JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Effects of Added Sodium Caseinate on the Formation of Particles in Heated Milk

ELIZABETH A. PARKER, LAURENCE DONATO, AND DOUGLAS G. DALGLEISH*

Department of Food Science, University of Guelph, Guelph, Ontario, Canada N1G 2W1

The effects of heat at temperatures in the range of 80−90 °C on mixtures of reconstituted skim milk powder (RSMP) and sodium caseinate have been determined. In the absence of caseinate, the action of heat on RSMP produces soluble complexes of whey proteins and κ-casein, as well as complexes of whey protein with the casein micelles. When sodium caseinate was added to RSMP at levels of 0.5 and 1.0%, the denaturation of the whey protein and the production of the soluble complexes in the serum were hardly affected, either in rate or in amount. However, during the heating, the caseinate disappeared from the serum. Further studies on model mixtures of the different components showed that it was probable that the bulk of the caseinate associated with the casein micelles during heating, probably by binding inside the surface layer of κ-casein, because no increase in the diameters of the casein micelles could be observed.

KEYWORDS: Milk; heating; casein micelles; whey protein complexes; sodium caseinate

INTRODUCTION

The chemistry of the effects of different heat treatments on milk has been extensively studied over the last 30 years. This research has shown that at temperatures above about 80 °C, the whey proteins denature and interact with the κ-casein of the micellar casein fraction (*1*−*3*). It is not established clearly what the precise mechanism is for this reaction, especially when the κ-casein is originally part of the casein micelles, although it is well-known that the interaction involves the formation of intermolecular disulfide bonds (*4*−*6*). The overall result of the heating is that the heated milk contains casein micelles, to which a portion of the denatured serum proteins are bound, as well as small (up to approximately 50 nm in diameter) particles that are composed of denatured whey proteins and κ-casein (*7*−*10*). These latter complexes (hereafter referred to as soluble complexes) are found in the serum of the milk and are not sedimented by the centrifugation conditions used for the removal of casein micelles from milk. The different amounts of these two types of complexes that are formed appear to depend strongly upon the pH (*7*, *11*, *12*) and possibly upon the temperature of heating, although this is less clear. The relative concentrations of whey protein and micellar casein also affect the amount of soluble complexes that are formed and the composition of the complexes (*9*).

Much less information is available on the properties of milks to which supplementary proteins have been added. However, in some industrial processes, especially in the manufacture of fermented products such as yogurts, it is customary to fortify the base milk with the addition of proteins in the form of skim milk powder, whey protein concentrate, sodium caseinate, or combinations thereof. The results of the interactions of whey proteins (both native, denatured, and in complexes from milk) with the casein micelles has been studied, although the main focus has been on its effects on the rheology of the acid gels formed from the heated milk (*13*, *14*), rather than on the effects at the molecular level. Some studies have been made on the effects of increasing the amount of whey protein in milk on the micellar (*15*) and soluble (*9*) complexes formed during heating of whey protein-fortified milks. These have established that there seems to be a limit to the amount of whey protein that can bind to the casein micelles at a given pH (*1*, *15*), so that the addition of whey protein tends to increase the amount of soluble complexes that are formed (*9*). It is also noteworthy that some of the κ-casein remains unreacted, even when whey proteins are added to the milk before heating (*9*).

Much less is known of the effects of heat on milk fortified by the addition of sodium caseinate. This ingredient is originally derived from the casein micellar fraction of milk but lacks the calcium and phosphate that characterize the native casein micelles. In solution, sodium caseinate exists in particles ranging from monomers up to small complexes, containing a few tens of molecules, with particle weights of about 500−800 kDa (*16*, *17*). In the presence of sufficient calcium ions, sodium caseinate can form larger particles, in effect forming calcium caseinate (*18*), based on precipitates of α_s- and β-caseins stabilized by κ-casein. What happens to the aggregation state of sodium caseinate when it is added to milk is not known, because effectively the proteins are placed in a solution containing some calcium and phosphate, which are in a soluble form and are at too low of a concentration to form crystalline material. It can be conjectured that the addition of sodium caseinate to milk before heating would create an additional amount of κ-casein for the reaction with the denaturing whey proteins. This could

* To whom correspondence should be addressed. Fax: +1-519-824-6631. E-mail: ddalglei@uoguelph.ca.

10.1021/jf0512306 CCC: $30.25
Published on Web 09/20/2005

also influence the interaction between the denatured whey proteins and the casein micelles. The research described below was intended to probe the interactions that occur between the different milk proteins when sodium caseinate is added to milk and the mixtures are heated.

MATERIALS AND METHODS

Milks and Heat Treatment. Skim milk powder (low-heat, Parmalat Foods, Inc., Toronto, Ontario, Canada) was made to a concentration of 11% (w/w) in Milli-Q deionized water. The reconstituted milk was stirred for approximately 1 h at room temperature and was then stored at 4 °C overnight before use. Mixtures of milk with sodium caseinate were prepared by dry-mixing appropriate amounts of skim milk powder with sodium caseinate (Alanate-180, Fonterra Milk Products, Lemoyne, PA), to give final concentrations of 0.5 and 1.0% (w/w) of caseinate and 11% SMP in the final reconstituted milks. The SMP/caseinate mixtures were hydrated and stored in the same way as for the milk alone.

The milks were heat-treated in test tubes (15 mL volumes) in a water bath at temperatures of 80, 85, or 90 °C, for times from 5 to 30 min. The time required to attain the final temperature was 3 min. The samples were then rapidly cooled to room temperature in ice water.

Separation of Casein Micellar and Serum Fractions. Because all of the studies described here focused on the materials in the serum of the reconstituted milk/caseinate mixtures, the casein micellar fraction was removed by centrifugation. Samples of the unheated or heated RSMP and its mixtures with sodium caseinate were centrifuged at 25000*g* using an Optima LE-80K centrifuge (Beckman Coulter, Inc., Mississauga, Ontario, Canada). Centrifugation time was 1 h, and temperature was 20 °C. This resulted in a complete separation of micellar material (which sedimented) and soluble complexes, which remained in the serum. Tests were made to ensure that the separation of the casein micellar fraction and the serum complexes did not depend upon the centrifugation time (i.e., that longer centrifugation did not result in the sedimentation of some of the complexes to be studied). It was evident from the lack of turbidity of the serum produced from unheated RSMP and from the results of size-exclusion chromatography (SEC) described below that no large particulate (casein micellar) matter remained in the serum of unheated RSMP. On the other hand, the soluble complexes were retained in the sera of heated milks after the centrifugation. We believe therefore that the centrifugation gives a true separation between the casein micelles and the soluble complexes in the serum.

