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Effect of the pH of Heating on the Qualitative and Quantitative Compositions of the Sera of Reconstituted Skim Milks and on the Mechanisms of Formation of Soluble Aggregates

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The effect of the pH of heating (6.3−7.3) on the composition of sera in reconstituted skimmed milks was investigated. A combination of SDS−PAGE analysis and size exclusion chromatography (SEC) combined with an original approach to the analysis of the SEC profiles was performed. The composition of the sera varied greatly when the pH of heating was adjusted below and above the natural pH of milk. The formation, composition, and concentration of heat-induced soluble complexes depended on the combination of the effect of adjusting the pH of the milk and the heat treatment. Two types of mechanism for the formation of soluble aggregates appeared to exist, depending on the pH of the milk. The first type results from the formation of WP/κ-casein aggregates at the surface of the micelle, and these were detached partially into the serum in larger amount as the pH increased up to 6.7, where it reaches a maximum. The second type of complexes, whose amount increased as the pH of heating increased from 6.7 to 7.3, may be formed between caseins (κ- but also perhaps some α_s-casein) and aggregated WP resulting in complexes that are smaller in size and with a higher κ-casein/whey protein ratio than the first type.

KEYWORDS: SDS−PAGE; SEC; α-lactalbumin; β-lactoglobulin; caseins; pH

INTRODUCTION

The thermal treatment of milk is an essential step in the processing of various dairy products. For example, in the yogurt process, heating at temperatures above 70 °C leads to a higher value of the pH at gelation, increases the firmness, and reduces the syneresis of the acid gel compared to the one formed from unheated milk.

During thermal processing, the whey proteins (WP, mainly β-lactoglobulin (β-lg) and α-lactalbumin (α-lac)) denature and aggregate with each other and with κ-casein (κ-cas) to form heat-induced protein aggregates that are known to have a marked effect on the properties of acid-induced gels (*1*−*3*). This interaction between denatured WP and κ-cas is known to involve the formation of disulfide bonds (*4*−*6*). Other interactions such as hydrophobic bonds (*7*) also seem to play a role in the initial formation of the complex.

The heat-induced complexes are located partly at the surfaces of the casein micelles and partly in the serum as small dispersed particles that remain in the supernatant after removal of the micellar fraction by centrifugation. The mechanism of formation of the soluble complexes is still not clear, although it has been shown that the partition of denatured WP between soluble aggregates and the micellar coating, as well as the protein composition of the complex, appear to be affected by the pH of heating (*8*−*16*). These authors showed that, over the pH range of 6.3 to 7.1, the complexes are mainly present on the surfaces of the casein micelles at pH lower than the natural pH of milk (6.6−6.7) (*8*, *9*, *16*), whereas at higher pH, most of the WP was associated with κ-cas as soluble complexes in the serum (*10*, *14*).

It has been also been shown that as the pH of milk is increased, the amount of caseins released from the casein micelles increases as a function of temperature (*14*, *15*, *17*−*20*). However, the different effects of pH and heat treatment on the mechanism of formation of the soluble complex do not seem to be fully understood. In the present study, we intend to give more explanations on these two combined effects on the mechanisms of formation of soluble complexes by determining the differences of composition of the sera of heated and unheated milk samples after adjusting the pH between 6.3 and 7.3, using size exclusion chromatography and SDS−PAGE techniques.

MATERIALS AND METHODS

Reconstituted Skim Milk Powder (RSMP). To ensure standard conditions and total rehydration, skim milk powder (low-heat, Parmalat Foods Inc., Toronto, ON, Canada) from a single batch was reconstituted to 110 g·kg^{-1} solids in Milli-Q water (which corresponds to a total protein content of 38.2 g·kg^{-1} based on the analysis provided by manufacturer) and stirred overnight at refrigeration temperature (4 °C). Sodium azide (0.2 g·kg^{-1}) was added at the time of solution to prevent bacterial growth. Prior to each experiment, the milk was tempered at ambient temperature until it reached approximately 24−25 °C.

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Heat Treatments at Different pH Values. The pH of samples of RSMP was adjusted to values between 6.3 and 7.3 by slow addition of 1 M HCl or NaOH to the milks while stirring. The pH was allowed to equilibrate for at least 2 h, and minor readjustments were made during that time. The milk samples were then transferred to glass tubes and heated for 10 min at 90 °C in a water bath allowing 2.5 min for the samples to reach the final temperature. After the heat treatment, the milk samples were immediately cooled to room temperature by immersion in an ice bath. The samples were stored for 2 h at ambient temperature after heat treatment before any further analysis.

Preparation of Supernatant (Serum) from Milks. To investigate the effect of the different treatments on milk on the formation of soluble complexes of casein and serum protein, samples of the differently treated RSMP were centrifuged at 25000*g* for 1 h at 20 °C in a Beckman Coulter Optima LE-80K ultracentrifuge, with rotor type 70.1 Ti (Beckman Coulter Canada Inc., Mississauga, ON, Canada). The adequacy of this method in removing casein micelles while retaining the serum complexes has been discussed previously (*21*, *22*). The supernatants were carefully removed from each centrifuge tube with a syringe and then filtered using a 0.45 μm filter (Millipore Corporation, Bedford, MA). Supernatants were analyzed within 1−2 days after preparation and stored at 4 °C. The total protein concentration of the supernatants was measured using the DC protein assay method (Bio-Rad, Mississauga, ON, Canada).

Size Exclusion Chromatography (SEC). One milliliter samples of each supernatant were analyzed by size exclusion chromatography (SEC) using a Bio-Rad Biologic Duo-Flow chromatography system (Bio-Rad Laboratories, Hercules, CA) equipped with a 1.0 mL sample loop. A Pharmacia XK 16/70 column was used, with a packed bed height of 67 cm. The packing material was S-500 Sephacryl high-resolution gel, with a nominal fractionation range of $40-2 \times 10^4$ kDa (Amersham Biosciences Inc., Baie d'Urfé, Quebec, Canada). Detection of the eluted peaks was by absorption at 280 nm. Samples were loaded onto the column and eluted at a flow rate of 1 mL/min. The total elution time for each sample was 180 min. The mobile phase was 20 mM Bis−Tris−Propane at a pH of 7.0 containing 0.2 g·kg^{-1} of sodium azide. The method is comparable to that used by Guyomarc'h et al. (*23*) but used a higher flow rate. To have exactly the same time scale in all runs where different columns were used, the elution profiles were fitted and normalized with a routine of the Sigmaplot program (version 8.0, SPSS Inc., Chicago, IL). This created profiles based on a standard relative elution time scale (T_R) from 0 to 2.7 that was used for all elution profiles. On this scale, a value of $T_R = 1$ was given to the breakthrough time (dead volume) of the column. This normalization of the time scale was important in allowing the fitting of the profiles described in a later section.

