 °C through a 10 kDa cutoff, 0.37 m^2 surface, hollow fiber membrane UFP-10-D-6 (A/G Technology, Needham, MA).

Preparation of Serum of Control (S0) or Heated (S1) Milk Mixtures or Heated Serum (S2). Control or heated (95 °C/10 min) milk mixtures were centrifuged at 19 000 rpm (33 025*g*) for 60 min in a Sorvall RC28S centrifuge equipped with a SS-34 fixed angle rotor (Kendro Laboratory Products Ltd, Hertfordshire, U.K.) at a temperature of 20 ± 1 °C. The supernatant serum was collected by gently pouring it from the tubes; the sera from control and heated mixtures were

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designated S0 and S1, respectively. A third serum (S2) was prepared by heating serum S0 at 95 °C for 10 min.

Preparation of Suspensions of Micelles of Control (M0) and Heated (M1) Milk Mixtures. Fractions of large micelles (M0 and M1) were separated from control and heated milk, respectively, by centrifugation at 15 000 rpm (20 600*g*) for 15 min at 20 ± 1 °C. This lower speed was intended to ensure that no soluble protein aggregates could cosediment with the micelles. After it was centrifuged, the nonsedimented milk was poured off and the pellets were left to drain for approximately 1 min. Ten milliliters of MUF was added to each pellet after draining, and the micelles were resuspended by stirring gently overnight at 4 °C. The dispersion was then completed by homogenizing each sample for 90 s in an Ultra-Turrax drive T25 (Janke & Kunkel GmbH, IKA Labortechnik, Stanfen, Germany) run at its lowest speed. This treatment was shown not to alter the average hydrodynamic diameter of milk micelles as measured by photon correlation spectroscopy on a preliminary experiment (not shown). The suspensions were again stirred overnight at 4 °C to complete the resuspension process.

Skim Milk Powder Enriched in CN Micelles or in WPs. Reconstituted milk was centrifuged at 12 000 rpm (14 750*g*) at 20 ± 1 °C for 2 h in the Sorvall centrifuge, equipped with a GSA rotor. This gave in each tube a solid white pellet topped with a thick layer of white interphase. Analysis of the protein in initial milk and in the final supernatant showed that these two white fractions contained about 87% of the CN, i.e., most of the CN micelles of the milk. About two-thirds of the supernatant above these fractions was pumped out and stored at 4 °C. The residual material was then homogenized as described above for the dispersion of micelles in serum, to give a milk enriched in CN micelles.

Two volumes of the serum that was removed after the centrifugation was mixed with one volume of reconstituted milk, and then, the protein concentration was brought slightly above that of milk by ultrafiltration of the mix to a concentration factor of 3. The retentate (WP-enriched milk) and MUF, as well as the CN micelle-enriched milk, were finally freeze-dried. Each dried sample was crushed into a powder and packed in double-sealed bags for storage at −20 °C.

Production of Dairy Mixes with Total Protein (TP) Contents of 4.7% with Varying CN/WP Ratio. The formulas of five dairy mixes at 4.7% (w/w) TP and varying CN/WP ratios were calculated, based on the known compositions of the constituents (skim milk powder, CN micelle-enriched milk powder, WP-enriched milk powder, dried MUF, and distilled water). In the mixes, the TP was maintained at 4.7%, the protein fraction derived from the skim milk powder was 2.1%, and the overall CN/WP ratios were 3.0, 3.9, 4.8, 5.7, or 6.6 w/w. The lactose concentration was 5.3%. Because the materials involved were all made from the same batch of skim milk powder, it was assumed that the adjustment of lactose with dried MUF allowed adjustment of all other diffusible serum components (minerals and salts) at the same time. The unique combination of constituents was calculated for each CN/WP ratio, and the mixes were reconstituted as described above with appropriate blends of the reconstituted powders. All of the mixes were pH 6.67 ± 0.02 after heating.

Other Materials. WPC 35 was purchased from Ingredia (Arras, France). Bis-tris propane (BTP), β-mercaptoethanol (β-ME), sodium lauryl sulfate (SDS), trifluoroacetic acid (TFA), NaN_3, and sodium chloride were purchased from Sigma (St. Louis, MO). Tris was purchased from Sigma-Aldrich (Gillingham, U.K.). Ethylenediaminetetraacetic acid (EDTA), bulk urea, methanol, and acetonitrile were from Fisher Scientific (Loughborough, U.K.). All chemicals were of analytical grade, and solvents were of far-UV grade. Pure samples of catalase, ferritin, thyroglobulin, and Blue Dextran 2000 were purchased from Amersham Biosciences (Bucks, U.K.), IgM was purchased from Sigma, λ-DNA was purchased from Promega (Southampton, U.K.), and two circular plasmids pRHL and pBK 4666 were produced and amplified at the Hannah Research Institute.

Heat Treatment at 95 °C for 10 min. It is common knowledge that WPs start to denature and to aggregate when the temperature exceeds 60−65 °C. Denaturation of β-lg and α-la follows different kinetics depending on the temperature (*25*, *26*). To ensure that the aggregates formed in heated skim milk were generated at the final heating temperature and not during the increase in temperature to 95 °C, a small-scale tubular heat exchanger was designed to perform a very rapid (<1 s) increase in temperature between 55 and 95 °C (**Figure 1**).