The sera were removed from the centrifuge tubes with a syringe and were then analyzed using SEC.

Model Systems of Proteins. Some studies were also made on the stability of the different protein components and of model mixtures during heating. These used the sodium caseinate, the casein micellar fraction from the centrifugation of unheated RSMP, and whey protein isolate (Alacen-895, Fonterra, Lemoyne, PA). The proteins were dispersed in permeate (MUF) that was prepared by ultrafiltration of 11% (w/w) RSMP, using a Millipore Prep-scale TFF cartridge (PLGC10K) obtained from Fisher Scientific (Mississauga, Ontario, Canada). The molecular weight cutoff of this ultrafilter (10 kDa) ensures that none of the intact milk proteins are present in the permeate.

Solutions (1%, w/w) of sodium caseinate and of WPI were made in the MUF. In addition, a mixed solution of 1% (w/w) WPI and 1% (w/w) sodium caseinate was prepared. Finally, a serum-protein-free milk was prepared by resuspending the casein micellar pellet from the centrifugation of RSMP in MUF, to give a serum-protein-free milk with the same micellar concentration as in the original milk. To this preparation, sodium caseinate was added to give a 1% (w/w) solution. Samples of all four of the model protein preparations were heated at 90 °C for 10 min and then were subjected to centrifugation as described above. Sera from heated and unheated preparations were then analyzed by SEC.

SEC. All of the sera from the differently treated milks were analyzed by SEC, basically as described by Guyomarc'h et al. (*9*). The separation was performed using a Pharmacia XK16/70 column packed with Sephacryl S-500 high-resolution gel beads (Amersham-Pharmacia Biotech, Baie d'Urfé, Quebec, Canada). This gel has a wide range of separation and allowed us to separate molecular whey proteins, aggregates of caseinate, and the soluble complexes, which all have different sizes. The chromatography equipment was a Bio-Rad Biologic Dual Flow Chromatography system (Bio-Rad Laboratories (Canada), Mississauga, Ontario, Canada), with detection of the eluted material by absorbance at 280 nm.

To run the experiments, 1 mL of the serum from heated or unheated milk/caseinate preparations after centrifugation was injected into the column and the sample was eluted with a buffer containing 20 mM bis-tris-propane (Sigma−Aldrich Canada Ltd., Oakville, Ontario, Canada), containing 0.02% sodium azide. The elution rate was 1 mL/min, and the total elution time was up to 160 min per sample.

Calculation of Relative Elution Times. In the course of these experiments, several different size-exclusion columns were used. Because these were of slightly different lengths, it was necessary to standardize the elution times to allow subtraction of the elution profiles to be performed as described in the Results and Discussion. This was achieved by, setting for each profile, the elution time of the maximum of the first peak in the profiles to a value of 1 and then calculating the relative elution times of all points on the profile relative to this. Many of the elution profiles shown below were standardized in elution time using this method.

Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC). This analysis used an HPLC system consisting of a Waters 700 Satellite WISP (injector and autosampler), Waters 600E System Controller, and a Waters 486 Turntable Absorbance Detector using BaseLine 815 software (Waters Ltd., Mississauga, Ontario, Canada). The column used was a Grace Vydac 214TP C_4 reversed-phase column (Mandel Scientific Company Ltd., Guelph, Ontario, Canada), which was maintained at a temperature of 40 °C. The flow rate of buffer was 0.3 mL/min. The sample size injected onto the column was 20 μL. Elution was by a gradient from 10 to 90% acetonitrile (Fisher Scientific, Whitby, Ontario, Canada) in Milli-Q water containing 0.01% trifluoroacetic acid (Sigma−Aldrich Canada Ltd., Oakville, Ontario, Canada). The protein was detected and quantified by absorption at 214 nm.

Measurement of Particle Sizes. Particle sizes of the material in the fractions from the SEC column were measured by dynamic light scattering using a Malvern 4700 system (Malvern Instruments, Southboro, MA) and autocorrelator, equipped with a He−Ne laser with a wavelength of 633 nm and nominal power of 12 mW. Samples of the effluent from the SEC column were collected directly into 1 cm square cuvettes, which were then placed in the measuring system. The scattering angle was 90°, and average diameters of the particles were calculated by the method of cumulants. For measurement of the particle sizes in heated and unheated RSMP, the milks were diluted by a factor

