Interactions between α-Lactalbumin and β-Lactoglobulin in the Early Stages of Heat Denaturation

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Interactions between whey proteins in mixed systems (10% w/w total protein) containing α-lactalbumin (α-la) and β-lactoglobulin (β-lg) heated at 75 °C for different times were studied using gel filtration chromatography and sodium dodecyl sulfate−polyacrylamide gel electrophoresis. On its own, α-la did not form aggregates, and β-lg formed large aggregates with no evidence for intermediates. However, the two proteins interacted to form soluble aggregates, as well as larger particles, by means of both disulfide bonds and hydrophobic interactions. Two main soluble products were identified: high molecular weight components with molecular masses in excess of 300 000 Da and intermediate aggregates with molecular weights in the region of 100 000 Da. The aggregation process was substantially affected, both quantitatively and qualitatively, by the relative proportions of α-la and β-lg: when the weight fraction of α-la in the mixture was <0.3, the smaller aggregates were not induced, whereas they were formed at higher weight fractions and were the only product at a weight fraction of α-la of 0.95. The composition of the aggregates changed during heating and was different for each mixture, but it always approximated the β-lg/α-la ratio of the initial mixture after about 8 min of heating. The different interactions observed at the different ratios of proteins may explain the enhancement of heat-induced gelation of β-lg by α-la.

Keywords: *Denaturation; α-lactalbumin; β-lactoglobulin; whey protein; heating; gelation; aggregation*

INTRODUCTION

Whey proteins are a major source of highly functional food proteins. Functional properties such as gelation, foaming, and emulsification are affected by the chemical and physical properties of the main protein constituents: β-lactoglobulin (β-lg), α-lactalbumin (α-la), and bovine serum albumin (BSA). This has led to extensive studies of individual whey proteins, in particular the heat denaturation and aggregation of β-lg (Laligant *et al.*, 1991; McSwiney *et al.*, 1994; Qi *et al.*, 1995; Iametti *et al.*, 1995), the apparently reversible heat denaturation of α-la (de Wit and Klarenbeek, 1984), and the thermal gelation of whey protein concentrates (Parris *et al.*, 1993; Hollar *et al.*, 1995; Monahan *et al.*, 1995). There is also extensive literature on the interactions between whey proteins and casein micelles when milk is heated.

The major whey proteins have different amino acid compositions, structures, and properties, and the functional behavior of an individual protein can be altered by the presence of the others. Some interactions between whey proteins have been identified; for example, it has been shown that addition of α-la or BSA to solutions of β-lg enhances the strength of the gels formed during heating (Matsudomi *et al.*, 1992, 1993; Legowo *et al.*, 1993). However, the mechanisms of the interactions between these proteins during gelation have not been fully elucidated, although it has been established that molecular complexes between the proteins are formed (Baer *et al.*, 1976).

It is known that soluble aggregates of polymerized whey proteins are formed during the early stages of heat-induced gelation and that subsequent polymerization results in the formation of a rigid gel network (Baer *et al.*, 1976; Parris *et al.*, 1991, 1993). It is possible that gel properties may be influenced by the formation of specific soluble aggregates formed by the interaction between whey proteins in the early stages of heating.

It is not established in what manner α-la and β-lg interact. They are known to both bind to casein micelles in milk (Dannenberg and Kessler, 1988; Corredig and Dalgleish, 1996) and to caseins in homogenized milk (Sharma and Dalgleish, 1993), at about the same rate, when the temperature is below about 80 °C. It is generally believed that α-la does not polymerize readily, since it does not form gels on heating at neutral pH; however, the presence of other proteins, specifically those containing free sulfhydryl groups (Calvo *et al.*, 1993), allows interactions to occur between α-la and the added protein. Even isolated κ-casein interacts with α-la (Doi *et al.*, 1983), although interaction with the κ-casein in casein micelles does not occur (Smits and van Brouwershaven, 1980). In the particular case of the interaction between α-la and β-lg, there is various evidence for soluble complexes. Zhu and Damodaran (1994) found that α-la/β-lg dimers formed during heating at 70 °C, but not at 90 °C. Parris *et al.* (1993) also found that a "soluble" (i.e., nongelled) product was formed, as did Matsudomi *et al.* (1992). Perhaps unexpectedly in view of the apparently unreactive nature of α-la, it was found to disappear rather more rapidly than β-lg from heated whey protein concentrate (Hollar *et al.*, 1995; Boye *et al.*, 1995), compared to the apparently similar reaction rates of the two proteins in milk. Although not specifically analyzed, similar trends may be apparent in the studies of whey protein concentrate by Zhu and Damodaran (1994) and Monahan *et al.* (1995).