For analyses of the protein compositions of the effluent at different positions on the profile, 3 mL fractions were collected directly at the times defined by the maxima of the Gaussian peaks used to describe the profile (see later in the section). The collected fractions were then concentrated to 100 μL using a Centrivap cold trap (Labconco, Kansas City, MO) for 3 h at 60 °C.

Gel Electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) was performed using a Bio-Rad electrophoresis unit (Bio-Rad Power Pac HC). Samples of the centrifugal supernatants were diluted to different extents (1:2, 1:3, 1:4, 2:3, depending on the samples) with water and then in a 1:2 ratio with the sample buffer (0.5 M Tris−HCl, pH 6.8, 20 g·kg^{-1} SDS, 190 g·kg^{-1} glycerol, 0.5 g·kg^{-1} β-mercaptoethanol, 0.1 g·kg^{-1} bromophenol blue) and heated for 5 min at 95 °C.

Samples collected from SEC and concentrated were diluted only with sample buffer (1:1). The same sample buffer without the mercaptoethanol was used to study the samples in nonreducing conditions, to evaluate how much protein was involved in covalent aggregates formed through disulfide linkages. In this case, samples were diluted only with sample buffer (1:2).

For the electrophoresis, the resolving gel contained 18% acrylamide (3.3% bis-acrylamide) in 0.4 M Tris−HCl at pH 8.9, and the stacking gel contained 4% acrylamide in 0.05 M Tris−HCl buffer at pH 6.7. The electrophoresis buffer was 0.7 M Tris−HCl, 0.45 M glycine at pH 8.3. Aliquots of 5 μL of the prepared samples were loaded onto the gels. For samples collected from SEC and concentrated, aliquots of 7 μL were loaded onto the gel. The electrophoretic separation was performed at 200 V for 40 min. The gels were stained with coomassie blue in 50% (v/v) methanol and 10% (v/v) acetic acid for 30 min with shaking and were destained with two changes of 1 h of a 45% (v/v) methanol and 10% (v/v) acetic acid solution and then one change for 12 h of a 4.5% (v/v) methanol and 1% (v/v) acetic acid solution.

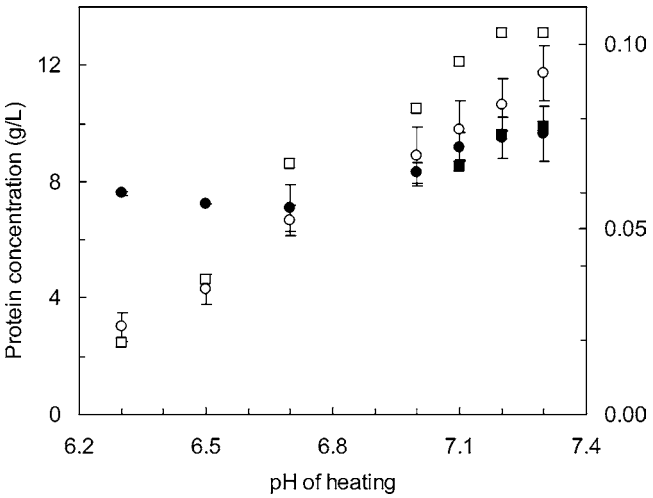

Figure 1. Concentrations of total protein in the sera of milks unheated and heated at different pH values and centrifuged: (○, ●) concentrations measured by protein assay; (□, ■) total integrated area of the Gaussian peaks used to fit the elution profiles. Filled symbols are the values for unheated milk.

The gels were scanned (Sharp JX-330 scanner, Pharmacia Biotech), and the integrated intensities of the protein bands were analyzed using Image Master ID Elite software (version 3 from Novell). Because protein staining densities may vary from gel to gel, all gels contained reference samples of 4 g·kg^{-1} WP isolate (WPI, Power Pro, Land O' Lakes Dairy Proteins Group, St. Paul, MN) and 3 g·kg^{-1} sodium caseinate (NaCas, Alanate-180, Fonterra Milk Products, Lemoyne, PA). The quantification of protein concentration from electrophoresis band intensities was analyzed according to the following procedure: for each supernatant sample, three bands of interest were identified corresponding to α-lac, β-lg, and caseins (κ-cas, β-cas, and α_s-cas were considered all together). The ratio of each band to the corresponding one in the reference protein band of WPI or NaCas was then calculated. Quantification was then made using calibration curves that were developed by plotting the concentration of pure protein samples of α-lac (Sigma, St. Louis, MO), β-lg (Protose, Teeswater, Canada), and NaCas as a function of the ratio of the intensity of the pure protein band over the intensity of the same protein in the reference sample (i.e., WPI or NaCas).

Measurement of Particle Sizes. Particle sizes of the effluent from the SEC column collected in fractions of 3 mL were measured by dynamic light scattering on a Zetasizer Nano ZS Malvern (Malvern Instruments, Southboro, MA). Samples were collected directly into 1 cm square cuvettes, which were then placed in the measuring system at 20 °C.

Replication of Experiments. All experiments were made at least in duplicate.

RESULTS

Protein Quantification of Sera from Milks Heated at Different pH. The total concentrations of proteins in the milk sera as a function of the pH for heated and unheated samples were measured using the assay procedure for total protein and are shown in **Figure 1**. At pH 6.7 and below, the average protein concentration in the serum from unheated milk was 7.0 ± 0.8 g·L^{-1}, which is in agreement with the average protein concentration measured in milk (*24*). As the pH of the unheated milk was increased, the concentration of protein in the serum increased linearly with pH up to 9.7 ± 0.9 g·L^{-1} at pH 7.3.

