Heat-Induced Aggregation of Whey Proteins Is Enhanced by Addition of Thiolated β-Casein

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Bovine β-casein has been chemically thiolated by reaction with thiolactone. The purified product was found to contain a variable number of free thiol groups in addition to a number of disulfide bridges. Gel electrophoresis under nonreducing conditions showed the presence of monomeric, dimeric, and tetrameric forms of the protein. Heating whey protein concentrate in the presence of this thiolated casein resulted in a significant increase in the rate of aggregation of all of the whey proteins with the exception of α-lactalbumin, where in general the rate of aggregation decreased. This enhancement of the heat-induced aggregation was found to vary with the concentration of thiolated casein. The results are discussed with relevance to decreasing the costs of manufacture of whey protein concentrate.

Keywords: *Whey protein; β-casein; thiolation; heating; aggregation*

INTRODUCTION

Whey proteins are major and potentially very valuable byproducts of both the cheese and caseinate manufacturing processes. They are rich in essential amino acids, and as a result of good foaming and emulsifying properties, whey protein concentrate is used in the manufacture of a variety of processed foods. However, isolation of proteins from cheese whey and acid whey is expensive due to their relatively low concentration. As a result, much of this whey protein, amounting to several hundred million kilograms of protein, is discarded every year (Banerjee and Chen, 1995) leading to significant environmental problems.

Bovine whey contains approximately 4 g L^{-1} β-lactoglobulin (βLG), 1 g L^{-1} α-lactalbumin (αLA), 0.9 g L^{-1} serum albumin (SA), and 1 g L^{-1} immunoglobulins (Ig). All of these globular whey proteins possess intramolecular disulfide bridges, 2 in the case of βLG, 4 in the case of αLA and 17 in the case of SA. Heat-induced interactions between the globular whey proteins are complex (see review by Jelen and Rattray, 1995). In general, it has been shown that incubating solutions of either βLG or SA at or above their transition temperatures, as determined by differential scanning calorimetry, results in a decrease in monomer concentration and the formation of disulfide-linked protein aggregates. In contrast, solutions of αLA are very heat-stable as determined by the lack of aggregate formation, although this protein does undergo a thermal transition at about 65 °C that is largely reversible (Rüegg *et al.*, 1977). Unlike βLG and SA, αLA has no free sulfhydryl group and cannot therefore form the intermolecular disulfide bridges that are to a large extent responsible for the polymer stability (although hydrophobic interactions appear also to be involved). Addition of either βLG or SA to solutions of αLA prior to heating results in aggregation of all of the proteins (Calvo *et al.*, 1993), the free sulfhydryl group on the βLG and SA apparently acting to break one or more of the disulfide bridges of the αLA molecules. Whether this leads to the formation of heteropolymers containing more than one whey protein or to homopolymers containing a single protein species is not yet known. The rate and extent of aggregation of the αLA appear to be proportional to the concentration of free sulfhydryl groups.

Production and utilisation of whey proteins have been reviewed (see the papers presented at two American Dairy Science Association Symposia and edited by Morr and Melachouris, 1984). Some isolation procedures make use of the heat-induced aggregation of the proteins, which occurs as a result of heating the whey above the transition temperatures (60−75 °C) of the component proteins. In the industrial process, the whey is typically heated to 90 °C and held at this temperature for at least 10 min. Aggregation is not instantaneous, the rate being dependent upon the temperature and holding time. The economics of concentrating whey proteins by this method is therefore determined largely by the cost of heating large volumes of liquid whey.

As part of an investigation into the effects of chemical modification on the behavior of milk proteins, we have produced β-casein containing a number of sulfhydryl groups both free and disulfide-linked. In view of the interest in whey protein denaturation, we have investigated the effect of this protein on the heat stability of whey proteins. The results of these investigations are given in this paper.

MATERIALS AND METHODS

β-Casein, phenotype A^1, was purified from the milk of individual Friesian cows in early to midlactation by ion-exchange chromatography (Leaver and Law, 1992). Thiol groups were introduced into the protein by stirring for 24 h at room temperature, at pH 7.5 with *N*-acetylhomocysteine thiolactone (Sigma Chemical Co. Ltd., Poole, Dorset, U.K.) as described by Benesch and Benesch (1958). At the end of the reaction period, the protein was purified by cation-exchange chromatography on Poros 50 HQ resin (PerSeptive Biosystems (UK) Ltd., Hertford, 12 cm × 2.5 cm i.d.) using a 0−1 M NaCl gradient in 5 mM bis-tris-propane/6 M urea, pH 7.0, over 100 min. The flow rate was 5 mL min^{-1}, and detection was at 280 nm. The thiolated β-casein was then dialysed extensively against distilled water at 4 °C prior to freeze-drying.