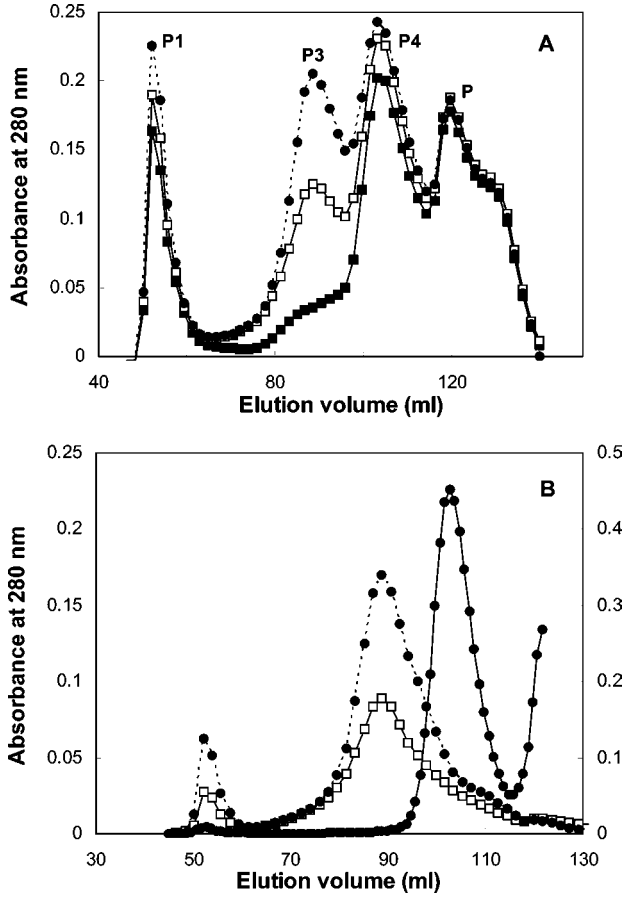

Figure 1. (A) SEC elution profiles of centrifugal sera from milks containing 0% (■ and —), 0.5% (□ and —), and 1.0% (● and - - -) added sodium caseinate. The labeling of the peaks (P1, etc.) is described in the text. (B) Difference elution profiles obtained by subtracting the profile for milk containing 0% sodium caseinate from those containing 0.5% (□, —, and left-hand scale) and 1% (●, - - -, and left-hand scale) added caseinate. The elution profile of 1% WPI in milk ultrafiltrate (●, —, and right-hand scale) is shown for a comparison.

of 500 into a buffer of 20 mM imidazole, 5 mM $CaCl_2$, and 50 mM NaCl before measurement.

RESULTS AND DISCUSSION

Elution Profiles of Milks and Milk/Sodium Caseinate Mixtures. The SEC elution patterns of a set of centrifugal sera from RSMP containing 0, 0.5, and 1% added sodium caseinate, are shown in **Figure 1A**. Several peaks are seen to elute, similarly to patterns described previously. The first peak to elute had previously been shown to contain no protein (*9*); this was confirmed using RP-HPLC of the fraction. The peak was very variable in size between samples and was probably composed of small fat globules, which are extremely difficult to separate from the serum after centrifugation. In principle, it would be possible to remove them by filtration, but we wished to avoid the possibility of removing other material along with this fraction. The second peak from these sera to elute (labeled P3 in **Figure 1A**) is evidently a consequence of the presence of the sodium caseinate; the peak is small in the original RSMP but increases proportionally with the addition of caseinate. This can be seen clearly in **Figure 1B**. The peak P3 is present in the original RSMP, and analysis by RP-HPLC showed it to consist of caseins, in agreement with previous analyses (*9*). Thus, it is clear that caseins elute somewhat earlier than the whey proteins.

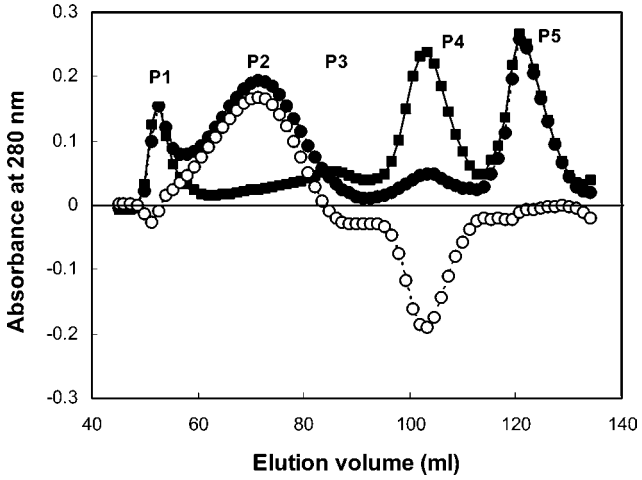

Figure 2. Typical SEC elution profiles of centrifugal sera from unheated and heated RSMP (■ and ●, respectively), with no added sodium caseinate, and the difference between the two profiles (○). Heating was at 80 °C for 30 min.

The third peak in the chromatograms (labeled P4) has been shown to consist of the native whey proteins of the milk (*9*), and the peak labeled P5 has been shown to be nonproteinaceous (*9*, *19*). It is therefore easy to identify the presence of the caseinate in the elution profiles, because it is clearly in a somewhat aggregated form that mainly elutes some time before the (monomeric or dimeric) whey proteins. It has been shown previously (*9*) that the median retention time of this material corresponded to particle weights of 500−800 kDa, that is, much smaller that that of casein micelles. This is consistent with the presence of small particles containing approximately 30 protein molecules (*16*, *17*), although it is clear from **Figure 1B** that the distribution of casein particles does include some monomers, which elute at the same time as the whey proteins. Lucey et al. (*17*) found a bimodal distribution of particles in sodium caseinate, but our results shown in **Figure 1B** suggest that in milk, at least, added sodium caseinate is in the form of complexes, with very few monomers. The SEC results show that, although the sodium caseinate is suspended in milk serum, which contains calcium ions, the latter do not appear to be in sufficient amount to cause any large particles or precipitate of calcium caseinate to form (otherwise, it would have been sedimented with the casein micelles when the milks were centrifuged after mixing). Therefore, in unheated mixtures, the added sodium caseinate seems not to undergo any interactions with either the whey proteins or the casein micelles.

When the milks are heated and then centrifuged, a new peak (labeled P2) appears in the centrifuged sera (**Figure 2**). At the same time, the peak P4 disappears, because the whey proteins of which it is composed become thermally denatured and react with each other and with the κ-casein in the milk. Previous work (*9*) has established that in milk the peak P2 is composed of complexes of whey protein with κ-casein, which are formed during the heating process, and we confirmed this by concentrating the complexes and analyzing them by RP-HPLC. Various authors have discussed the formation of these soluble complexes as a function of the pH of heating of the milk and the distribution of denatured whey proteins between these soluble complexes and bound to the casein micelles (*7*−*9*, *11*, *12*). At the normal pH of milk, where we are working, it is generally agreed that the denatured whey protein is partly found in the soluble complexes and partly attached to the casein micelles. Likewise, some of the κ-casein dissociates from the micelles to help to

form the soluble complexes (*9*, *20*, *21*). These soluble complexes isolated by the use of SEC appear from light-scattering measurements to be a maximum of 50 nm in diameter, and electron microscopy shows that they have a range of sizes between about 20 and 50 nm (Rodriguez and Dalgleish, unpublished observations). The fact that these particles elute considerably earlier than the caseinate complexes confirms that the caseins aggregate little, if at all, as a result of being suspended in the milk.