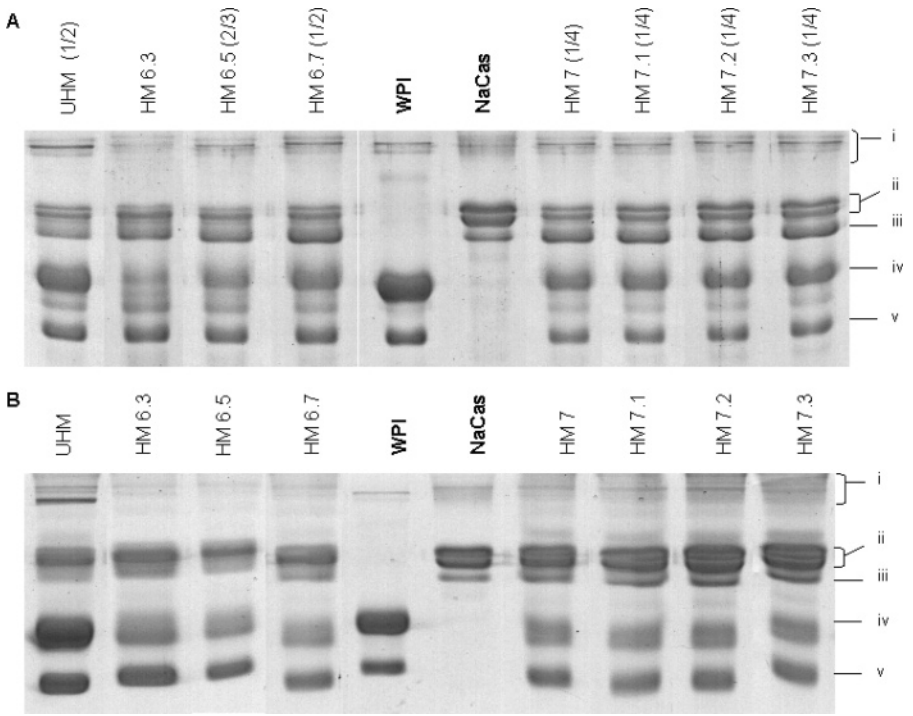

Figure 2. SDS–PAGE patterns of the sera in milks heated at different pH values and centrifuged: (**A**) under reducing conditions; (**B**) under nonreducing conditions. In (**A**) the dilution factors of the different sera used to prepare the samples for electrophoresis are given. The bands in the gels are identified as (i) minor WP; (ii) α_s and β-cas; (iii) κ-cas; (iv) β-lg; (v) α-lac.

After heating at pH 6.7, there was no significant change in the total protein concentration in the serum compared to that of unheated milk. After heating at lower pH, the protein concentration in the serum decreased to 3.0 ± 0.5 g·L^{-1} at pH 6.3; in contrast, the protein concentration of the serum increased to $\sim 12.0 \pm 1.0$ g·L^{-1} after heating at pH 7.3. The increase in concentration of proteins in the heated sera between pH 6.3 and 7.3 appeared to be approximately linear with pH, and no discontinuities were observable. The pH of heating considerably affected the protein content of the serum. The difference of concentration between heated and unheated samples shows that there was an increase in the protein concentration in the serum when the pH of the milk was higher than 6.7 although the distribution of the different proteins in the serum changed significantly (see below).

To analyze the differences in protein concentration in the serum more precisely, quantification of the different sera was performed using SDS−PAGE. **Figure 2A** shows an electrophoresis gel obtained under reducing conditions, which allows the determination of all of the protein present in the supernatants of the milk, and **Figure 2B** shows a similar gel run on the same sera in nonreducing conditions, which allows the determination of the proteins that are not involved in covalent aggregates formed through disulfide linkages (for these, the aggregates are too large to migrate into the resolving gel). Therefore, the difference between protein concentrations calculated from bands obtained in reducing and nonreducing conditions can be interpreted as the concentrations of proteins that are involved in the soluble complexes via disulfide bonding (*4*−*6*). In **Figure 2**, different dilution factors were used for the different sera, so as to obtain a better resolution and more even staining of the bands. Consequently, the different intensities of the bands cannot be interpreted simply visually as differences in concentrations. Quantification of the intensity of the protein bands was made after scanning and using calibration curves as described in the methodology. Since no pure samples of individual caseins were available, calibration curves of caseins were made using the total integrated area of all of the casein bands. The effects of heating on the serum composition were determined by comparison with the composition of the serum from unheated milk.

In all of the samples run on the reducing gel, major bands of α-lac, β-lg, and caseins were identified as well as minor bands of immunoglobulin-G, lactoferrin, and bovine serum albumin. These minor bands were hardly seen in the nonreducing gels. The concentration of α-lac in all heated sera was approximately constant at around 0.87 ± 0.3 g·L^{-1} (results not shown); only a small difference, within experimental error, was found between reducing and nonreducing conditions at pH 6.5 and 7, while at pH > 7.0 there seemed to be no difference between reducing and nonreducing analysis. This seemed to suggest a rather small involvement of this protein in the formation of the heat-induced complexes. The quantification of the intensities of bands from β-lg and casein in heated and unheated sera is shown in **Figure 3A**. For nonheated systems, results are shown for pH $\geq$ 6.7 only since no significant difference was found between the results at lower pH and those at the normal pH of the milk. In reducing conditions, a large increase (from 1 ± 0.4 to 3.6 ± 0.6 g·L^{-1}) of β-lg concentration was observed in the sera as the pH of heating was increased from 6.3 to 6.7, after which the value remained constant. In comparison, the concentration of β-lg originally present in the sera of unheated milk at all pH values was constant at 4.2 ± 0.6 g·L^{-1}. The effect of heating milk at pH values from 6.3 to 7.3 is accompanied by an increase in the amount of β-lg from $\sim$25% to an average of $\sim$85% in the serum at pH 6.7 and up to an average of $\sim$96% at higher pH. Since the speed of centrifugation did not cosediment the soluble aggregates with the casein micelles, we can assume that at pH 6.7 about 25% of the β-lg was bound to the casein micelles

