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Denaturation and Aggregation of Serum Proteins and Caseins in Heated Milk

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Skimmed milk was heated in a specially constructed apparatus for times between 1 and 20 min, at set temperatures in the range 75–90 °C. The extent of denaturation and loss of the serum proteins, principally β-lactoglobulin, was estimated by gel filtration chromatography in nondissociating buffers. The amount of aggregated β-lactoglobulin/κ-casein complex was estimated by gel filtration in buffer that dissociated all micellar components apart from those which were linked by disulfide bonds. The results demonstrated that β-lactoglobulin denatured by a pseudo-first-order process and that there was a direct relationship between the formation of aggregate and the amount of serum protein lost. Only disulfide-linked polymers were necessary to explain the products formed during heating in this temperature range.

Extensive studies have demonstrated that when milk is heated at temperatures in excess of about 70 °C, the serum proteins β-lactoglobulin and α-lactalbumin are denatured (Dannenberg and Kessler, 1988b; de Wit and Klarenbeek, 1981; Lyster, 1970). They may then bind to the casein micelles (Smits and van Brouwershaven, 1980; Singh and Fox, 1987) or simply self-aggregate to form polymeric products. In isolation, heated whey proteins form gels, but this does not occur in milk, presumably because the presence of casein micelles disrupts the incipient gel structure and also because specific interactions between the denatured whey protein and the κ-casein of the micelles are possible, although the details of this latter reaction, especially its temperature dependence, have not been completely established.

Several investigations have shown that the denaturation of β-lactoglobulin is complex: agreement on the order of the reaction has not been achieved. Dannenberg and Kessler (1988a,b) suggest that a reaction order of 1.5 is best to fit the rate of denaturation, but other groups favor either a first-order (de Wit and Swinkels, 1980; Luf, 1988) or a second-order reaction (Lyster, 1970; Hillier and Lyster, 1979; Mauji and Kakuda, 1986). Despite these differences, there appears to be general agreement that the reaction gives a nonlinear Arrhenius plot (Dannenberg and Kessler, 1988a,b; Lyster, 1970; Luf, 1988; Manji and Kakuda, 1986). At temperatures below about 90 °C, a linear Arrhenius plot is obtained, yielding an activation energy (E_a) of 260–310 kJ/mol, while above that temperature, a second linear portion is found with a much reduced slope, giving E_a of about 50 kJ/mol.

The denatured β-lactoglobulin polymerizes via disulfide bonds and binds to κ-casein (Smits and van Brouwershaven, 1980; McKenzie et al., 1971). It has been suggested that initially the aggregates may link by hydrophobic interactions rather than by intermolecular disulfide formation (Smits and van Brouwershaven, 1980; Haque and Kinsella, 1988), but it seems clear that the formation of final aggregate is caused by the latter type of covalent bond formation, either between β-lactoglobulin molecules or between β-lactoglobulin and κ-casein. The details of the aggregation process have been studied in model systems containing β-lactoglobulin and κ-casein rather than in milk itself. A number of factors can be identified as important: (i) the rate at which the serum proteins are denatured; (ii) the rates of aggregation of the denatured proteins; (iii) the sizes of the polymer products formed; and (iv) the rate of binding of single molecules or aggregates to the casein micelles. In available studies, little has been done on the relation between denaturation of serum protein and the rate and extent of aggregate formation. The work described in this paper was designed to study this in more detail.

EXPERIMENTAL PROCEDURES

Milk was obtained from the Institute's farm and was skimmed by centrifugation and filtration through a glass fiber filter pad.

Heating of the milk utilized the equipment described previ-

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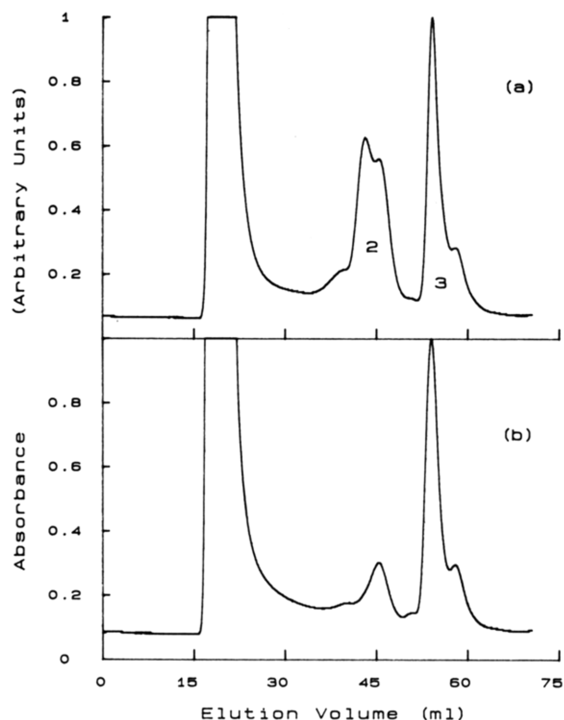

Figure 1. Chromatography of intact milk on Superose 6 Prep Grade column using Ca/imidazole/NaCl buffer. (a) Unheated milk; (b) milk heated at 85 °C for 8 min. Numbered fractions are described in the text.

ously (Dalgleish et al., 1987a), which allowed samples of milk, heated for defined times and temperature, to be prepared. Milk was brought to the required temperatures (75, 80, 85, or 90 °C) and was held for times from 1 to 20 min at these temperatures, 5-mL samples being taken at 1-min intervals. The samples were immediately cooled in ice to room temperature and were retained at that temperature until analyzed.