It is evident from **Figure 2** that there is no change in the P5 fraction of the SEC chromatogram as a result of heating. In addition, no systematic changes in P1 with heating were found; variations in this region of the chromatogram were random and were dependent upon the exact method of decantation of the serum from the micellar fraction after centrifugation, so that greater or lesser amounts of small fat globules were incorporated in the sera, which were subjected to SEC. It is evident that the profiles from the chromatography become more complex as a result of heating, and to attempt to clarify the changes in the compositions of the sera, we used a subtraction method to try to identify which parts of the SEC profiles were changing (as was also done to show the contribution of the sodium caseinate in **Figure 1B**). This involved subtracting the elution profile of the sera from unheated milk or milk/caseinate mixtures from those of the sera from the heated systems. A typical difference profile is shown in **Figure 2**, where it can be seen that the difference between the profiles of heated and unheated RSMP is resolved into two peaks, relating to the loss of whey protein (change in P4) and the growth of the soluble aggregates (change in P2). Essentially, P1 and P5 do not show changes as a result of heating and are eliminated from the difference profile. Even at the most extended heating conditions (30 min at 90 °C), P4 does not completely disappear. This suggests that the peak in the original serum may not be completely composed of whey proteins that denature and participate in aggregation reactions.

Milks Containing No Added Sodium Caseinate. Figure 3 shows the protein-containing bands in the difference SEC for RSMP with no added caseinate, heated at 80 or 85 °C for times from 5 to 30 min. There are clearly only two components in these difference SEC plots. Two effects are evident; first, the whey protein fraction P4 decreases and the aggregate fraction P2 increases with increasing heating time. Second, it can also be seen that the position of the maximum in P2 shifts to shorter elution times as the heating time increases; this is evidence that the sizes of the aggregate particles increase somewhat with the increased heat load. The changes in both the whey protein and the aggregates are complete after about 20 min at 85 °C, but at 80 °C, the difference peaks are still changing after 30 min, because of the slower denaturation of the whey proteins at this lower temperature. The shift of the maximum of P2 with time is also more clearly seen at 80 °C, simply because the reaction is slower.

The processes that we observe in SMP without added sodium caseinate are therefore the denaturation of whey protein accompanied by the growth of soluble whey protein/κ-casein particles whose size is controlled by the time of reaction and temperature of heating. It seems clear that, under these conditions at least, there is no indefinite aggregation of the whey proteins, because, although the peak of aggregate particles does move in a manner suggesting that they increase somewhat in size, there is little evidence of material being lost because of increasing aggregation at long heating times. The size of the peak reaches a limit and then stops growing, and it is in a fixed position, as soon as the loss of whey protein from the original

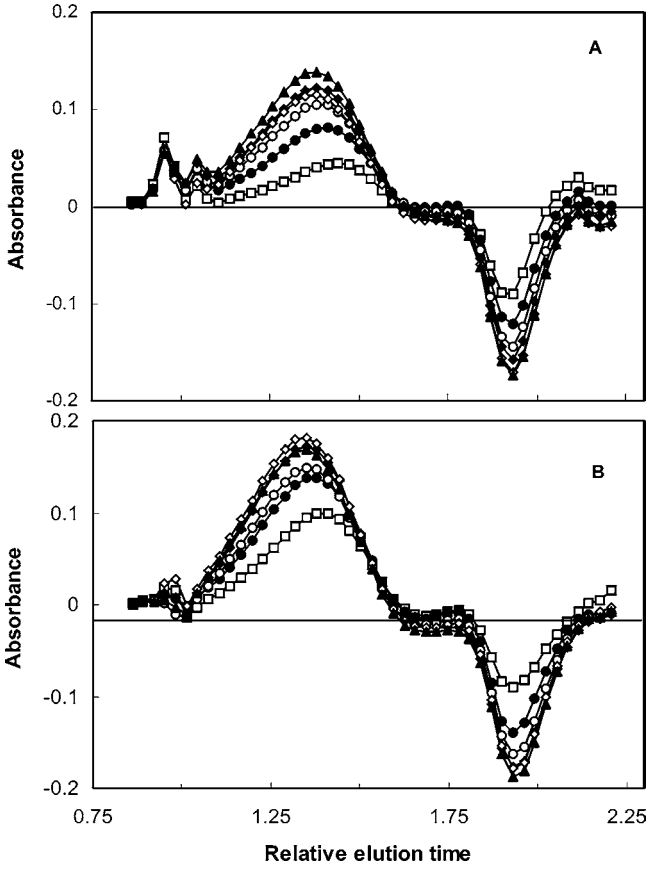

Figure 3. Difference SEC elution profiles of sera of RSMP heated at 80 °C (A) or 85 °C (B) for 5 (□), 10 (●), 15 (○), 20 (◇), 25 (◆), and 30 (▲) min.

P4 is complete. Therefore, there is no evidence that the aggregate particles themselves aggregate to form larger complexes, at least in the short term. Thus far, the results agree with what has been observed previously for the behavior of heated milks (*7*, *9*, *11*).