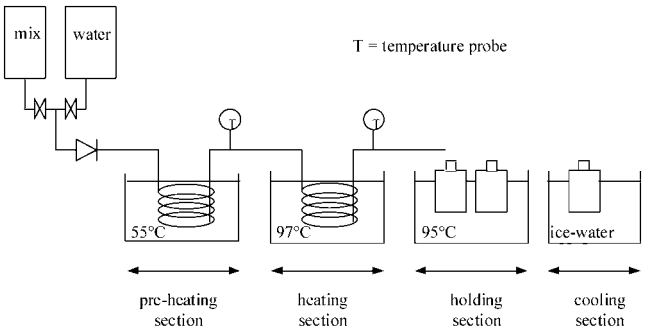

Figure 1. Diagram of the small-scale heat exchanger with instantaneous increase in temperature. The preheating and heating sections were coiled tubular heat exchangers in water baths. The preheating section brought the product to 55 °C in 1 min; the heating section then brought it to 95 °C in <1 s. The product was distributed in a 250 mL Schott bottle held at 95 °C in the holding section and cooled in ice water after complete heat treatment.

The plant was designed by Danone Vitapole and assembled by Techno-Fluides Industries (Verriéres-le-Buisson, France). The pump (2 RB 10) was from Fristam Pompes S. A. (Noisy-le-Sec, France). The temperature probes were from Chauvin-Arnoux (Vaulx-en-Velin, France). The heating fluid was water contained in thermostated water baths (Grant Instruments, Cambridge, U.K.) and covered with polypropylene beads. Tanks and tubing were in food compatible stainless steel 316L and were from Inoxtubes (Coulommier, France). The preheating section consisted of an 8.3 m length of tubing with an external diameter of 6.4 mm and a wall thickness of 0.7 mm. At a flow rate of 10 L h^{-1}, the flow was laminar and the residence time was 1 min. The heating section consisted of a 3 m length of tubing of 1.6 mm external diameter and 0.3 mm wall thickness. The flow was turbulent in this section, and the residence time was 0.9 s. The outlet tubing (feeding the bottles of the holding section) was an insulated tube of 170 mm length and 12 mm internal diameter; the flow was laminar, and the residence time was 7 s. In the holding section, the milk was incubated at 95 °C in filled 250 mL Schott bottles for 10 min ± 45 s; the timing was started when the bottle was half full. After the required holding time, the bottles were cooled rapidly in ice water. The possible extra changes in protein aggregation during cooling were assumed to be negligible, considering that a heat treatment of 95 °C for 10 min denatures 100% of β-lg and about 90% of α-la (*25*, *26*).

Size Exclusion Chromatography (SEC). Separations were performed at room temperature by loading 500 μL of sample onto a Sephacryl S-500 HR Hi-Prep 16/60 column (Pharmacia Biotech, Milton Keynes, U.K.). The Sephacryl S-500 has a separation range from 40 to 10^5 kDa. Different buffers were used to separate different reaction products. For the separation of milk sera under nondissociating conditions, the buffer used was 0.1 M Tris, 0.5 M NaCl, and 10 mM NaN_3, pH 7.0 (*27*). For the separation of suspensions of milk micelles in MUF under dissociating conditions, the buffer used was 2.5% SDS, 7.63 mM Tris, 0.76 mM EDTA, and 10 mM NaN_3, pH 8.2 (*28*). For these analyses, the micellar pellets were resuspended and mixed with the running buffer in a 1:1 ratio before applying the sample to the column, to dissociate the CN micelles and isolate covalently bonded protein aggregates. All buffers were filtered through 0.2 μm filters before use. The flow rate in all cases was 0.5 mL min^{-1}, and absorbance of the eluate was monitored at 280 nm. Fractions were collected every 10 min from 70 min onward.

Samples were injected on to the column with a Spark Holland Basic Marathon autosampler (Thermo Separation Products Inc., San Jose, CA). The flow rate of the mobile phase was regulated by a P-3500 pump (Amersham Biosciences). The UV-M detector and the Frac-100 fraction collector were also from Amersham Biosciences. The integrator was a Datajet, from SpectraPhysics (Thermo Separation Products Inc.).

The system was driven by a Liquid Chromatography Controller LCC-500 (Amersham Biosciences). Data were collected and stored with the TSP P-1000 software version 3.0 (Thermo Separation Products Inc.).

Calibration of the SEC Column. Pure samples of the molecular weight standard materials described above were each dissolved in Tris/NaCl buffer, injected on the column, and eluted with the same buffers. Each experiment was carried out twice. The λ-DNA was excluded from the gel and allowed determination of the void volume. Circular DNAs had molecular masses (MM) exceeding 5 million Da, and their loose coiled conformation made them good candidates as globular high molecular mass standards, as proteins are scarce in this range. The K_{av} value for each point was calculated by

$$K_{av} = (V_e - V_0)/(V_t - V_0)$$

where V_t is the total volume of the column, V_0 is the void volume, and V_e is the elution volume for the sample. The data were plotted against MM on a semilog scale and gave a straight line of equation $K_{av} = -0.1786 \times \mathrm{Ln(MM)} + 2.9724$, with $r^2 = 0.951$. This line was for the standards used. Proteins with MM < 40 kDa all eluted at the same K_{av} and were not separated.