In none of the above studies were the complexes between α-la and β-lg studied in detail. Therefore, we undertook to study in more detail the reaction inter-

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mediates formed during the interactions between α-la and β-lg during heating, using different mixtures of the two proteins. It was hoped that by this means details of the reaction mechanism could be clarified.

MATERIALS AND METHODS

Proteins and Chemicals. Samples of commercial α-la and β-lg were provided by Protose Separations, Inc., Teeswater, ON. These commercially available fractions were not completely pure individual proteins (being mixtures of α-la and β-lg), but for preparation of most of the mixtures described below they were used without further purification. Pure fractions of the proteins were isolated using ion exchange chromatography, on a column of Q-Sepharose Fast Flow (Pharmacia Biotech, Baie d'Urfé, PQ). The individual purified protein fractions were dialyzed exhaustively against deionized water and freeze-dried. The β-lg was the natural mixture of genetic variants A and B. These purified proteins were used for the samples containing weight fractions of α-la of 1.00, 0.95, and 0.

Deionized water was purified using a Milli-Q system (Millipore Ltd., Mississauga, ON).

All chemicals were of reagent grade and purchased from Fisher Scientific Ltd., Mississauga, ON. Phosphate buffers were prepared from dibasic salts, and the pH was adjusted to pH 7.0 with 5 M HCl.

Sample Preparation. All of the protein mixtures, containing different proportions of α-la and β-lg, were dissolved in 20 mM phosphate buffer, pH 7.0, to give a total final protein concentration of 10% (w/w). The mixtures used contained fractions of α-la of 1.00, 0.95, 0.68, 0.49, 0.30, and 0 of the weight of the protein; the compositions of the mixtures were confirmed using sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE; see below). Samples (3 mL) of the mixtures were then heated at a temperature of 75 °C in a water bath for periods of time ranging from 1 to 10 min, after a warm-up period of 90 s, and were then immediately cooled to room temperature in an ice bath. The samples were then stored at 4 °C until they were required for analysis by gel permeation chromatography. The temperature of 75 °C was selected because it gave kinetics on a suitable time scale for the experiments and because it is close to the denaturation temperature of β-lg and above that of α-la.

Fractionation of the Aggregated Material. The gel permeation chromatography of the samples was performed using a prepacked column (1 × 30 cm) of Superose 12, attached to a Fast Protein liquid chromatography (FPLC) system (Pharmacia Biotech). The particular column packing was selected because it could separate aggregated material from the monomers in the molecular mass range 1 000−300 000 Da. The monomer molecular masses of α-la and β-lg are 14 200 and 18 300 Da, respectively, but since the β-lg is a dimer at pH 7.0, its effective molecular mass is twice that of the monomer.

During heating, many of the protein mixtures aggregated or gelled somewhat, although we avoided heating times at which extensive gelation occurred. The whole sample containing these extensively aggregated materials could not be loaded onto the chromatographic column, because of the risk of blockage; therefore, all heated samples were passed through a 0.22 μm cellulose nitrate filter before they were loaded onto the column. This filtration was sufficient to remove all of the gelled material present in the samples. After filtration, a 100 μL sample of the heated protein mixture was loaded onto the column and eluted by a buffer containing 20 mM phosphate, pH 7.0, at a flow rate of 0.8 mL min^{-1}. The elution profile of the proteins was detected by absorbance at 280 nm. Typically, the profiles showed three or four major fractions (depending on the mixture): two fractions of aggregated materials and unreacted β-lg and α-la. The high molecular mass fractions were collected as they eluted from the column. They were then dialyzed exhaustively against deionized water and freeze-dried before being analyzed by SDS−PAGE. Each heating experiment was repeated at least twice.