Milks Containing Added Sodium Caseinate. Figure 4 shows the SEC difference profiles of sera from the heating of RSMP containing added 0.5% sodium caseinate. In these difference SEC profiles, it can be seen that there is a contribution in the region of P1; this arises because, as explained above, this region depends upon the presence or absence of fat globules in the serum and, in **Figure 4A**, the serum from untreated milk contained very little of this material. However, the peak was still found to contain no protein. More significantly, there is now a third main component in the difference SEC, centered on the position of P3 in the original profiles of parts **A** and **B** of **Figure 1**. This implies that there is a change in the state of the caseinate as a result of the heating, at either 80 or 85 °C (if the caseinate had remained unaltered in the serum, there would be no peak in the difference profiles). In other respects, the behavior of the SMP/caseinate system is very similar to that of the SMP alone. There seems to be little change in the rate of disappearance of the whey protein (P4) between the two mixtures, and there is a similar buildup of P2. The amounts of P2 produced in the two systems are also almost identical; the maximum heights of the difference peaks after 30 min of heating at 80 and 85 °C were 0.156 ± 0.029 and 0.164 ± 0.034 for the milk with 0 and 0.5% caseinate, respectively. This suggests that, even though the addition of caseinate to the milk causes additional κ-casein to be present in the serum before heating, it does not seem to have any significant effect, either on the denaturation and disappearance of whey protein or the reactions

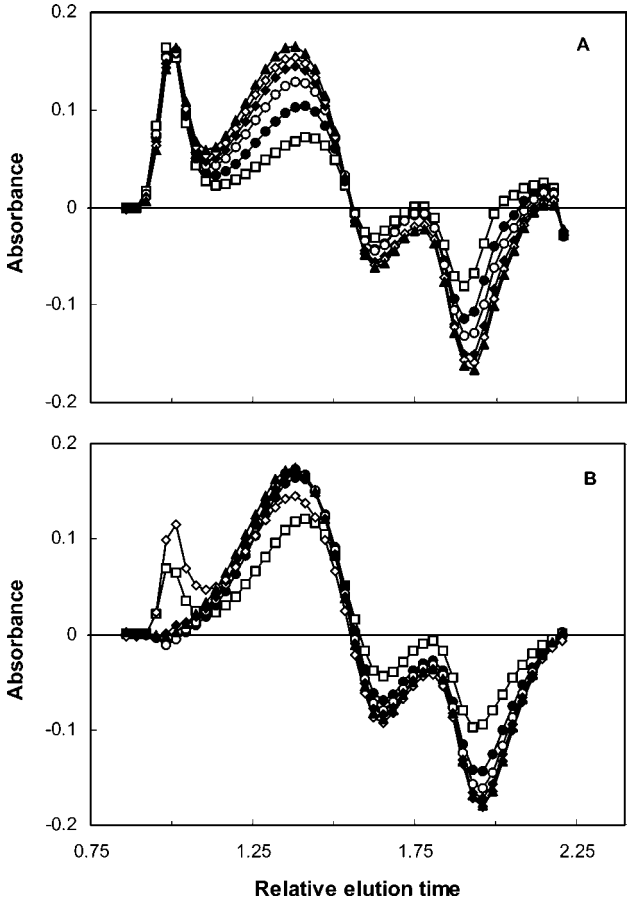

Figure 4. Difference SEC elution profiles of sera of RSMP containing 0.5% added sodium caseinate, heated at 80 °C (A) or 85 °C (B) for 5 (□), 10 (●), 15 (○), 20 (◇), 25 (◆), and 30 (▲) min.

that produce the soluble complexes found in P2. The presence of sodium caseinate did not alter the composition of the complexes in the P2 peak; even with 1% caseinate, the peak was shown by RP-HPLC to contain only whey proteins and κ-casein. The observation of the third peak in the region of P3 of the difference elution profile shows, however, that the added sodium caseinate disappears from the milk serum in the course of heating, approximately in parallel with the loss of whey protein. The profiles seem to indicate that the extent of the loss is temperature-dependent. The cause of the loss of caseinate from the serum will be discussed later.

Results for heated mixtures of 1% sodium caseinate added to milk are almost identical with those of the mixture containing 0.5% caseinate and are not shown here. As was seen for the lower concentration of caseinate, there was a buildup of the P2 fraction and a loss of both caseinate and whey protein fractions. Again, the presence of the added caseinate did not seem to greatly affect the formation of the P2 fraction, either in rate or amount. This can be more clearly seen in **Figure 5**, where the height of P2 at its maximum is plotted against the decrease in the height of P4, for all of the casein concentrations, times, and temperatures used. Although the results are rather spread, it can be seen that there is no systematic trend between milks containing different amounts of added sodium caseinate. It is clear that the height of P2 follows the disappearance of P4, as is to be expected because the particles in P2 are directly formed from the denatured whey proteins. Analysis of all of the results at all temperatures and concentrations of added sodium caseinate showed that, although there appeared to be a small caseinate-dependent increase of P2 after the maximum time of heating,

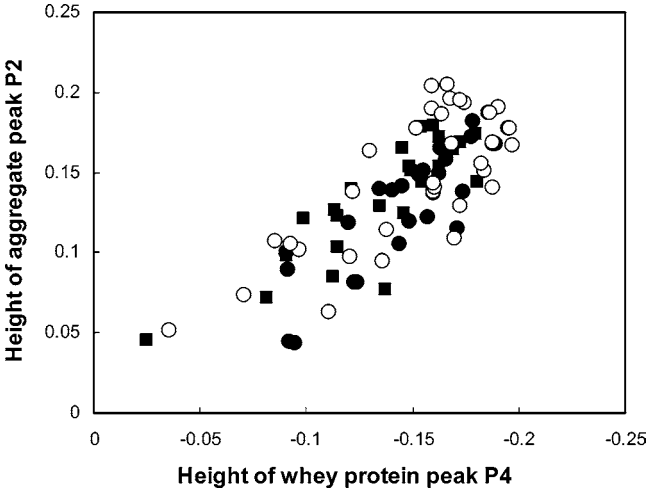

Figure 5. Maximum height of the aggregate peak P2 relative to the loss of the whey protein peak P4 in chromatograms of heated milk/caseinate mixtures. ●, no added caseinate; ■, 0.5% added caseinate; and ○, 1.0% added caseinate.

this increase was not significant. The peak height of P2 with 1.0% caseinate was 0.185 ± 0.027, which may be compared with the results for 0 and 0.5% caseinate given in the preceding paragraph.

The relative lack of effect of added sodium caseinate on the amount of complex P2 formed and the fact that the particle size of the soluble complexes (as estimated from the elution time at the maximum of P2) does not depend significantly upon the amount of added sodium caseinate may suggest that the whey protein/κ-casein complexes that comprise P2 can only be formed by direct interaction of the denatured or denaturing serum proteins with the casein micelles themselves. This would imply that complexes are formed on the micellar surface and subsequently dissociate.