Dialysis. Fractions collected from the SEC column were desalted by dialysis of one volume fraction in 14 mm diameter, 12−14 kDa cutoff dialysis sacks (Medicell International Ltd., London, U.K.) against 100 volumes of distilled water. Dialysis of fractions collected in nondissociating conditions was performed at +4 °C for 40 h with four changes of water; for fractions collected in dissociating conditions, the dialysis was performed at room temperature for 96 h with nine changes of water. This temperature was used to prevent crystal formation of SDS. NaN_3 (0.05%) was added to the water to prevent bacterial growth, except in the last two batches of water. The fractions were then poured into plastic containers, frozen, and finally freeze-dried in a EC Supermodulyo (Edwards, North Walsham, U.K.) at −55 °C and 0.6 mbar over 2 days.

Sample Preparation. Protein dissociation and cleavage of disulfide bonds in the fractions of heated milks from the SEC column were carried out, before analysis by reversed phase high-performance liquid chromatography (RP-HPLC), by resuspending the freeze-dried material in 50 μL of distilled water + 200 μL of denaturing buffer (7 M urea + 20 mM BTP, pH 7.5) to which was added 5 μL mL^{-1} of β-ME immediately before use.

RP-HPLC. Protein composition of the fractions was determined by RP-HPLC using an adaptation of the method of Visser et al. (*29*, *30*). The column was a 25 cm length, 0.46 cm inner diameter, Apex wide-pore C18 column, with a bead diameter of 7 μm (Jones Chromatography, Hengoed, U.K.). Buffer A was 0.1% v/v TFA in ultrapure deionized water, degassed. Buffer B was 0.1% v/v TFA in acetonitrile, degassed. The sample loop size was 10 μL, the oven temperature was 46 °C, and the detector wavelength was 214 nm. The flow rate was 1 mL min^{-1}, and a gradient of 33−47% of buffer B was used to elute the proteins.

The equipment consisted of an AS 3000 automatic injector, a P 2000 binary gradient pump, and an UV 2000 UV/vis detector, all from Spectra-Physics Analytical (San Jose, CA). This equipment was driven automatically by the PC 1000 software version 3.0 (Thermo Separation Products) through a SN 4000 interface (Spectra-Physics). Protein mass concentration (mg mL^{-1} of prepared 50 μL sample) was calculated from integrated peak areas.

RESULTS

Protein Aggregates in the Serum Fractions. Figure 2 shows the gel permeation profiles of the serum of heated milk (S1) and of the heated serum of control milk (S2) as compared with the profile of the serum of control milk (S0). In the control serum, no protein material of large molecular mass was found. The two peaks at 240 and 270 min were found in all serum samples and were consistent in size. They were also consistent with the only two peaks found in MUF (not shown), which had been identified as orotic acid and other small plasma solutes,

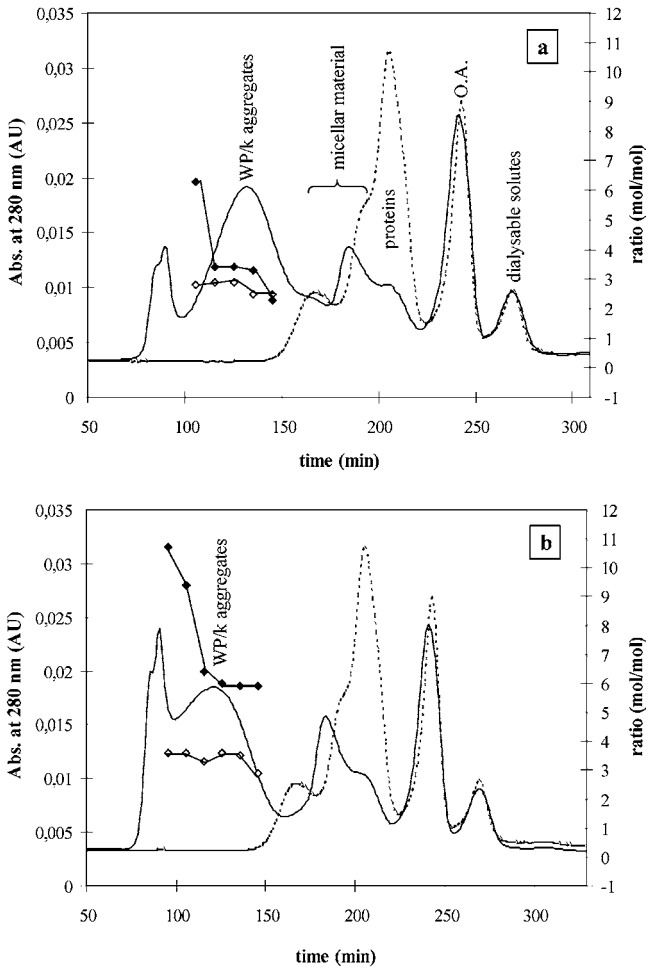

Figure 2. Elution profiles obtained by SEC on a Sephacryl S-500 HR Hi-Prep 16/60 column in native (nondissociative and nonreducing) conditions. Serum of control milk (S0) (– – –); (**a**) serum of heated milk (S1) (—); (**b**) heat-treated serum from control milk (S2) (–); molar ratio of β-lg:α-la (◇); molar ratio of WPs (β-lg + α-la):κ-CN (◆). OA stands for orotic acid.