Electrophoretic Analyses of the Complexes. Electrophoresis (SDS−PAGE) was carried out under both reducing and nonreducing conditions. Reducing electrophoresis was performed in the presence of 2-mercaptoethanol, to break the disulfide bridges between the molecules. Comparison of the electrophoretic patterns in reducing and nonreducing conditions is widely used to identify the presence of disulfide bonds holding together aggregates of proteins (Matsudomi *et al.*, 1992; Zhu and Damodaran, 1994; Monahan *et al.*, 1995).

The lyophilized samples from the chromatography were redissolved in 200 μL of buffer, containing 10 mM Tris/HCl and 1 mM EDTA, pH 8.0. These solutions were divided into two parts to allow electrophoresis to be run under reducing and nonreducing conditions. For reducing conditions, 150 μL of 20% SDS, 100 μL of 2-mercaptoethanol, and 50 μL of 0.01% bromophenol blue were added to the sample, and for nonreducing conditions, the 2-mercaptoethanol was replaced by 100 μL of Tris buffer pH 8.0. These samples were heated in a boiling water bath for 5 min with constant stirring. After cooling, 1 μL samples were loaded onto a 20% homogeneous gel and run on a rapid electrophoresis system (Phastsystem, Pharmacia Biotech) at 15 °C. Each sample was analyzed in triplicate. The gels were stained with a Coomassie blue solution containing 30% (v/v) methanol, 10% (v/v) acetic acid, and 0.1% (v/v) Phastgel Blue R, destained with methanol/acetic acid solution, and preserved with a glycerol solution to prevent drying and deterioration.

The gels were scanned using an Ultrascan X laser densitometer (Pharmacia Biotech) at a wavelength of 633 nm. The integrated densities of the α-la and β-lg bands were converted into protein quantities by comparison with standard calibration curves, which were constructed using known amounts of purified α-la and β-lg [1% (w/w) protein solution] in reducing electrophoresis. The relation between the optical density and the protein quantity was determined by simple linear regression.

RESULTS

Even before the analysis by chromatography, the heated mixtures showed visual evidence of interactions between the proteins. A ring of aggregated proteins was deposited on the vial at the surface of the heated solutions, and the amount of this increased with heating time. After ca. 8 min, the mixtures in which α-la was predominant (but not the mixtures containing α-la at weight fractions of 1.0 and 0.95) formed a gel (one of the final stages of interaction between the proteins), whereas full gelation did not occur before ca. 10 min with the mixtures in which β-lg was predominant. For this reason, solutions containing α-la in weight fractions of 0.68 and 0.49 were heated for up to 8 min and the other ones for up to 10 min, since the gelled samples could not be analyzed by chromatography.

The Superose 12 gel filtration medium gave a good separation of α-la and β-lg (Andrews *et al.*, 1985). It also allowed separation of any aggregated material from the unreacted whey proteins. No substantial changes were observed between the unheated solutions and solutions heated for 2 min (the time required for the sample in the vial to reach the desired temperature), and so the first samples for analysis were collected after heating for at least this time. In nearly all cases, aggregate peaks were observed at the exclusion limit of the column (nominally 300 000 Da), indicating that some material was present with molecular masses in excess of this value. In some cases, a second peak of less aggregated material was observed between the excluded material and the position of the β-lactoglobulin.

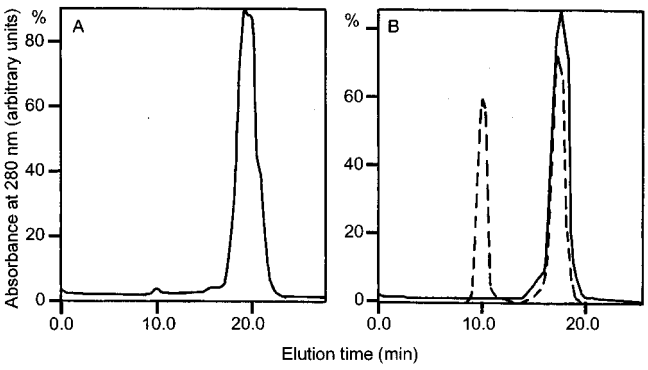

Figure 1. Analysis of the purified protein fractions after heating, using gel permeation chromatography: (A) α-la, unheated (solid line) and heated for 8 min (broken line) (no difference is apparent); (B) β-lg, before (solid line) and after (broken line) heating for 8 min at 75 °C.