Studies of Heated Model Systems Containing the Different Proteins. The disappearance of the sodium caseinate from the milk serum is difficult to explain, because it involves caseins (α_{s1}- and β-caseins) that are not expected to be affected by the denaturation and subsequent reaction of the whey proteins. To study this further, a series of experiments was undertaken in which the behaviors of the individual components of the sodium caseinate/casein micelle/whey protein system were heated separately or in different combinations (**Figure 6**). When WPI (1%), suspended in milk ultrafiltrate (MUF), was heated, the whey protein mainly vanished from the serum (it formed large particles, precipitated, and was sedimented by the centrifugation step). Only very small amounts of soluble complexes were found, relative to the amount of original protein (**Figure 6A**). This is of course typical behavior for whey proteins suspended in a medium of moderate ionic strength (*22*, *23*) and confirms that the soluble complexes that we observe in milk cannot derive from whey proteins alone. Sodium caseinate (1%) suspended in MUF and heated showed very little change in the SEC profile of the centrifuged serum (**Figure 6C**). A small amount of the protein was lost during heating and centrifuging, but no soluble complexes were formed. This suggests that permeate and therefore the serum of milk to which caseinate is added contain insufficient calcium and/or phosphate to form any "micellar" particles with added caseinate, even when heat is applied. Interestingly, the maximum in the elution profile did not coincide with those of the difference SECs in **Figure 1B** nor in **Figure 6D** but suggested that the caseinate complexes in the

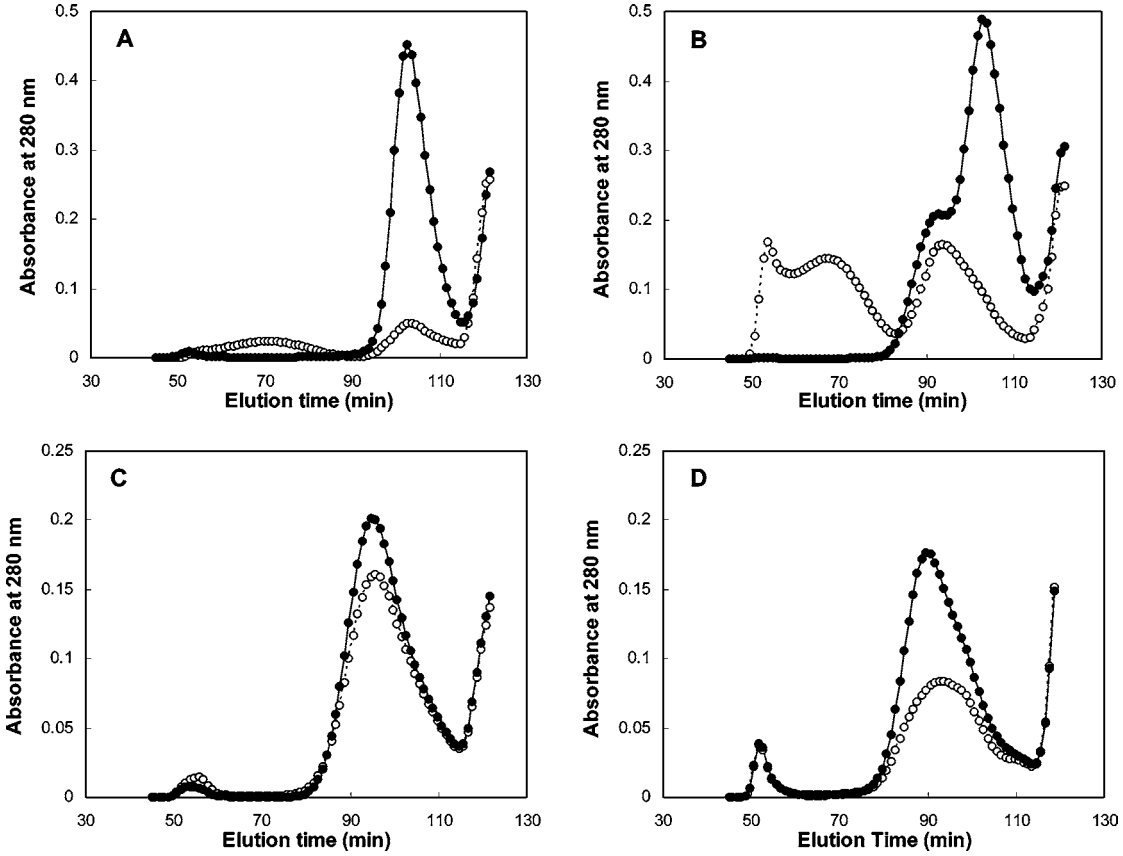

Figure 6. SEC elution profiles of the sera of heated mixtures of the components of the milk/whey protein/caseinate system. In all cases, ● and ○ represent unheated and heated mixtures, respectively. (A) 1% (w/w) WPI dispersed in milk ultrafiltrate, (B) 1% (w/w) WPI and 1% (w/w) sodium caseinate mixture, (C) 1% (w/w) sodium caseinate dispersed in milk ultrafiltrate, and (D) 1% (w/w) sodium caseinate/casein micellar mixture.

MUF were smaller than those in the original RSMP or the whey protein-free micellar preparation.

A mixture of WPI (1%) and sodium caseinate (1%) dispersed in MUF reproduced some of the features that were seen in the caseinate/milk mixtures (**Figure 6B**). In this case, some soluble complexes were produced and remained in the serum after centrifugation. They were, however, larger (i.e., had a considerably shorter elution time) than those that were formed in the caseinate/milk mixtures, so that their elution profile was different. This indicates that the κ-casein of the sodium caseinate is capable of reacting with at least some of the denatured serum proteins to form soluble complexes. The larger size of the complexes compared with those in heated milk can probably be explained because, in milk, the concentration of whey proteins is about 0.7%, rather than the 1% used here, and also that the 1% dispersion of sodium caseinate contains less than half of the κ-casein that is present in milk or milk/caseinate mixtures. It was noteworthy that, in the course of the heating of the WPI/caseinate mixture, the peak corresponding to the whey proteins completely disappeared from the SEC profile but the casein peak decreased only by a small amount, presumably because it lost its κ-casein fraction, while the other caseins remained in an unaltered state. Therefore, the caseinate particles mainly stayed small and resisted centrifugation, even though at least some of their κ-casein had been removed. The fact that this peak remained in the chromatograms shows that the disappearance of the caseinate from heated milks does not arise simply because of, for example, destabilization of α_{s1}- and β-caseins as a result of removal of the κ-casein from the sodium caseinate complexes.