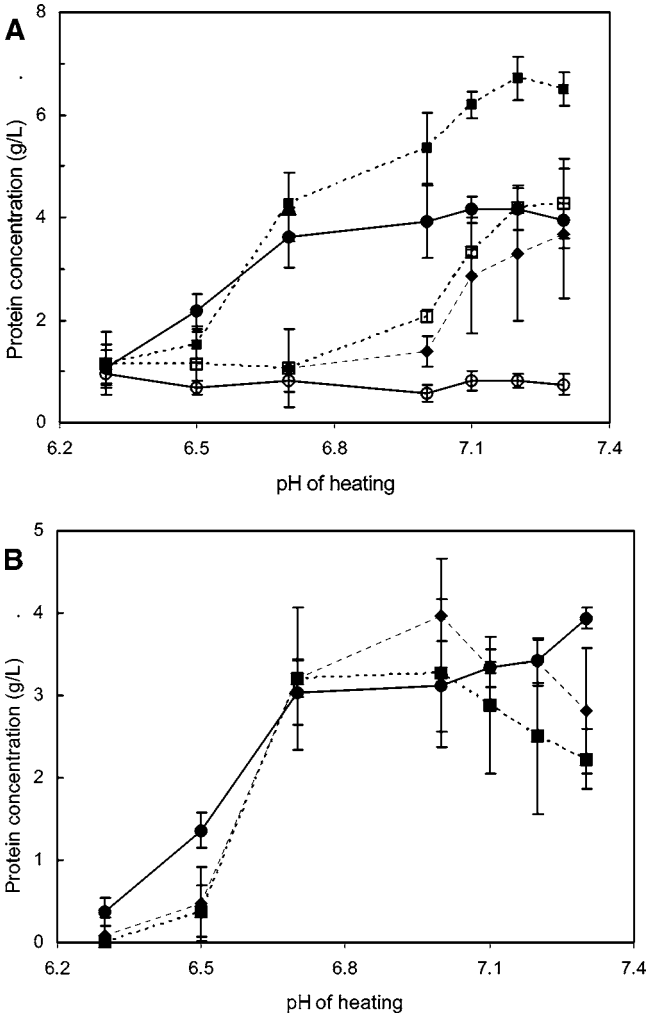

Figure 3. Quantification of the intensity of scanned bands in the SDS–PAGE gels. Reference lanes were pure samples of WPI (4 g·kg^{-1}) and NaCas (3 g·kg^{-1}). (**A**) Concentrations of β-lg in heated (○, ●) and unheated (▲) samples; total caseins in heated (□, ■) and unheated (◆) samples in reducing (filled symbols) and nonreducing (empty symbols) conditions. (**B**) Difference of concentrations between reducing and nonreducing conditions for β-lg (●) and total caseins (■) in heated samples. Difference of concentrations between unheated and heated samples in reducing conditions for total caseins (◆).

forming sedimentable aggregates. This value is lower than that found by previous authors, who found values between 50% and 100% (*1*, *8*−*10*, *15*), but is close to the lower values found by other authors (*14*, *23*). The nonreducing gel gave a β-lg concentration that was approximately constant around 0.8 ± 0.2 g·L^{-1} at all pH values. From this quantification, the amount of β-lg involved in the complex via disulfide bonds can be estimated and is shown in **Figure 3B**. As the pH of heating increased, the concentration of β-lg involved in the complex increased. A similar trend was attributed to the decrease of the casein micelle-bound aggregates formed between WP and κ-cas (*10*, *14*, *15*).

The concentration of caseins in unheated sera of milks whose pH was adjusted between 6.7 and 7.3 increased from ~ 1.1 ± 0.8 to 3.7 ± 1.3 g·L^{-1}, which corresponds to an increase of about 4 ± 3 to 12 ± 4 % of the total concentration of casein present in the milk. These values are comparable with what is reported by Menard et al. (*14*) who estimated from RP-HPLC analysis of the serum that less than 5% of the total κ-cas was released at pH 6.6 and 7.1 but that this amount increased to 10% when the pH was adjusted to 7.6, the rate of dissociation of the other caseins being lower than that of κ-cas. Other studies suggest that lower dissociation occurs in these conditions; Anema and co-workers (*15*, *21*) estimated from SDS−PAGE analysis that from 5% to 8% of the total casein was in the serum when the pH of the milk was adjusted between 6.3 and 7.1 at 20 °C. Differences between our results and those described can be explained possibly by the different methods of analysis, by the preparation of the sample, and probably also because the starting milks were different.

When milk was heated, the casein concentration in the serum measured in the reducing gel at pH 6.3 was similar to that of unheated milk. Between pH 6.5 and 7.0 a first increase in the concentration from 1.5 ± 0.3 to 5.3 ± 1 g·L^{-1} was measured, and then the concentration increased further up to 6.5 ± 0.5 g·L^{-1} at higher pH values. Heating at higher pH values increased the amount of caseins in the serum as was described previously (*15*, *16*). However, taking account of the difference between the amount of caseins released by the pH in unheated milk and assuming that this dissociation is irreversible, the amount of additional caseins released by the effect of heating increased up to pH 7 and decreased slightly for higher pH (**Figure 3B**). This result differs from those of previous reports showing a more linear increase of casein release with pH of heating (*15*, *16*). It can be noted, however, that the ratios of intensities of the different caseins present in the sera of heated milk were different from those measured in the corresponding unheated milks. At all pH values, the ratio of intensities of the κ-cas band relative to that of the α_s- and β-caseins was equal to 0.5 for unheated samples, whereas it varied from 1 to 0.7 in heated samples due to an increase in the intensities of κ-cas at all pH values and α_s- and β-caseins for pH ≥ 7. Thus, at all pH values the sera were enriched in κ-cas compared to the other caseins. The dissociated caseins never showed the distribution typical of the total casein composition, such as in the sodium caseinate reference sample.