Heating of Individual Proteins. The sample of purified α-la did not gel when heated, and the chromatograms (Figure 1A) showed a single peak, with no major difference between the unheated and the heated (8 min) samples, indicating that α-la does not aggregate when heated alone. This agrees with the results of de Wit and Klarenbeek (1984), who have shown that, although α-la is susceptible to heat-denaturation (with a transition temperature of 68 °C), the process is somewhat reversible because of the high degree of renaturation of the protein in the absence of other sulfhydryl-containing proteins (Calvo *et al.*, 1993). A similar result has been found by Matsudomi *et al.* (1992).

On the other hand, the sample of purified β-lg showed two peaks after heating (Figure 1B): one from unreacted β-lg, and the other of high molecular mass components, eluting at the void volume of the column, that was not present in the original solution. During heating, the area of the aggregate peak increased while the area of the β-lg peak decreased, although they could not be quantified because of the necessity for filtration of the samples. This protein is known to be highly reactive when heated above 65 °C. It has been proposed that heat denaturation exposes sulfhydryl groups previously buried in the native molecule and leads to protein copolymerization (Roefs and de Kruif, 1994). In agreement with other studies (Matsudomi *et al.*, 1992; McSwiney *et al.*, 1994), no products of intermediate molecular mass were found in any of the samples of heated β-lactoglobulin, suggesting that the denatured protein molecules react very rapidly to give products of high molecular mass, passing through any intermediate stage with great efficiency.

Heating of Mixtures of α-la and β-lg. The mixtures of α-la and β-lg after heating showed three different sets of patterns on gel permeation chromatography, depending on their composition. When β-lg was predominant (weight fraction of α-la = 0.30), the chromatogram showed three peaks (Figure 2A), from unreacted α-la and β-lg, and highly aggregated material eluting at the void volume. The chromatogram of this mixture was similar to that of the pure β-lg; with heating, the area of the peak corresponding to the aggregated material increased while peak areas of both α-la and β-lg decreased, but no peaks of intermediately sized aggregate were observed at any of the heating times.

When α-la and β-lg were both present in similar amounts (mixtures with weight fractions of α-la of 0.49 and 0.68), four peaks were present in the chromatogram (Figure 2B): α-la, β-lg, intermediate aggregate, and high molecular mass components, as found by Matsudomi *et al.* (1992). The peak of intermediate aggregate was rather broad and, therefore, was likely to contain a range of sizes of aggregates, centered around molecular masses of approximately 100 000 Da. As the heating time increased, the areas of unreacted α-la and β-lg decreased while the area of the large aggregate peak increased and the intermediate peak increased in size and gradually shifted toward higher molecular mass, although it always remained distinct from the aggregated material eluting at the void volume of the column.

When α-la was the predominant protein, in the mixture with weight fraction = 0.95 (Figure 2C), virtually no large aggregate material was formed within 10 min of heating. Intermediate aggregate was produced, in quantities that increased slowly with heating time. As with the earlier examples, this aggregate peak shifted to larger molecular mass as the heating time increased. Since no large aggregate was formed, it was evident that the small amount of β-lg present was not acting as a catalyst for the polymerization of α-la.

From these observations, there seemed to be two types of aggregation mechanism occurring in the heated α-la/β-lg system. In one, the high molecular mass material was formed directly and without buildup of appreciable amounts of intermediates. In the second, intermediates containing approximately 10−15 protein molecules (depending on the heating time and composition of the mixture), and which are themselves reactive, are created by the interaction of β-lg and α-la. There is evidently a synergistic interaction between α-la and β-lg. The presence of β-lg enables α-la to react and when the α-la/β-lg ratio is low, the α-la is enabled to integrate into the highly aggregated material without going through an intermediate stage.