respectively, by Andrews et al. (*31*). Although in theory the column was not capable of separating these small molecules, it can be seen that a good separation was achieved. The main peak from unheated serum eluted at 205 min, i.e., the retention time of individual proteins of MM < 40 kDa (not shown) and was identified as the soluble protein fraction, i.e., mostly WPs and the soluble fraction of CN. The peak eluting at 170 min contained small quantities of all of the CNs. The average mass ratio β-CN:α_{s1}-CN:α_{s2}-CN:κ-CN was about 10:9:1:8, with the highest ratio of κ-CN at the beginning of the peak. The size of the material was estimated to be 8.5×10^5 Da. The shoulder on the major peak at 190 min contained mainly α_{s1} and β-CNs with a ratio between 2:3 and 1:1, in particles whose mass was estimated to be 4×10^5 Da. These two latter peaks had some similarity to the submicelles F2 and F3 described by Ono et al. (*32*) and Ono and Obata (*33*), composed of κ and β-CN and of α_{s1} and β-CN, respectively, with respective particle masses of 2 and 5×10^5 Da (*34*), although our molecular masses were larger than those quoted in these references. The κ-CN-rich peak at 170 min could also be very small micelles that were not separated by ultracentrifugation or could possibly (although this is less likely) result from reassociation of some CNs during storage at 5 °C. It can be seen that the amounts of these CN-containing components were small relative to the total amount

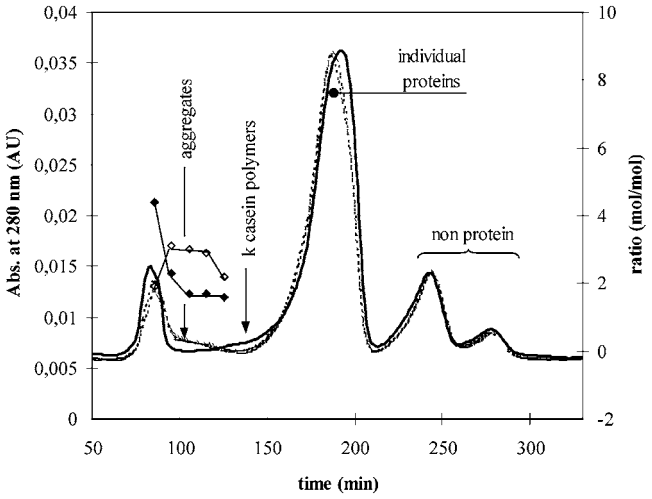

Figure 3. Elution profiles obtained by SEC on a Sephacryl S-500 HR Hi-Prep 16/60 column in dissociative (nonreducing) conditions. Suspension of micelles of control milk (M0) (—); suspensions of micelles of heated milk (M1) (– – – and – – –); molar ratio of β-lg:α-la (◇); molar ratio of WPs (β-lg + α-la):κ-CN (◆).

of CN in the milk; RP-HPLC analysis showed that they represented less than 10% of the total CN of the milk.

A void volume peak appeared on most SEC profiles (not shown for control serum in **Figure 2**). It did not contain any protein material and was not further identified.

Orotic acid is very stable to heat and to chemical changes (*35*). It also gave a well-resolved peak on separation by Sephacryl and was therefore a good material to use as the internal standard to compare profiles of sera from the same milk. **Figure 2** thus shows that the amounts of the native WPs decreased considerably during heating, while a large symmetrical peak appeared at retention times between 100 and 160 min. In both the serum of heated milk (**Figure 2a**) and the heated serum of control milk (**Figure 2b**), analysis by RP-HPLC of these fractions detected only κ-CN and the WPs β-lg and α-la. In another experiment, the serum of a heated dairy mix was separated in both native and dissociating conditions. The results showed that in dissociating conditions, a large WP/κ-CN peak remained that eluted at shorter elution times (due to the binding of SDS molecules; see also **Figure 3**), with no large increase in the area of the individual protein peak (not shown). Formation of heat-induced aggregates of κ-CN and WPs based on hydrophobic interactions and disulfide bonds when milk is heated at temperatures exceeding 60−70 °C is now well-established, and our results on the lack of effect of SDS on the dissociation of the complexes indicated that disulfide exchanges were the main mechanism of aggregation in our conditions. Smits and Van Brouwershaven (*5*) and Pearse et al. (*36*) suggested that a soluble form of these aggregates existed in milk serum, and soluble aggregates were also invoked by Creamer et al. (*37*), Patocka et al. (*12*), and Beaulieu et al. (*14*) to explain the occurrence of linear particles in the serum phase of heated milk or of WPC/milk mixes or by Lucey et al. (*8*), O'Kennedy and Kelly (*10*), and Schorsch et al. (*11*) to account for some of the gelation properties of heated milk. The κ-CN/WP aggregate particles were identified by Singh and Creamer (*17*) in the serum of milk that had been heated at 120 °C, using a Sephacryl S-1000 column and SDS−polyacrylamide gel electrophoresis analysis of the fractions in the presence of β-ME.

After the milk was heated for 10 min at 95 °C, cooled, and reheated at 95 °C for a further 10 min prior to centrifugation and separation of the serum, the profile of the reheated serum superimposed on the profile is shown in **Figure 2a**. This showed that further heating did not change the size or the retention time of the aggregate peak and therefore that the aggregation reaction, as well as the denaturation of the proteins, must be complete by the end of 10 min at 95 °C.