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Purified β-casein was analyzed before and after thiolation by reverse-phase HPLC on a C18 Apex WP column (Jones Chromatography Ltd., Hengoed, Mid-Glamorgan, U.K.; 25 cm $\times$ 4.6 mm i.d.) using a gradient of acetonitrile in 0.1% TFA.

The sulfhydryl content of the thiolated protein was determined using Ellman's reagent (Ellman, 1959) in 6 M guanidine hydrochloride in accordance with the instructions supplied by the manufacturer (Pierce, Rockford, IL). Bovine βLG was used as a standard protein in this determination.

SDS−polyacrylamide gel electrophoresis (SDS−PAGE) was performed on 20% homogeneous PhastGels (Pharmacia Ltd, Milton Keynes, Bucks, U.K.) with and without the addition of 2-mercaptoethanol in order to obtain an estimate of the molecular weight of the thiolated protein.

Whey protein concentrate was prepared from bulk milk obtained from the Institute herd of Friesian cows. Caseins were removed from skimmed milk by isoelectric precipitation at pH 4.6. The pH of the supernatant was adjusted to 7.0, and after extensive dialysis against distilled water at 4 °C, the protein solution was freeze-dried.

Heat-induced aggregation of whey proteins in the presence and absence of thiolated β-casein was determined by gel permeation FPLC (Law *et al.*, 1993). Whey protein was dissolved at a concentration of 10 mg mL^{-1} in imidazole buffer (20 mM, pH 7.0). Portions (10 mL) of this stock whey protein solution were mixed with equal volumes of either a solution of thiolated β-casein (2 mg mL^{-1} in imidazole buffer) or imidazole buffer alone. In those incubations where the level of thiolated β-casein was varied, the ratio of the solution of the thiolated protein and of the buffer was changed accordingly. The final concentration of whey protein in the reaction mixtures (5 mg mL^{-1}) was approximately the level of these proteins in whey. The mixture was immersed in a water bath, and after leaving for 1 min to allow the mixture to reach the temperature of the bath, aliquots were removed at intervals and cooled rapidly to 20 °C. The pH was then adjusted to 4.6 to precipitate any casein and associated denatured whey proteins, and after filtering through a 0.22-μm filter, the filtrate was used for gel permeation chromatography. Aggregation was determined by measuring the decrease in the integrated peak areas of each of the component whey proteins.

RESULTS AND DISCUSSION

Analysis of the purified, thiolated β-casein by reverse-phase chromatography showed that all of the native protein had been converted to a slightly more hydrophobic product. SDS−PAGE (Figure 1) showed that whereas in the presence of 2-mercaptoethanol the modified protein migrated as a single component with a mobility very similar to that of the native protein, in the absence of the thiol-reducing agent, three major bands were observed. The molecular weight of these three bands was estimated to correspond to the monomeric, dimeric, and tetrameric forms of the protein. These multimers are presumably stabilized by intermolecular disulfide bridges.

Estimating the number of free thiol groups using Ellman's reagent gave values varying between 5 and 9 per mole depending upon the particular batch of protein. Attempts to measure the number of disulfide bridges using the same reagent were unsuccessful due to the very slow development of color. A similar problem was encountered when SA, which contains 17 disulfide bridges, was used as a standard protein. This suggests that the thiolated β-casein probably contains a number of S−S bridges. Thiolactone reportedly reacts with lysine residues, of which there are 11 in β-casein. It is therefore probable that all of the available lysine residues in β-casein had reacted, and the observed variation in the average number of SH groups is due to differences in the number of S−S bridges.