Nature of the Intermolecular Bonds. When the aggregated peaks were subjected to nonreducing electrophoresis, the results were quite similar for all protein mixtures. In addition to the well-established bands from α-la and β-lg, there were several bands from polymers, some of which were so large in molecular weight that they could hardly or not at all migrate into the stacking part of the polyacrylamide gel. This agrees with observations on whey protein concentrates (Parris *et al.*, 1993; Hollar *et al.*, 1995; Monahan *et al.*, 1995). The major difference found between the first and the second peaks of high molecular mass components isolated by chromatography was that the second (lower molecular mass) fraction contained aggregates able to migrate into the stacking gel, confirming that this fraction is composed of smaller aggregates. When any of the samples was reduced with 2-mercaptoethanol and run by SDS−PAGE, the bands from polymeric aggregated materials were completely dissociated into the monomers of α-la and β-lg.

This showed clearly that α-la and β-lg were both present in the aggregates formed from the mixture of two proteins and also that β-lg formed the polymers with α-la partly, but not exclusively, through thiol−disulfide interchange during heating. It is assumed that these are true complexes, rather than mixtures of homopolymers of the proteins, since we have seen that α-la, on its own or in combination with small amounts of β-lg, is not capable of forming polymers under these experimental conditions. The role of sulfhydryl groups in the

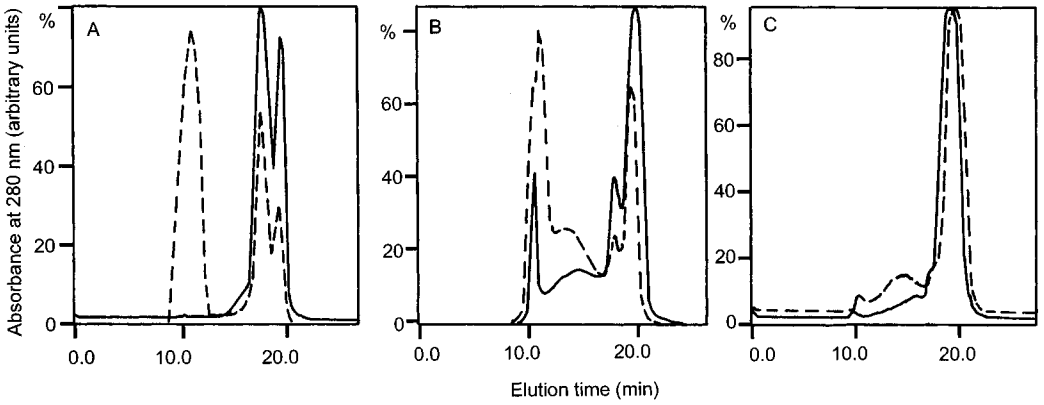

Figure 2. Analysis of the products of reaction of different mixtures of α-la and β-lg: (A) mixture with weight fraction of α-la of 0.30, unheated and heated for 8 min; (B) mixture with weight fraction of α-la of 0.49, after heating for 3 or 8 min; (C) mixture with weight fraction of α-la of 0.95, after heating for 3 or 8 min. In all cases, the broken lines are from samples heated for 8 min.

interactions between proteins during heating, such as κ-casein and β-lg, β-lg and BSA, β-lg and α-la, has already been emphasized (Shimada and Cheftel, 1989; Matsudomi *et al.*, 1992, 1993; Monahan *et al.*, 1995). At pH 7.0, β-lg exists as a stable dimer in which the thiol group is sheltered in a hydrophobic environment, probably near the interface between the two monomeric units. Above 65 °C, extensive conformational transitions occur in the molecule, which expose the reactive −SH group, which may then participate in thiol/disulfide interchange reactions with α-la, as shown by the presence of aggregated material in the electrophoresis.

However, disulfide bonds were not the only means of interaction between β-lg and α-la. The presence of substantial bands of monomeric α-la and β-lg in the nonreducing gels indicated that hydrophobic interactions (which were disrupted in the presence of SDS) were also involved in linking the proteins, similarly to the hydrophobic bonds in the heat-induced κ-casein/β-lg complex (Doi *et al.*, 1983; Jang and Swaisgood, 1990) and in α-la/β-lg complexes (Parris *et al.*, 1993; Matsudomi *et al.*, 1992; Monahan *et al.*, 1995).