Finally, the micelles from a sample of milk were collected by centrifugation and were dispersed in MUF to their original concentration. Sodium caseinate (1%, w/w) was added to this whey protein-free milk sample, and the mixture was heated. This resulted in a partial removal of the caseinate peak from the SEC of the centrifuged serum (**Figure 6D**). Although the removal of the caseinate from the serum was not as complete as from the heated milk/caseinate mixtures, the partial loss of the casein from the serum demonstrates that the "molecular" caseins are capable of interacting with the casein micelles during the heating of the milk/caseinate mixtures. As was pointed out earlier, the elution time of the maximum of the peak of the sodium caseinate was shorter in the serum from the caseinate/MUF/casein micelle mixture than in the others, suggesting that the behavior of the caseinate is modified in the presence of the micelles. The position of elution does, however, agree well with the position in milk/caseinate mixtures (**Figure 1B**). No specific mechanism is immediately apparent to explain this behavior, but it may possibly be caused by the redistribution of calcium between the casein micelles, the serum, and the added casein molecules. Because no effect was observed when the caseinate was dispersed in MUF, it would seem that the casein micelles could act as a reservoir of Ca for transfer to the caseinate, although this transfer must be far from an equilibrium state, because there is no tendency of the caseinate to form large particles or to sediment.

These four mixtures exhibit several features of the reactions that occur during the heating of the sodium caseinate/milk mixtures. It is clear that both caseins (specifically κ-casein) and whey proteins must be present to form the soluble complexes; it also seems that the form of the κ-casein (whether it is in micelles or in caseinate particles) is not critical to the success of the complex-forming reactions. Therefore, it is rather surprising that the presence of the κ-casein derived from sodium

caseinate does not seem to affect the rate of formation, the size, or the quantity of the soluble complexes, as was shown by the results in **Figures 4** and **5**. This may suggest that there are other factors apart from the simple quantity of available κ-casein that govern the production of the soluble complexes. The observation could be explained by postulating a preferential reaction between the κ-casein of the casein micelles and the denaturing whey proteins, but we have no detailed evidence of this.

Although the results shown in **Figure 6D** confirm that the added sodium caseinate can form complexes with the casein micelles when the milk is heated, it was found that the sizes of the casein micelles themselves did not show a significant increase during heating. The concentration of the casein naturally in milk is approximately 2.5%; therefore, the addition of 1% caseinate represents a significant extra amount to be added to the casein micelles. However, measurements using dynamic light scattering of the particle sizes in unheated and heated milks, with and without added caseinates, showed no significant effects of either heating and/or caseinate addition. The results of Anema and Li (*24*) showed an increase in the diameters of micelles heated at a pH lower than that of milk but that this increase is less at the normal pH of the milk. Nevertheless, our results differed somewhat from theirs in that we observed no increase at all in any of the milks or milk/caseinate mixtures.

Therefore, our results suggest that the caseinate binds to the casein micelles but that there is no evidence of a significant change in the sizes of these particles as a result. This apparent contradiction may possibly be explained by the structure of the surface of the casein micelle. Detailed electron microscopy (*25*) has recently suggested that the surface of the casein micelle is quite rough and that deep fissures exist, which extend below what will be the hydrodynamic surface (measured by DLS). In addition, we know that the surface of the casein micelle is covered by a hairy layer composed of the macropeptide portion of κ-casein. The added sodium caseinate may bind inside the layer that forms the hydrodynamic diameter of the casein micelle. An alternative way of looking at the problem is to estimate what the coverage of the surfaces of the casein micelles would be if all of the caseinate adsorbed as a monolayer. The surface area of the casein micelles in 1 mL of milk can be estimated to be about 3−4 m^2, depending upon what the diameter of the average micelle is taken to be. The added sodium caseinate (1%), if it all binds to the micellar surface, would give a surface coverage of about 2.5 mg/m^2. Studies of emulsions have shown that this is just enough to form a monolayer of adsorbed casein (*26*). Assuming that we can compare the surfaces of casein micelles and emulsion droplets, a monolayer of casein molecules would be able to bind to the interstices of the surface and not greatly increase the micellar diameters.

In conclusion, it seems clear that all of the molecules in sodium caseinate added to milk (at least up to a concentration of 1%) are capable of undergoing heat-induced reactions. The κ-casein of the caseinate can participate in the formation of complexes with the denaturing whey proteins and in doing so may prevent some of the micellar κ-casein from being dissociated. We may conclude this because the addition of caseinate does not proportionally increase the size of the peak because of soluble complexes in the SEC. Conversely, the other caseins (α_s and β) appear to interact with the casein micelles. If the interior of the micelles [generally reasoned to be hydrophobic (*27*, *28*)] is accessible via the irregularities in the surface, the hydrophobicity of the individual casein molecules from the caseinate may allow them to bind in positions that are effectively below the hydrodynamic surface of the micelles. Hence, the added casein molecules can bind to the casein micelles without causing changes in the hydrodynamic diameters.

LITERATURE CITED

(1) Corredig, M.; Dalgleish, D. G. The mechanisms of the heat-induced interaction of whey proteins with casein micelles in milk. *Int. Dairy J.* **1999**, *5*, 233−236.

(2) Corredig, M.; Dalgleish, D. G. Effect of temperature and pH on the interactions of whey proteins with casein micelles in skim milk. *Food Res. Int.* **1996**, *29*, 49−55.

(3) Oldfield, D. J.; Singh, H.; Taylor, M. W.; Pearce, K. N. Heat-induced interactions of β-lactoglobulin and α-lactalbumin with the casein micelle in pH-adjusted skim milk. *Int. Dairy J.* **2000**, *10*, 509−518.