In the nonreducing gel of heated milks, the concentration of casein was close to the values estimated in nonheated systems. It remained constant and low (1.1 ± 0.1 g·L^{-1}) in milks heated at 6.7 and below, while above pH 6.7, the concentration increased progressively to around 4.3 ± 0.9 g·L^{-1}. The amount of caseins involved within the soluble complexes in covalent (disulfide-bound) aggregates that are thought to be κ-cas mainly but may also involve α_{s2}-caseins (*23*), estimated by the difference between the concentrations in the reducing and nonreducing gels, increased up to ~3.3 ± 0.9 g·L^{-1} as the pH of heating varied from 6.3 to 7, then the concentration decreased to ~2.2 ± 0.4 g·L^{-1} for higher pH (**Figure 3B**). The decrease of casein concentrations observed can be related to the increase in the amount of α_s-cas and β-cas measured in nonreducing conditions. It is interesting to note that the nonreducing SDS−PAGE shows that there is noncovalently bound κ-cas in increasing amounts in the sera of heated milks as the pH is increased above 7.0 (**Figure 2B**). At pH 6.7 and below, no band from noncovalently linked κ-cas appears.

These results suggest that the heat-induced dissociation of κ-cas is more sensitive to heat treatment than shown previously (*14*, *19*, *20*) compared to the dissociation of α_s-cas and β-cas, which were found to be dissociated mostly by increasing the pH of milk and also by heating at pH ≥ 7.

Elution Profiles of Sera from Milks Heated at Different pH Values. To elucidate the nature of the particles produced by the proteins at different pH values, the SEC chromatograms for serum samples produced by centrifugation of milk unheated and

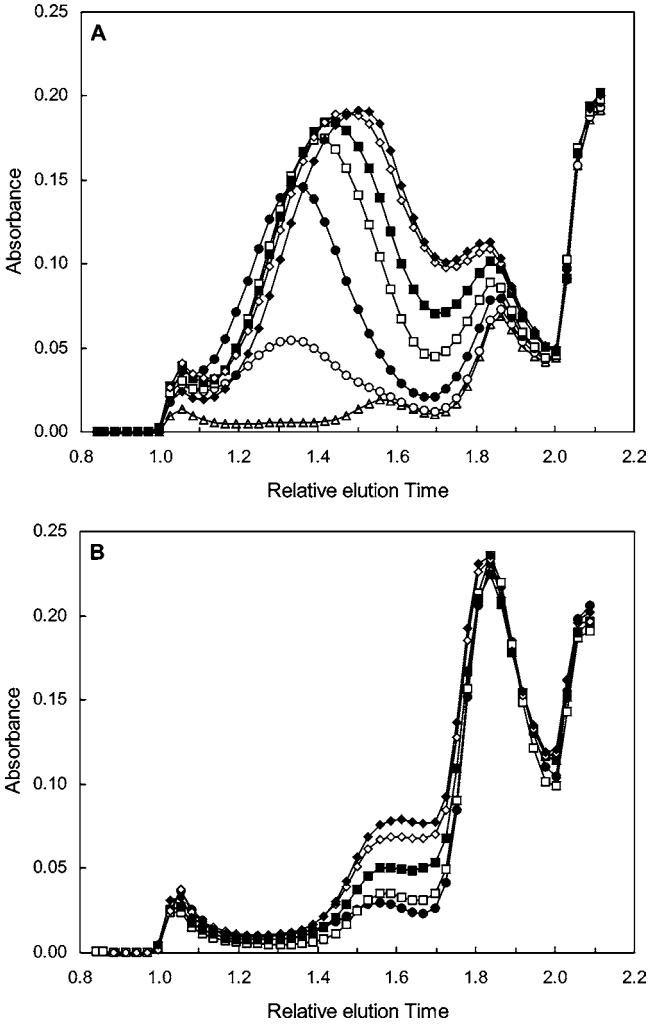

Figure 4. (**A**) Elution profiles of sera of milk samples heated at pH 6.3 (△), pH 6.5 (○), pH 6.7 (●), pH 7 (□), pH 7.1 (■), pH 7.2 (◊), and pH 7.3 (◆). (**B**) The corresponding samples (same symbols) from milk adjusted to different pH values from 6.7 to 7.3.

heated at different pH values from 6.3 to 7.3 are shown in **Figure 4**. For unheated samples (**Figure 4B**), the profiles obtained at pH 6.3 and 6.5 were very close to that at pH 6.7 and are not presented here. The chromatograms showed five peaks, similarly to patterns described previously (*15*−*17*). The first peak ($T_R = 1.0$) is present in all of the sera; it is small and contains very little protein (*17*) and is probably composed of very small fat globules that redisperse after centrifugation and filtration (0.45 μm). A second peak ($T_R = 1.3-1.5$) is observed in all of the sera of heated milks, except at pH 6.3 (**Figure 4A**). Previous studies (*14*, *21*−*23*) have established that this peak is composed of complexes of WP and κ-cas that are formed during the heating process. A third peak ($T_R = 1.6$) occurs in unheated milk, and SDS−PAGE analysis (see later in the text) showed that it contained small quantities of caseins, significant amounts of β-lg, and traces of α-lac. The fourth major peak ($T_R = 1.8$) has previously been identified as containing the native WP (*23*). It is much smaller in the sera of heated milks but does not completely disappear from the serum after heating. These general observations suggest that some of the WP identified in the third peak may be somewhat aggregated as a result of protein powder preparation and therefore elute at a shorter time than the main native WP band. A final peak that elutes at about $T_R = 2.3$ was identified; it does not contain protein or change with either heating or pH (*23*), and for clarity is omitted from all of the SEC profiles shown here.

The SEC profiles of unheated milks were very similar for all pH values except the third peak, which increased in size as the pH was increased above 6.7. This must be caused by the increasing amount of caseins released. The chromatograms of the sera of heated milk show that the behavior of the milks varied considerably depending on the pH at which the heating was performed. At pH 6.3, the profile of the serum was similar to that of unheated milk, except that the peak at $T_R = 1.8$ was very much diminished as for the higher pH of heating. For pH > 6.3, the area of the peak attributable to the soluble complexes increased and progressively shifted to longer elution times (*22*). The shape of the elution profile changed in a rather complex manner between pH 6.3 and 7.2, but the differences between the profiles of sera from milks heated at pH 7.2 and 7.3 were fairly small.