According to our calibration plot, the sizes of the aggregates ranged between 1.2×10^6 Da and the exclusion limit of Sephacryl S-500 i.e., $\sim 10^8$ Da. Analysis of the time derivative of the absorbance between 100 and 150 min indicated that the maxima of the peaks were at 130 min for S1 (**Figure 2a**) and at 120 min for S2 (**Figure 2b**). The distribution therefore gave a maximum for aggregates weighing about 3.7 and 5.4×10^6 Da, respectively. There are few estimates of the sizes of heat-induced aggregates; one, in solutions of WPs alone, has been estimated at $\geq 2 \times 10^6$ Da (*38*), from measurements by dynamic light scattering. This figure is in reasonable agreement with our estimates. The molar ratios of WP:κ-CN (**Figure 2**, black diamonds) and β-lg:α-la (**Figure 2**, white diamonds) were calculated for each fraction from RP-HPLC data. The patterns were very consistent for the three repetitions of this experiment and showed that the WP:κ-CN ratio had a constant value across the peak of aggregated protein, although it was high in and around the excluded peak, where only minute amounts of protein were found (possibly because of lower sensitivity of the RP-HPLC for κ-CN than for the WPs), and decreasing rapidly in the latest fractions (due to contamination with the next "submicelle" peak). The β-lg:α-la ratio was also relatively consistent across the peak (**Figure 2**) and was also consistent between S1 and S2 on each repetition of the experiment. However, the β-lg:α-la ratio varied from 2.7 to 3.5 mol:mol in repeated experiments, mainly because of the difficulty of resolving the two WPs on RP-HPLC analysis. The WP:κ-CN ratio was noticeably larger in S2 (**Figure 2b**) than in S1 (**Figure 2a**). At the plateau, the ratio of the fractions was ~3.6 mol:mol in S1 and ~6.6 mol:mol in S2 (taking 3 β-lg + 1 α-la for the WPs); the standard deviation across the three repetitions was ~0.5. Repeatability was better because β-lg and α-la were integrated together for this calculation. Quantitative RP-HPLC analysis of the milk proteins in total mix of CN:WP ratio of 4.8 (i.e., as in milk) and in serum and micellar phases before and after heating also allowed us to estimate that serum κ-CN increased by 8−11% of its initial mass amount on heating while serum β-lg decreased by 9−12%. These results altogether showed that heat-induced κ-CN/WP aggregates were larger and contained less κ-CN and more WPs when the serum was heated separately from micelles. In the latter case, the κ-CN involved in the aggregates had to be present in the serum fraction before heating, i.e., in the submicelle peak or in the individual protein peak, respectively, eluting at 170 and 205 min observed in the S0 fraction. Conversely, this confirms that during heating of milk, the κ-CN incorporated in the soluble aggregates must have partly originated from the CN micelles.

The amount of WP transferred to the CN micelles during these experiments, by comparing the amounts of WPs in the sera of heated and unheated milks, was less than the estimates made by other authors (*5*, *18*, *19*, *39*−*42*). On the other hand, the amount of κ-CN released is in reasonable agreement with the published values. Because we worked at the normal pH of the milk, similar to other studies, it is probable that the difference in the measured WP arises from the heating method that we used, where the milk is passed through very narrow tubes in the preheating stage, where it experiences a very rapid rise in temperature and then is kept in agitated glass bottles in a water

bath. Quantitative RP-HPLC protein analysis of the mix before and after heating showed that some deposition of the WPs occurred during heating. We therefore corrected the amount of initial β-lg (i.e., available for binding onto micelles) in the calculation of the loss of serum β-lg due to its association with micelles. This correction significantly decreased the percent values of β-lactoglobulin bound to the micelles. Previous studies as referred to above have taken no account of protein losses during heating; therefore, direct comparisons are difficult to make.

Micellar Aggregates. Figure 3 shows the gel permeation profiles of a suspension of the large micelles of heated milk (M1) as compared to the profile of a suspension of micelles of control milk (M0), separated in dissociating (SDS/EDTA) buffer. In control milk, no protein material was found in the first fractions, including the void volume peak. The two peaks at 245 and 285 min were found in all samples and were respectively identified as orotic acid and other small plasma solute, similar to the serum samples. The main peak eluted at 190 min, which was the retention time of standard individual proteins in the presence of SDS (not shown). It contained all of the CN types with a mass ratio β-CN:α_{s1}-CN:α_{s2}-CN:κ-CN of about 4.2:3.8:2:1, consistent with it being composed of dissociated CN micelles. Small amounts of WPs were also present, probably after they were trapped in the serum contained within the micellar pellet after centrifugation. In samples from heated milk, the WPs could also have been dissociated from the micelle-bound aggregates, although no significant variation of the single WP content could be noticed between heated and control milk samples. The peak was also preceded by a shoulder between 130 and 160 min. This fraction contained only κ-CN and small amounts of α_{s2}-CN and was interpreted as being composed of polymers of these CNs (*43*, *44*). The presence of κ-CN in the main peak suggested that there were also significant amounts of unpolymerized κ-CNs in the micelles. The sum of the amounts of κ-CN in the corresponding fractions of SEC profiles of micelles of control milk suggested that more than 50% of the micellar κ-CN was found in the main peak (therefore in monomeric form) but the use of better ranged SEC columns of more appropriate range for this particle size (e.g., Superose 6 from Amersham Bioscience) would be required for more precise estimation. Groves et al. (*45*) reported that 10−12% of each κ-CN form (monomer to octamer) were present in milk, and other studies also estimated monomeric κ-CN to be in minor amounts (*43*).