(4) Jang, H. D.; Swaisgood, H. E. Disulfide bond formation between thermally denatured β-lactoglobulin and κ-casein in casein micelles. *J. Dairy Sci.* **1990**, *73*, 900−904.

(5) Cho, Y.; Singh H.; Creamer, L. K. Heat-induced interactions of β-lactoglobulin A and κ-casein B in a model system. *J. Dairy Res.* **2003**, *70*, 61−71.

(6) Livney, Y. D.; Dalgleish, D. G. Specificity of disulfide bond formation during thermal aggregation in solutions of β-lactoglobulin B and κ-casein A. *J. Agric. Food Chem.* **2004**, *52*, 5527−5532.

(7) Anema, S. G.; Li, Y. Effect of pH on the association of denatured whey proteins with casein micelles in heated reconstituted skim milk. *J. Agric. Food Chem.* **2003**, *51*, 1640−1646.

(8) Anema, S. G.; Li, Y. Association of denatured whey proteins with casein micelles in heated reconstituted skim milk and its effect on casein micelle size. *J. Dairy Res.* **2003**, *70*, 73−83.

(9) Guyomarc'h, F.; Law, A. J. R.; Dalgleish, D. G. Formation of soluble and micelle-bound protein aggregates in heated milk. *J. Agric. Food Chem.* **2003**, *51*, 4652−4660.

(10) Beaulieu, M.; Pouliot, Y.; Pouliot, M. Thermal aggregation of whey proteins in model solutions as affected by casein/whey protein ratios. *J. Food Sci.* **1999**, *64*, 776−780.

(11) Vasbinder, A. J.; de Kruif, C. G. Casein−whey protein interactions in heated milk: The influence of pH. *Int. Dairy J.* **2003**, *13*, 669−677.

(12) Vasbinder, A. J.; Alting, A. C.; de Kruif, K. G. Quantification of heat-induced casein−whey protein interactions in milk and its relation to gelation kinetics. *Colloids Surf., B* **2003**, *31*, 115−123.

(13) Lucey, J. A.; Singh, H. Formation and physical properties of acid milk gels: A review. *Food Res. Int.* **1998**, *30*, 529−542.

(14) Anema, S. G.; Lee, S. K.; Lowe, E. K.; Klostermeyer, H. Rheological properties of acid gels prepared from heated pH-adjusted skim milk. *J. Agric. Food Chem.* **2004**, *52*, 337−343.

(15) Dalgleish, D. G.; van Mourik, L.; Corredig, M. Heat-induced interactions of whey proteins and casein micelles with different concentrations of α-lactalbumin and β-lactoglobulin. *J. Agric. Food Chem.* **1997**, *45*, 4806−4813.

(16) Waugh, D. F.; Creamer, L. K.; Slattery, C. W. Core polymers of casein micelles. *Biochemistry* **1970**, *9*, 786−795.

(17) Lucey, J. A.; Srinivasan, M.; Singh, H.; Munro, P. A. Characterization of commercial and experimental sodium caseinates by multiangle laser light scattering and size-exclusion chromatography. *J. Agric. Food Chem.* **2000**, *48*, 1610−1616.

(18) Srinivasan, M.; Singh, H.; Munro, P. A. Adsorption behaviour of sodium and calcium caseinates in oil-in-water emulsions. *Int. Dairy J.* **1999**, *9*, 337−341.

(19) Andrews, A. T.; Taylor, M. D.; Owen, A. J. Rapid analysis of bovine milk proteins by fast protein liquid chromatography. *J. Chromatogr.* **1985**, *348*, 177−185.

(20) Anema, S. G.; Klostermeyer, H. Heat-induced, pH-dependent dissociation of casein micelles on heating reconstituted skim milk

at temperatures below 100 °C. *J. Agric. Food Chem.* **1997**, *45*, 1108−1115.
(21) Anema, S. G.; Li, Y. Further studies on the heat-induced, pH-dependent dissociation of casein from the micelles in reconstituted skim milk. *Lebensm.-Wiss. Technol.* **2000**, *33*, 335−343.
(22) Gezimati, J.; Creamer, L. K.; Singh, H. Heat-induced interaction and gelation of mixtures of β-lactoglobulin and α-lactalbumin. *J. Agric. Food Chem.* **1997**, *45*, 1130−1136.
(23) Havea, P.; Singh, H.; Creamer, L. K. Characterization of heat-induced aggregates of β-lactoglobulin, α-lactalbumin, and bovine serum albumin in a whey protein concentrate environment. *J. Dairy Res.* **2001**, *68*, 483−497.
(24) Anema, S. G.; Lowe, E. K.; Lee, S. K. Effect of pH at heating on the acid-induced aggregation of casein micelles in reconstituted skim milk. *Lebensm.-Wiss. Technol.* **2004**, *37*, 779−787.
(25) Dalgleish, D. G.; Spagnuolo, P. A.; Goff, H. D. A possible structure of the casein micelle based on high-resolution field-emission scanning electron microscopy. *Int. Dairy J.* **2004**, *14*, 1025−1031.
(26) Fang, Y.; Dalgleish, D. G. Dimensions of the adsorbed layers in oil-in-water emulsions stabilized by caseins. *J. Colloid Interface Sci.* **1993**, *156*, 329−334.
(27) Holt, C.; Timmins, P. A.; Errington, N.; Leaver, J. A core−shell model of calcium phosphate nanoclusters stabilized by β-casein phosphopeptides, derived from sedimentation equilibrium and small-angle X-ray and neutron-scattering measurements. *Eur. J. Biochem.* **1998**, *252*, 73−78.
(28) Horne, D. S. Casein interactions: Casting light on the *Black Boxes*, the structure in dairy products. *Int. Dairy J.* **1998**, *8*, 171−177.

Received for review May 26, 2005. Revised manuscript received August 8, 2005. Accepted August 18, 2005. The authors acknowledge the financial support of the Ontario Dairy Council and the Natural Sciences and Engineering Research Council of Canada.

JF0512306