The total integrated areas under the SEC chromatograms between T_R 1.0 and 2.0 can be compared with the total concentration of protein in the sera presented previously. **Figure 1** (square symbols) shows that the area under the elution profile increased linearly with the pH, and in fact the area of the peaks in the SEC and the protein content as measured directly correlated well ($r^2 = 0.98$ for heated and $r^2 = 0.96$ for unheated systems). Therefore, it is clear that the SEC appears to be giving the details of the size distribution of all of the protein material in the sera of the milks.

Gaussian Analysis of the SEC Profiles. We attempted to make an analysis of the shapes of the elution profiles shown in **Figure 4**. Our assumption was based on the belief that, if the sera contain a number of distinct types of particles of different sizes, they should elute as a series of bands with approximately Gaussian band shapes. Therefore, we fitted the elution profiles with seven overlapping Gaussian peaks (named G1−G7) whose positions in the elution profile were fixed with 2% maximum variation from a defined position. The fitting was done using a PeakFit program (version 4.12, Framingham, MA). The best fitting profiles were obtained with the autofit peaks I residuals option. Comparison of pairs of elution profiles at different pH values showed that a set of seven peaks centered around T_R values of 1.96 (G1), 1.85 (G2), 1.72 (G3), 1.54 (G4), 1.35 (G5), 1.12 (G6), and 1.05 (G7) were necessary to make a fit with a r^2 coefficient varying from 0.990 to 0.998 for all of the SEC profiles. Some of the fitted profiles using this method are shown in **Figure 5A**, where it can be seen that the calculated fits reproduced the shapes of the measured profiles closely. Although this fitting procedure is empirical, it allows us to describe and discuss quantitatively certain regions of the profiles.

The major peaks observed in the SEC of the sera of the heated milks were essentially fitted by the peaks G2, G3, G4, and G5 and for unheated milk by the peaks G2, G3, and G4. The areas of all of these peaks changed at the different pH values, and this is shown in **Figure 6**. The area of the peak G2 decreased very much between unheated and heated milks for all pH values. The area of peak G4 in heated samples showed a slow increase with pH up to 6.7 and a steep increase at the higher end of the pH range, whereas in unheated samples the area of this peak was very small and little variation with pH was observed. Identical behavior to that of peak G4 was found for peak G3. In heated samples, the peak area of G3 was about one-half of the value in the unheated samples, and it seems that the area of this peak increased more in unheated than heated samples at pH ≥ 7. At the other end of the elution profile for heated samples, the area of G5 increased rapidly with pH of heating

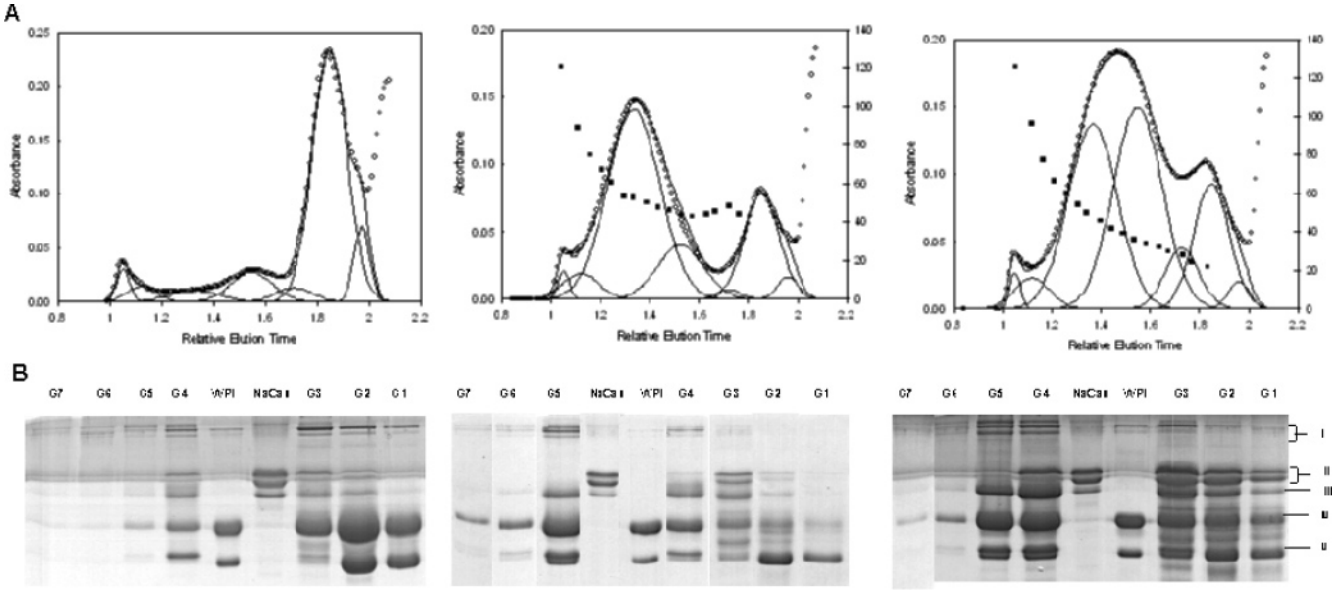

Figure 5. (**A**) Fitting by seven Gaussian peaks of the elution profiles of sera of milk samples unheated at pH 6.7 (left) ($r^2 = 0.994$) and heated at pH 6.7 (middle) ($r^2 = 0.992$) and 7.2 (right) ($r^2 = 0.997$); for details see text. In all of the figures, thin lines are the fitting peaks, heavy solid lines are the total fitted profile, and (○) represent the measured profiles. The left-hand scale and the symbols (■) in the results for pH 6.7 and 7.2 show the sizes (*Z*-average diameters) of the particles eluting at different points in the elution profile. (**B**) SDS−PAGE of the samples collected from the SEC column at the maxima of the seven Gaussian peaks G1−G7. Identification of bands in the gel is the same as in **Figure 3**. Reference lanes contained pure samples of WPI (4 g·kg^{-1}) and NaCas (3 g·kg^{-1}).