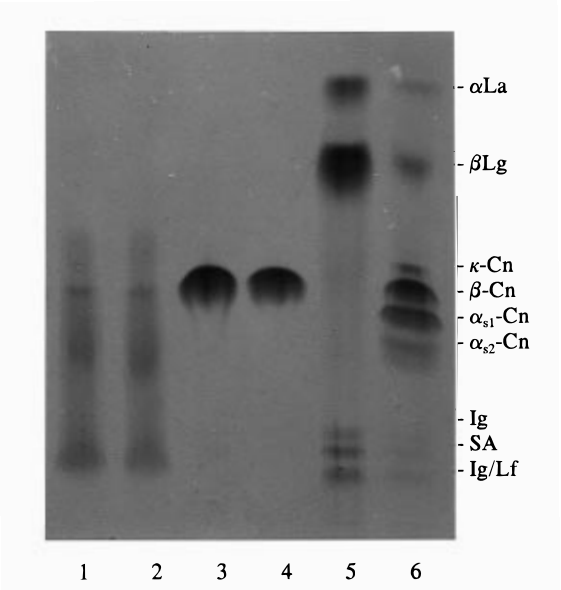

Figure 1. SDS−polyacrylamide gel electrophoresis of thiolated β-casein with and without 2-mercaptoethanol. Tracks: 1 and 2, thiolated β-casein without 2-mercaptoethanol; 3 and 4, with mercaptoethanol; 5, whey protein concentrate; 6, skimmed milk.

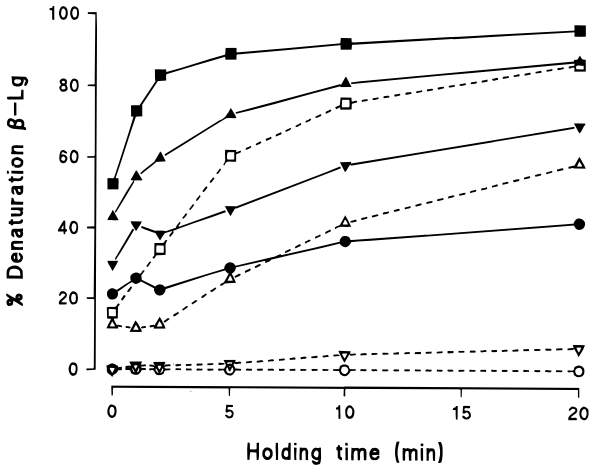

Figure 2. Extent of aggregation of the βLG component of whey protein heated at 60 (○), 70 (▽), 80 (△) and 90 °C (□) in the presence (closed symbols, continuous line) and absence (open symbols, dashed line) of thiolated β-casein.

Heating whey protein at a variety of temperatures in the presence of thiolated β-casein resulted in a significant increase in the rate of aggregation of all of the proteins except for αLA. These results are summarized in Figures 2−5. Some aggregation of whey proteins occurs during the initial 1-min equilibration period, particularly at the higher temperatures. While at all temperatures the rate of aggregation was higher in the presence of the thiolated β-casein, this was more obvious at 60 and 70 °C. In the absence of the thiolated protein, aggregation was slow at 70 °C and almost totally absent at 60 °C. As a control, the experiments were repeated substituting native β-casein for the thiolated derivative. No enhancement in the rate of aggregation was observed. Presumably the high level of free sulfhydryl groups on the thiolated β-casein causes it to interact with the whey proteins, resulting in the formation of intermolecular disulfide bridges leading to aggregation.

In contrast to the other whey proteins, the rate of aggregation of αLA was reduced at 80 and 90 °C in the presence of thiolated β-casein. Heat-induced aggrega-

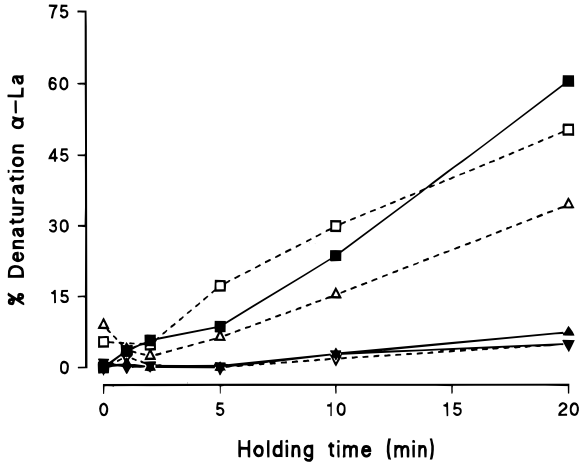

Figure 3. Extent of aggregation of the αLA component of whey protein. Symbols as in Figure 2.