A comparison between micelles of control and heated milk could again be made using orotic acid as the internal standard. **Figure 3** shows that the polymers of κ-CN virtually disappeared on heating, while material of high molecular size appeared after the exclusion peak, between 100 and 125 min. Analysis showed that this material contained κ-CN, the WPs β-lg and α-la, and small amounts of α_{s2}-CN in some fractions. No other CNs were found in this part of the chromatogram. As in the serum, this peak contains heat-induced protein aggregates, as established by Singh and Creamer (*17*).

α_{s2}-CN occurs in milk as a dimer with two intermolecular disulfide bridges, and it is possible for it to interchange disulfide bonds with other proteins on heating (*43*, *46*, *47*). Minor association of this CN to the aggregates has indeed been reported (*48*, *49*). However, we cannot exclude the possibility that our result was an artifact of the RP-HPLC method (*29*, *30*). In 4.7% TP mixes, however, the occurrence of α_{s2}-CN was very consistent across the aggregate peak, although only in small amounts (not shown). The occurrence of α_{s2}-CN tended to increase with elution time, and in some cases, the CN was only present in the later fractions, i.e., in smaller aggregates. This may suggest that aggregation of α_{s2}-CN molecules with κ-CN and WPs might somehow limit the size of the aggregate, possibly because α_{s2}-CN can only form dimers rather than the larger polymers formed by κ-CN. The fact that α_{s2}-CN was found only in micellar aggregates and not in the serum may arise from the deeper position of this CN in the micelle and by the 10−13 serine phosphate residues, which may link the CN with the micelle structure. As opposed to κ-CN, located on the micelle surface and containing only one phosphate group, α_{s2}-CN is unlikely to dissociate on heating (*40*, *41*).

Interaction of the SDS with protein material altered calibration of the Sephacryl column. Individual proteins thus eluted earlier in SDS/EDTA buffer than in nondissociating conditions. By comparing the masses of serum aggregates in native and dissociating conditions, the weight of SDS attached to the aggregates was estimated at about 1:1. On this basis, we may conclude that the micellar and soluble WP/κ-CN complexes were rather similar in size. Also, the fact that heat-induced aggregates were still of considerable size in the dissociating buffer indicated that disulfide bonds were important in building the aggregates, rather than only by hydrophobic interactions.

As in the serum aggregates, the WP:κ-CN ratio was rather constant across the aggregate peak, except for extreme fractions where sensitivity or resolution of the HPLC method could account for some variation. Values of the ratio taken at ∼105 min (taken as being the maximum point of the size distribution) varied between 2.3 and 3.8 mol:mol in repeated experiments (taking 3 β-lg + 1 α-la for the WP composition) and were therefore on average smaller than the aggregates that had been found in the serum. Micellar aggregates of WPs and κ-CN therefore contained less WP (or more CN) than serum ones. The β-lg:α-la ratio was rather constant across the peak and varied between 1.6 and 2.7 mol:mol. This was also lower than in the serum aggregates, possibly because in them the β-lg may be partly aggregated through hydrophobic interactions, which will be disrupted by SDS. This will also explain why the WP:κ-CN ratio was also lower than in the serum fractions, where any hydrophobically associated complexes will remain intact.

Even after heating, there were considerable quantities of κ-CN that had not participated in the formation of complexes, since the peak eluting at 190 min contained significant quantities of the protein. Therefore, not all of the micellar κ-CN may be capable of reacting with the WPs. The idea is supported by values of the WP:κ-CN ratio well above 1:1 as determined in this work (from the amount of WP transferred to the micelles, the total κ-CN would be in excess of the amount of WP). It is clear, however, that the peak attributed to polymeric κ-CN on SEC profiles disappeared after heating (**Figure 3**). It seems therefore that a portion of the monomeric and/or low polymer κ-CN in the micelles is not capable of reacting with WP during heating.

Effect of the CN/WP Ratio on Aggregate Composition and Size. Sera from the five mixes at 4.7% TP with CN/WP ratios ranging from 3.0 to 6.6 g:g were analyzed by SEC in nondissociating conditions before and after the mixtures were heated. **Figure 4** compares typical profiles of the sera of the five heated mixes with the profile of control unheated mix at ratio CN/WP of milk, i.e., 4.8 g:g. Other control profiles did not differ in detail from the one shown. There seemed to be however more material eluting at 170 and 190 min in the profiles of control mixes than in milk (**Figure 2**), probably because of