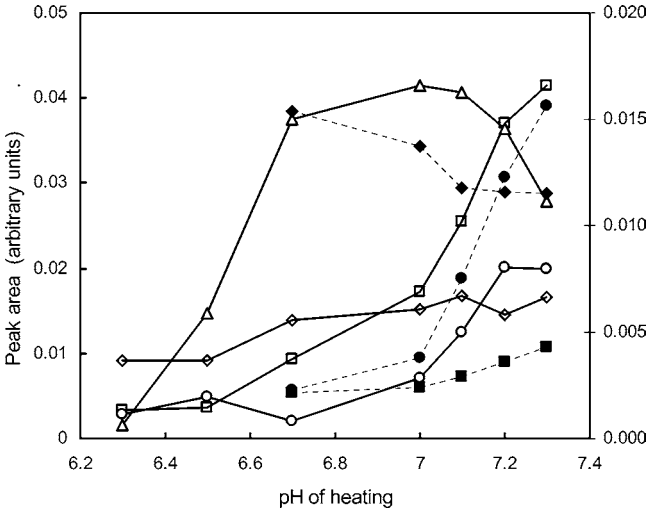

Figure 6. Changes in the area of the Gaussian peaks used to fit the elution profiles, as functions of the pH. Values are shown for the peaks G2 (◇,◆; left-hand scale), G3 (○, ●; right-hand scale), G4 (□, ■; left-hand scale), and G5 (△; left-hand scale) for unheated (filled symbols) and heated (empty symbols) samples.

until pH 6.7 and then leveled off and even decreased somewhat at higher pH values.

These results appeared to show that the curve fitting of the SEC profiles provides a way of describing changes in the eluting materials. Analysis of the composition of the material eluting from SEC at the positions of the different Gaussian peaks was made by SDS−PAGE by collecting and concentrating 3 mL samples at the predicted maximum peak positions. The results for heated sera at pH 6.7 and 7.2, and for unheated milk at pH 6.7, are presented in **Figure 5B**, showing that the Gaussian bands corresponded to significant changes in the compositions of the material eluting at these points. The analysis of the intensities of the bands (not presented here) showed that for all pH, in heated and unheated samples G1 and G2 contained WP with a higher amount of α-lac in G1 and higher amounts of β-lg in G2. At pH > 6.7, G2 also contained significant amounts of all of the caseins in heated samples. The sizes of G1 and G2 decreased considerably when the milks were heated, as the whey proteins denatured and formed complexes. Peak G3 contained WP and all types of caseins. In heated samples, the content of WP was lower than that of unheated samples and was very low at pH ≤ 6.7 but noticeable at higher pH. Significant amounts of all of the caseins were identified in this area for heated samples at pH above 6.7 but not at lower pH. In all sera, a small amount of β-cas and higher amounts of κ-cas and α_s-cas were observed in G3.

Peak G4 in unheated samples contained a small amount of WP and also some caseins (mainly α_s and κ) as in peak G3 but in lower quantities. However, in heated samples, the amount of WP was higher and even at pH 7.2 the κ-cas was very much the dominant casein, although some α_s-cas was also present (our methodology cannot distinguish between the different α_s-caseins). In heated milk, peak G5 was similar to G4 in composition, except that κ-cas was the only casein present whatever the pH of heating. The ratio between κ-cas and WP was estimated from the intensity of the bands to be around 0.2−0.3 for G5, which is approximately that found by Guyomarc'h et al. (*23*) in similar experiments at pH 6.7. For G4 this ratio increased to approximately 0.4−0.7. These ratios are close to the range given by Jean et al. (*25*) of 0.5−0.8 for the serum of heated milk at pH 6.7. Peaks G4 and G5 from heated milks also contained significant amounts of the minor whey proteins. Peaks G6 and G7 contained small traces of β-lg only.

From this analysis, the evolution of the areas of the different peaks described in **Figure 6** can be interpreted as the variation in the composition of the eluting material. A comparison of **Figures 3** and **6** shows that the behavior of the protein concentrations in different sera measured by the SDS−PAGE bears an interesting similarity to that of the individual Gaussian peaks used to fit the SEC profile. It appears that the overall behavior of the casein fractions involved in the covalent complex

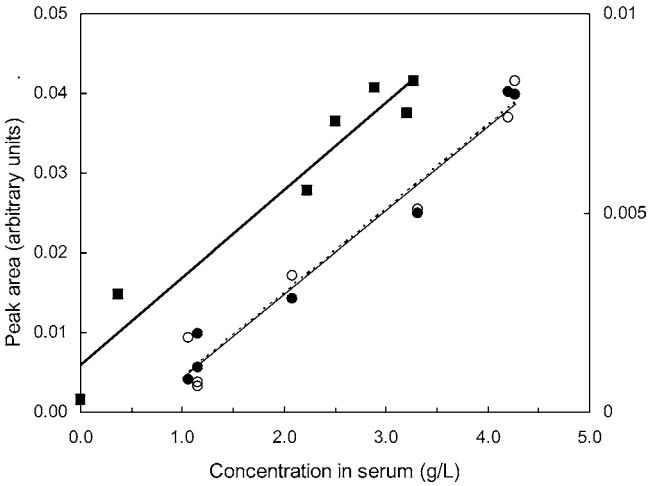

Figure 7. Relation between area of the Gaussian peaks used to fit the elution profiles and the concentrations of proteins determined by SDS–PAGE analysis. Area of peak G5 as a function of the difference of concentration of total casein measured between reducing and nonreducing conditions (■) ($r^2 = 0.94$); area of peak G4 as a function of the concentration of total caseins in nonreducing conditions (○) ($r^2 = 0.96$); area of peak G3 as a function of the concentration of total caseins in nonreducing conditions (●) ($r^2 = 0.97$).

(**Figure 3B**) resembles the variation of G5 as the pH changes up to pH 7. On the other hand, the behavior of the concentration of caseins seen in nonreducing gels for heated samples (**Figure 3A**) strongly resembles that of the areas of peaks G3 and G4. The changes observed from the two techniques strongly parallel one another, and this is demonstrated clearly in **Figure 7**, where the concentrations of the different protein fractions are plotted against the integrated areas of the Gaussian peaks. The plots are linear, and indeed the plots involving either G4 or G3 have very similar slopes. It seems, therefore, that the peaks seen in the Gaussian fitting are representative of different fractions of the protein and particles present in the serum of the milk after centrifugation.