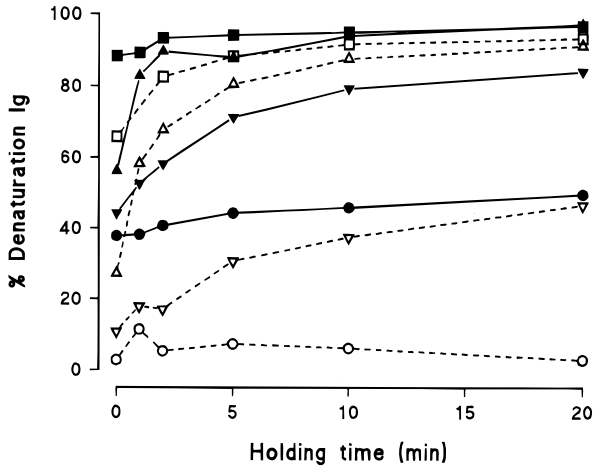

Figure 4. Extent of aggregation of the Ig component of whey protein. Symbols as in Figure 2.

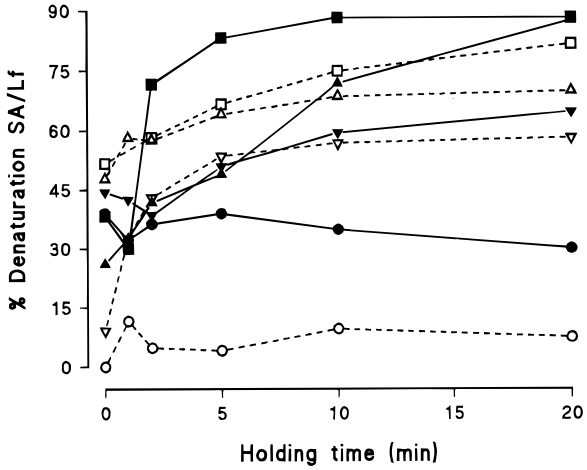

Figure 5. Extent of aggregation of the SA/Lf component of whey protein. Symbols as in Figure 2.

tion of solutions of pure αLA is very slow even in the presence of casein micelles (Calvo *et al.*, 1993). However, the addition of either βLG or SA, which unlike αLA have a single free thiol group, greatly enhances αLA aggregation. In the case of whey protein concentrate, the addition of the thiolated β-casein greatly increases the level of free thiols in the mixture but also increases the rate of removal, via aggregation, of the more reactive whey proteins, effectively "mopping-up" the free thiols

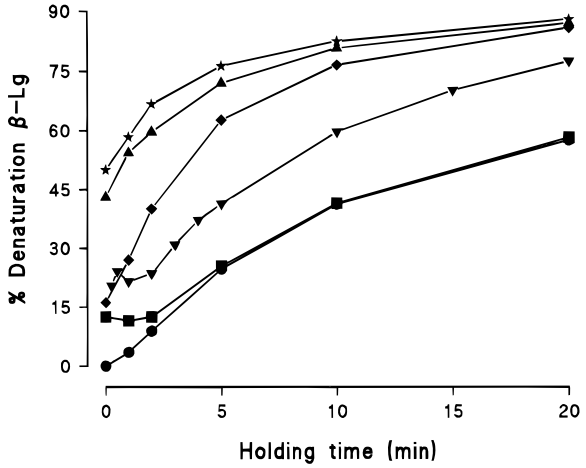

Figure 6. Extent of aggregation of the βLG component of whey at 80 °C in the presence of various levels of thiolated β-casein. Final concentrations of added thiolated β-casein were 0 (■), 0.5 (♦), 1 (▲), and 2 mg mL^{-1} (★). Also shown is the aggregation of βLG in whey protein heated at 80 °C in the presence of 1 mg $mL^{-1}$of native β-casein (●) and in milk heated at this temperature (▼).

and decreasing the rate of aggregation of the less reactive αLA.

The enhanced aggregation resulting from addition of the thiolated β-casein was dependent upon the level at which it was added to the whey protein solution. Aggregation of the βLG component at 80 °C in the presence of 0.5, 1, and 2 mg mL^{-1} thiolated β-casein is shown in Figure 6. Similarly, the rate of aggregation of βLG in heated milk was found to be intermediate between that of solutions of whey protein concentrate at the same level in the presence and absence of the thiolated β-casein. This is probably due to free sulfhydryl groups of the micellar κ-casein enhancing the aggregation of the whey proteins.

CONCLUSIONS

The relatively low concentration of protein in whey means that recovering it by heat-induced aggregation/flocculation is a relatively expensive technique. Incorporation of proteins containing high levels of free thiols would enable the heating temperature/holding time of the process to be significantly reduced, resulting in substantial savings in manufacturing costs. While it is unlikely that the use of chemically thiolated proteins would be permitted in the manufacture of foods for human consumption, it may be possible to use proteins that are naturally rich in cysteine groups to enhance the aggregation.

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