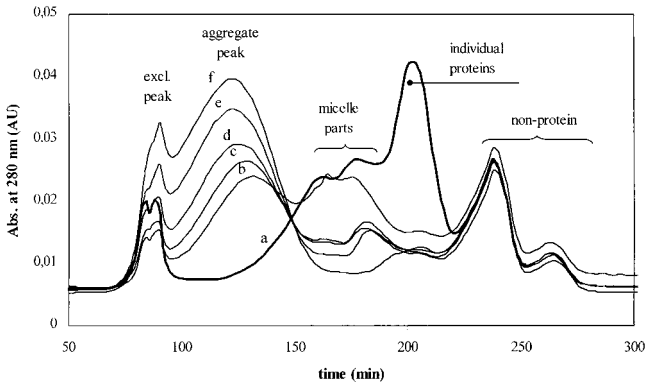

Figure 4. Effect of the CN:WP ratio of 4.7% TP milk mixes on the gel permeation profile of the corresponding serums, separated on a Sephacryl S-500 HR Hi-Prep 16/60 column in native (nondissociative and nonreducing) conditions. Serum of unheated mix with CN:WP = 4.8 (a); serum of heated mix with CN:WP = 6.6 (b); serum of heated mix with CN:WP = 5.7 (c); serum of heated mix with CN:WP = 4.8 (d); serum of heated mix with CN:WP = 3.9 (e); serum of heated mix with CN:WP = 3.0 (f).

the higher TP content of the mixes and subsequent consequences on viscosity and sedimentation efficiency.

Figure 4 shows that the lower the CN/WP ratio, the earlier the aggregate peak eluted, showing that as the proportion of WP in the mix increased, so did the average size of the soluble aggregates. The time derivative of absorbance showed that the maximum of the aggregate distribution shifted from 3.5×10^6 to 5×10^6 Da as the CN/WP ratio varied from 6.6 to 3.0. Because the plasma fraction of the mixes was kept constant with MUF, orotic acid was used as internal standard and this showed that at the lower CN/WP ratios greater amounts of soluble aggregates were formed. Micellar aggregates did not seem to share these trends. Profiles of micellar suspensions from the five mixes appeared to superimpose on one another (not shown) and on the curve of **Figure 3**. In comparison, the separation of sera from the two extreme mixes (CN:WP 3.0 and 6.6) in dissociating conditions showed that the height of the aggregate peak does vary with protein composition in the presence of SDS (not shown) and therefore that the superimposition of the profiles of micellar material showed some consistency in the quantity and/or size of the micellar WP/κ-CN aggregates no matter what was the CN/WP ratio. This would confirm the conclusions of Corredig and Dalgleish (*39*) that there is a limit to the extent to which WPs can bind to CN micelles. As was found in the analysis of the micellar fraction of milk, the dissociated CN micelles in the mixture contained unreacted κ-CN, even at the highest ratios of WP to CN. HPLC data also indicated that 20−35% of the total κ-CN found in control micelle profiles consistently remained in the main peak of individual proteins after heating.

All of the fractions of the aggregate peaks in the sera of all heated mixtures contained the two WPs, α-la and β-lg, and κ-CN. All of the observed aggregate peaks appeared to be monomodal, and the consistency of the β-lg:α-la ratio and of the changes in the WP:κ-CN ratio across the peaks suggested that the aggregated proteins might be formed from the polymerization of some basic unit containing the three proteins. No aggregates composed only of WPs were positively found, except with heated 4% WPC 35 solution (not shown). This agreed with previous findings that when CN is present only WP/κ-CN aggregates can be formed (*50*), although our results could not positively exclude the occurrence of a mixture of coeluting WP and WP/κ-CN aggregates.

Calculation of the WP:κ-CN and of the β-lg:α-la ratio confirmed the patterns observed in milk. In all cases, high WP:κ-CN ratios were found in the exclusion peak, which then rapidly decreased to plateau levels across the aggregate peak. The β-lg:α-la ratio was fairly constant across the peaks and was, quantitatively, also relatively consistent across the mixes and with the values found in milk. It varied between 2.7 and 3.5 mol:mol across the mixes and repetitions and was independent of the CN/WP ratio. This further confirmed the ratio of approximately 3:1 between β-lg and α-la in the aggregated materials, no matter what was the original protein composition. Values of the WP:κ-CN ratio increased regularly from 2.2 to 2.9 mol:mol (taking 3 β-lg + 1 α-la for the WPs) as the CN/WP ratio decreased from 6.6 to 3.0. Variation across repetition was $\pm$0.2 for each mix. This showed that the more WP in the system, the more WP was involved in the aggregates. This result seemed true also for micellar WP/κ-CN aggregates, albeit the relationship was not as clear as in the sera. Values of the ratio were lower in these 4.7% TP dairy mixes than in milk, suggesting that TP content of the milk, as well as the CN/WP ratio, could be an important factor in defining the compositions of heat-induced aggregates.

DISCUSSION

This study showed a clear relationship between the available κ-CN, especially micellar κ-CN, and the protein composition of the aggregates. In milk, the WP:κ-CN ratio of serum aggregates was much higher when the serum had been heated apart from the micelle fraction (S2) than in the presence of micelles (S1), and only in heated WPC, no κ-CN was found in the aggregates. In 4.7% TP dairy mixes with a varying CN:WP ratio, the WP:κ-CN ratio in the aggregates increased when the proportion of CN decreased. Protein composition of the aggregates across the peak separated by SEC also showed that the proportion of involved κ-CN was rather consistent no matter the aggregate size. These observations suggested that the WPs:κ-CN ratio in the aggregates is determined at first from the protein composition in the medium. Conversely, α_{s2}-CN, which was also involved in (micellar) aggregates, only occurred in smaller ones, which suggests that it somehow limited aggregate size. Another limitation might be evidenced by the fact that increasing the CN:WP ratio from 3.0 to 6.6 led to a decrease of the WP:κ-CN ratio from 2.9 to only 2.2 mol:mol. An explanation may be sought in the balancing effects of the opposite transfers of denatured WPs toward the micelle phase and of κ-CN toward the serum and in a possible limitation in the interaction between WPs and κ-CN (*39*). Anema and Li (*47*) reported for instance that adding WP in milk enhanced dissociation of κ-CN, which would tend to bring the WP:κ-CN ratio of possible serum aggregates down.