Composition of the Aggregated Materials. The production of the peaks of highly aggregated material excluded from the column followed similar patterns for each mixture: the amounts of aggregated α-la and β-lg increased with heating time, but there were different rates for the incorporation of the two proteins. It was found that the ratio β-lg/α-la in the peak was higher during the early stages of the reaction than the overall β-lg/α-la ratio in the mixture (Figure 3). As the reaction proceeded, the α-la must have reacted more rapidly since the β-lg/α-la ratio decreased until it was approximately equal to that in the original mixture. This was especially notable in the mixture with weight fraction of α-la of 0.68, where a 2-fold decrease in the β-lg/α-la ratio was observed, but the change in ratio was present in all of the mixtures studied. Thus, the earliest aggregation steps in these mixtures produced polymers richer in β-lg than the average, and the incorporation of α-la into the aggregates in the early stages was somewhat slower.

The ratio β-lg/α-la of the proteins in the aggregate peak of low molecular mass was generally smaller than that of the first peak, confirming that this intermediate peak contained more α-la. The weight ratio decreased with heating time similarly to that of the first peak. It should be remembered, however, that these intermediate fractions were not present in all of the mixtures.

The protein bands for the solution containing weight fraction = 0.95 of α-la were not quantified, because of lack of material, but qualitatively the SDS−PAGE showed that there was little β-lg in the aggregates.

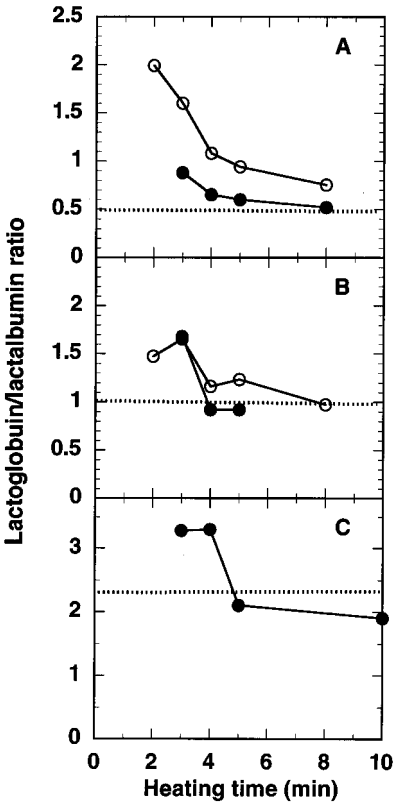

Figure 3. Analysis of the intermediate aggregates (where formed) and high molecular mass aggregates from mixtures of α-la and β-lg at different heating times: (A) mixture with weight fraction of α-la of 0.68; (B) mixture with weight fraction of α-la of 0.49; (C) mixture with weight fraction of α-la of 0.30. In (C), no intermediate material was found. In (A) and (B) the open symbols represent the aggregate of high molecular mass and the solid symbols are from the intermediate aggregates. The broken lines show the overall β-lg/α-la ratios in the mixtures.

An additional experiment was performed with a solution containing a mixture with weight fraction of α-la of 0.62, but at pH 6.0 rather than pH 7.0. The results (not shown) confirmed that the aggregation mechanism and the gelation behavior were different from those at pH 7.0: the weight ratio β-lg/α-la in the aggregated peaks was higher than at pH 7.0, the solution gelled after only 7 min, and there was less evidence for disulfide bond formation in the aggregated product.

DISCUSSION

The results described above confirm previous observations that α-la does not aggregate, even when it is denatured by heating at neutral pH. Moreover, the incorporation of small amounts of β-lg did not cause gelation of the α-la, but only moderate aggregation, which suggests that the action of β-lg is not simply catalytic. Effectively, if the reaction is to be considered as a polymerization (Roefs and de Kruif, 1994), the chain termination steps must be more efficient than the chain propagation by sulfhydryl/disulfide exchange. The qualitative analysis of the intermediate-sized peak in the chromatogram suggests that β-lg did not simply self-aggregate but that α-la was also a major component of the complex. Since at least part of the complex was hydrophobically linked, rather than via disulfides, it may be that the activated sulfhydryl in denatured β-lg is not capable of interacting with the disulfides in α-la to form free sulfhydryl groups in that protein.