From the overall results, the increased amount of soluble caseins arising from the adjustment of pH elute mainly in peak G3. As the milks were heated, part of the caseins released by the pH (mainly κ-cas) is involved in complex formation, and elutes in G4. In peak G5 complexes are formed with mainly κ-cas. Its area reached a maximum at pH 6.7 and then decreased as the pH of heating increased. The variation of composition of G4 and G5 and the evolution of the area of these peaks as the pH of the milk increased suggest that the mechanisms of formation of the soluble complex differ as the pH of the milk changes.

Sizes of the Particles Eluting from the SEC Column. In **Figure 5A**, the sizes of the particles eluting at different points for the sera of heated milk at pH 6.7 and 7.2 are shown. The measurements showed that the particles eluting near the void volume of the column (G6/G7) had sizes in the region of 120 nm. In the region of G3, the size measured was about 30 nm. In the result from pH 6.7, G3 is small and the particle size measurement becomes unreliable, but the sample from pH 7.2 has a much larger peak at G3 and contains enough particles to give a more reliable estimate. The first material eluting (in the position of G6/G7) corresponds to highly aggregated WP and perhaps some small fat globules. In the region of G5 the average size is 60 nm and, as shown by SDS−PAGE, corresponds to aggregates of mainly κ-cas and WP. The area of the peak G4 is more significant for milks heated at pH > 6.7, and the average size in this region is 35 nm. The size obtained for particles in G4 and G5, which are the peaks corresponding to the soluble aggregates in the SEC profile, are in agreement with results obtained from previous studies (*22*, *23*, *25*, *26*). In G4, electrophoresis analysis showed that the proportion of κ-cas relative to that of WP was higher than in G5. This measurement confirms previous studies showing that an increase in the κ-cas/WP ratio results in a decrease in the size of the complex (*23*). That is, there is increased utilization of κ-cas in complex formation with an increase in the smaller particles as the pH of heating increases.

The particles in the region of G3 were smaller than 30 nm and contained significant amounts of WP at pH ≥ 6.7, also caseins. The sizes obtained suggest that caseins are probably aggregated in small complexes, although some monomers appear that elute at essentially the same position as the WP. The state of the κ-cas in these complexes is not defined, and we do not know if it could be involved in small casein particles.

DISCUSSION

In agreement with other research groups, we find that heat treatment of milk combined with pH adjustments induces modification of the serum composition. However, the methods that we used to describe and compare the composition of sera from heated to unheated systems after adjusting the milk at different pH values have given new information on the effects of heat treatment and pH on the formation of soluble aggregates. In previous studies, it has been shown how the number, size (*10*, *13*, *22*), and possibly composition (*25*) of soluble aggregates vary, depending on the conditions of heating, but in general it appears that the assumption has been made that one single mechanism of formation of the complex exists. Our observations suggest that when milk is heated at 90 °C at different pH values there may be two distinct mechanisms, one of which predominates at the normal pH of milk (6.7) and below and the other that occurs at higher pH values. This is different from the apparently continuous change in mechanism as the pH of heating is raised, as suggested by the results of Anema and co-workers (*11*, *12*, *17*).

The analysis of the SEC profile by seven Gaussian peaks showed that the increased amount of soluble complexes formed in the serum as the pH was increased was explained by the variation of the peaks G4 and G5. These appear to define two types of complexes that may possibly result from different mechanisms of formation. Heating milk at pH ≤ 6.7 mainly causes the WP to denature and either bind to the casein micelles (at pH 6.3) or to form complexes with the κ-cas of the casein micelles that then dissociate into the serum. These are the complexes that elute in peak G5. As the pH of heating is increased, the complexes increasingly dissociate from the micelles (*23*, *26*). Evidence for this is the increase in the area of the peak G5 in the SEC, accompanied by very little change in peak G4 (**Figure 4**). The relative constancy of the position of G5 in the SEC suggests that the particles formed in this process are all about the same size, no matter what the pH.

This mechanism continues to be operative above pH 6.7, because G5 never disappears from the SEC profiles, but another mechanism appears to become important at higher pH of heating, where the SEC profiles are dominated by the peak G4, which contains smaller particles than those identified in G5, although they are also composed mainly of WP and κ-cas. This may be a result of the increasing amount of κ-cas that has undergone alkaline-induced dissociation before the heating is done. We have seen (**Figure 7**) that the area of the peak G4

scales very well with the amount of casein concentration not involved in the covalently bonded aggregates (nonreducing SDS−PAGE). This is marked by the release by the pH adjustment of α_s-cas and β-cas from the micelles, as well as significant additional release of κ-cas by both effect of pH and heating. The κ-cas liberated may interact more freely in the serum with the denaturing WP to form the smaller complexes. However, the presence of κ-cas in the longest-eluting fractions in the SEC (seen in peaks G3 and G2) demonstrates that in the heated samples not all of the κ-cas in the sera is reacted with any of the other proteins; this was also apparent in the SDS−PAGE gel in nonreducing conditions (**Figure 2B**), confirming that it is not part of disulfide-linked protein complexes.

Our results in this higher pH range are not comparable with those of Anema and co-workers (*15*, *21*). Their results show that after heating at 90 °C, most of the α_s-cas was associated with the casein micelles, no matter what the pH. We find that it increases in the serum from pH 6.7 to 7.3. Conversely, although they find that the κ-cas concentration in the serum continuously increases with the pH of heating, our results suggest that the disposition of the nonmicellar κ-cas is not uniform; it may appear in complexes with the WP (peak G4 and G5) but also may exist in a free state or in very small molecular complexes (peaks G2 and G3). Our results, therefore, are not completely in agreement with the mechanisms proposed by Anema and Li (*21*) for the behavior of milks when heated, since we see more caseins other than κ cas in the supernatants.

In summary, we conclude that there are two mechanisms operating on the behavior of the proteins in heated milks and that these two mechanisms are pH-dependent.

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