This study showed for the first time that there can be significant amounts of κ-CN in the micelle, which are unreactive to heat-induced aggregation with WPs. This may call in question the idea of a coverage of the micelle with κ-CN aggregates, as well as the "titration" of κ-CN by denatured WPs (*51*), but supported our values of the WP:κ-CN ratio in the aggregates. The monomeric κ-CN seems to be the unreactive species, possibly because of its reduced sulfhydryls (no intermolecular disulfide bridges) or its higher glycosylation degree (*45*). One further note we can make is that in SEC separation of heated samples, RP-HPLC profiles of fractions corresponding to the individual proteins (i.e., where unreactive κ-CN was found) showed a specific and consistent pattern. While κ-CN otherwise appeared as a series of early eluting small peaks, in these

fractions, only the latest peak was still present, identified as low glycosylated κ-CN (*30*). Because it opposed the fact that polymerized κ-CN seemed preferably involved in the aggregates through the inverse relationship between polymerization and glycosylation degrees of κ-CN (*45*), these results can only be taken as elements for further investigations.

Another interesting result was the consistency of the β-lg:α-la molar ratio in the aggregates, no matter the size or the dairy medium. The ratio was about 3 β-lg:1 α-la across the aggregate peak found in milk, in 4.7% TP dairy mixes of varying CN:WP ratio, and in heated WPC (not shown). In model solutions, β-lg seemed to associate into a trimer prior to association with κ-CN (*52*, *53*). Our results strongly suggested that primary aggregates of the two WPs were formed stoichiometrically and independently of the medium composition in protein and then aggregated with themselves and with κ-CN. This supported the view of Corredig and Dalgleish (*20*) and Elfgam and Wheelock (*14*, *54*, *55*), who proposed that β-lg and α-la formed primary aggregates prior to their association with κ-CN. Our observations of the compositions across the aggregate peak did not indicate that primary aggregates of WPs reacted together to give final aggregates containing only WP but suggested that κ-CN was present in all of the aggregates. The fact that these primary aggregates were not positively detected in our study indicated that they might be of too small dimensions for our methods and/or too reactive; the latter option is more likely since no intermediate peak (other than submicellar material) was found on SEC profiles between the aggregate peak and the individual proteins retention time of 205 min. It is however not possible to exclude that the β-lg:α-la ratio of three reflected in fact the proportions of the two proteins in initial milk, all denatured and aggregated independently to one another after 10 min at 95 °C, albeit β-lg:α-la was closer to 2 in our control milk and that small amounts of unreacted WPs, especially α-la, were still found in the individual protein peak after heat treatment. Other authors indeed found that the β-lg:α-la ratio changed during heat treatment (*19*, *50*, *56*). Corredig and Dalgleish (*39*) found that β-lg:κ-CN and α-la:κ-CN increased during heating following distinct time curves and tended toward plateau values as denaturation of the two WP reached 100%. Such relationships could artificially render the β-lg:α-la ratio constant when only analyzing protein composition of the milk after complete heating at 95 °C for 10 min.

Our results therefore provided further knowledge of the formation of heat-induced aggregates of WPs, κ-CN, and, to a lesser extent, α_{s2}-CN. We propose that β-lg and α-la formed primary aggregates, which further aggregate with κ-CN following a ratio that is determined by the initial composition of medium in WPs and (κ-)CN. The process of this determination (effect of medium composition on kinetics, on statistics of chaotic encounters, etc.) is still unknown. The absence of aggregates of intermediate size suggested that aggregation occurs rather quickly, but it also deprived us of an insight on the aggregation pathway. The fact that the WP:κ-CN ratio was constant across aggregate sizes, however, seemed to exclude a polymerization of the WP without including κ-CN, if any polymerization. However, it should be remembered that in these experiments the particle size distribution produced after the heating was stable and did not change with further heating.

The distribution of the heat-induced aggregates isolated by SEC was generally maximum for MM around 4×10^6 Da. Not considering possible attached water, this represented more than 200 molecules of 19 kDa each (β-lg or κ-CN) and could go up to thousands of molecules for the largest aggregates. The aggregation degree was therefore very high, and taking the diameter of β-lg or κ-CN to be of 3 nm (*57*), one can estimate the aggregates to be either globular particles of more than 10 nm diameter or hundreds of nanometers long linear particles. It is interesting to notice that this dimension for a protein globular structure matched other particle sizes reported in milk, e.g., for submicelles (*33*, *58*), κ-CN polymers (*59*), or β-CN micelles (*60*). In milk, the WP:CN micelle number ratio is 1000 to 10 000. This means that several units or several tens of aggregates of tens of nanometers in diameter can be formed for each micelle (180 nm average diameter). Such figures suggested a relatively equilibrated balance between the population of micelles and the acid precipitable aggregates formed in heated milk, in terms of both size and density. The consequences of this in terms of gelation properties of milk will be reported elsewhere.

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