At the other extreme, pure β-lg showed no intermediate material; only large aggregates were formed, which were eluted at the void volume of the gel permeation column. This suggests that the β-lg molecules once denatured by heating are highly reactive in polymerization and do not form low molecular mass complexes as chain termination occurs during polymerization. This also seems to be the case in the mixtures with α-la in which β-lg is the main component, since no intermediate particles were seen to be formed, although the amount of α-la decreased and it was obviously involved in polymer formation. The proportion of β-lg/α-la in the complexes was high, as befitted the concentration in the mixture, but the time dependence suggests that the β-lg started to aggregate first and then to react with α-la; it must be assumed that since α-la does not form aggregates on its own, it must form complexes with the β-lg.

The mixtures with weight fractions of α-la of 0.68 and 0.49 show the intermediate species with low molecular mass separated by chromatography. They may be regarded either as possible polymerizing units from which the final gel is constructed or as polymers in which chain termination has occurred and which cannot grow further (although in some cases they were observed to polymerize after being isolated). What is evident about these complexes is that they are neither of the same molecular mass nor of the same composition. This makes it apparent that there is no fixed stoichiometry of the interaction between α-la and β-lg, since complexes isolated at different heating times have different compositions (both the high molecular mass and intermediate fractions), as do the fractions isolated from different mixtures.

We have not yet been able to develop these observations to allow a calculation of the reaction mechanism. Possibly, different mechanisms are operational in the different mixtures. At high concentrations of β-lg, or at short heating times, complexes rich in β-lg are formed, suggesting either that β-lg reacts more readily with itself than with α-la or that complexes are favored in which more than one β-lg helps to unfold the α-la. On the other hand, the mixtures in which α-la predominates have been shown to form aggregates in which there is considerably less β-lg than α-la, so there is no absolute requirement for β-lg to be present in more than a 1:1 ratio with α-la. Nevertheless, even in these mixtures, the early aggregates are richer in β-lg than are the later ones. So, it seems evident that the earliest stages of the reaction involve the formation of complexes of several molecules of β-lg with one of α-la. How these complexes then facilitate the subsequent reaction is not clear; perhaps it is simply the case that the appearance of "reactive" α-la, whether by reaction with β-lg or by other means, is slower than the formation of "reactive" β-lg, even though the formal denaturation temperature of α-la is lower than that of β-lg. However, it has been suggested that the denaturation of α-la takes place via a number of steps (Apenten, 1995), and only the last of these may produce a fully reactive molecule. It does not seem possible for the formation of the complexes to simply occur via a chain propagation mechanism involving sulfhydryl/disulfide interchange, because such chains would have to begin with β-lg and be carried on by α-la; the lack of large products in the reaction of the mixture containing α-la at a weight fraction of 0.95 shows that chain termination is efficient in such a system. Thus, α-la seems not to be an efficient chain propagator, and the reaction must be more complex than the proposal by Roefs and de Kruif (1994).

It is evident from these results and those of others that intermolecular disulfides may not be the main agents holding together the protein molecules but that other interactions are equally important. It is difficult to estimate the relative amounts of material that is disulfide-linked and equally difficult to estimate the fractions of the different α-la/β-lg complexes which are linked by disulfide bonds.

CONCLUSION

Molecules of α-la and β-lg can interact directly with each other during the early stages of heating to form soluble aggregates mainly through disulfide bonding and hydrophobic interactions. The nature of the aggregation process and the products formed is highly dependent on the weight ratio of the two whey proteins in the mixtures, since intermediates are formed only when approximately equal amounts of the two proteins are present. The composition of the products depends on the ratio of the two proteins and suggests that the reaction does not proceed by forming complexes of fixed stoichiometry between the proteins. This aggregation process is responsible for the enhancement of the heat-induced gelation of β-lg by α-la, although it is not possible at this stage to define an overall mechanism.

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