The samples were analyzed by using gel permeation chromatography on a column (30 × 1.6 cm) of Superose 6 Prep Grade beads connected to a FPLC chromatographic apparatus (Pharmacia, Milton Keynes, U.K.), similarly to the method of Creamer et al. (1978). Two different methods of using the column were employed. In the first, 200 μL of heated milk was applied directly to the column and was eluted at room temperature with a buffer containing 20 mM imidazole, 5 mM $CaCl_2$, and 50 mM NaCl at pH 7.0. This buffer maintained the casein micelles intact during their passage through the column, with minimal dissociation. The elution profile showed a series of three major fractions, one of which decreased when the milk was heated (Figure 1). During the column chromatography, the absorbance at 280 nm of the eluted material was measured at 3-s intervals, and these values were stored on computer for later analysis.

The individual fractions were collected as they eluted from the column, dialyzed exhaustively against distilled water, and lyophilized. They were then analyzed by polyacrylamide gel electrophoresis using sodium dodecyl sulfate (SDS-PAGE) on 20% homogeneous gels, using a Phastsystem (Pharmacia U.K., Ltd.), according to the manufacturer's instructions. Figure 2 shows that the major fraction 1 contained a mixture of casein micelles and serum proteins, the latter having been denatured, and that fraction 2 contained undenatured serum proteins. The column that was used did not fully separate β-lactoglobulin and α-lactalbumin, and the shoulder which eluted ahead of the smaller serum proteins was identified from the electrophoresis as bovine serum albumin. The fraction designated 3 in the profile (Figure 1a) consisted of nonprotein material from the milk, which was completely lost during dialysis.

The second method required dissociation of the casein micelles by the addition of EDTA and urea (Dalgleish et al., 1987b), maintaining disulfide-linked material intact. To samples (1.5 mL) of the heated milk were added 1.5 mL of a buffer

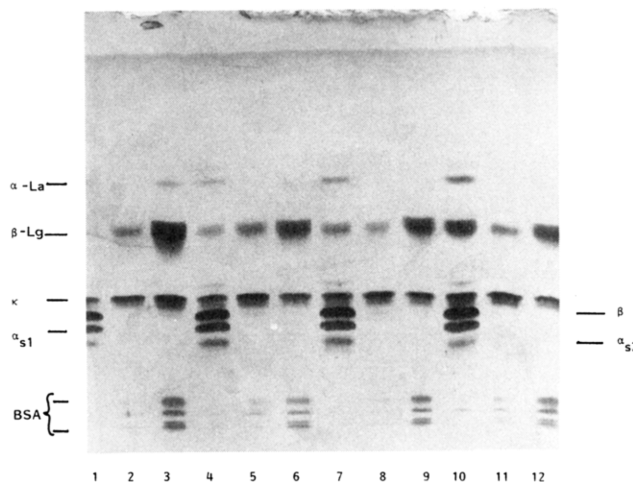

Figure 2. SDS-PAGE analysis of the fractions from the Ca chromatography of milk heated at 85 °C. Tracks 1 and 3, fractions 1 and 2 from milk heated for 3 min; tracks 4 and 6, fractions 1 and 2 from milk heated for 6 min; tracks 7 and 9, after heating for 9 min; tracks 10 and 12, after heating for 12 min. The proteins were identified by running standards (not shown).

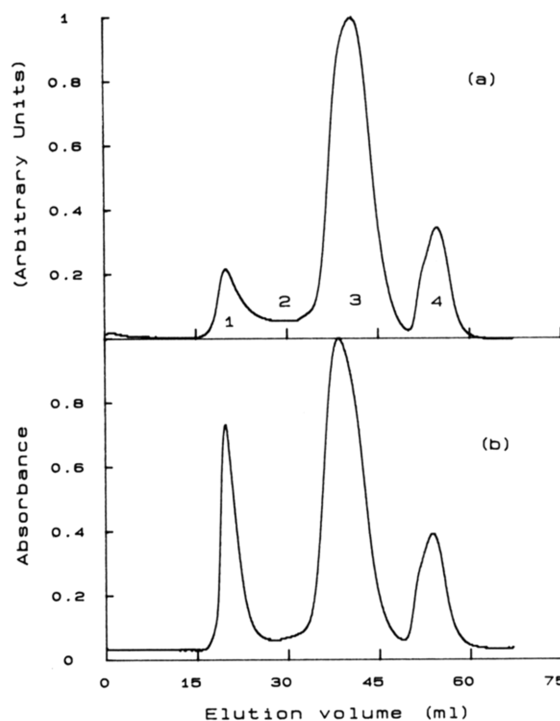

Figure 3. Chromatography of milk treated with urea/EDTA and eluted with urea/EDTA buffer. (a) Unheated milk; (b) milk heated at 85 °C for 8 min. Numbered fractions are described in the text.

containing 6 M urea, 50 mM EDTA, and 20 mM imidazole, pH 7.0, and 200 μL of 250 mM EDTA (at pH 7.0), together with 0.1 g of solid urea. This treatment caused the milk samples to become almost clear, although turbidity of the treated milk samples increased with the time and temperature of heating. Samples (200 μL) of the treated milk were applied to the column, which had previously been equilibrated with the urea/EDTA buffer, and were eluted with this buffer. The absorbance was measured and stored as before. The elution profile (Figure 3) consisted of four fractions, which were shown by SDS-PAGE to contain (1) aggregated material that was disulfide linked (otherwise, the urea/EDTA buffer would cause dissociation) and that contained κ-casein, which was combined with β-lactoglobulin and some α-lactalbumin when the samples were derived from heated milk (Figure 4); (2) a rather poorly defined region between the major components 1 and 3, which contained only polymeric κ-casein and, in some samples, small

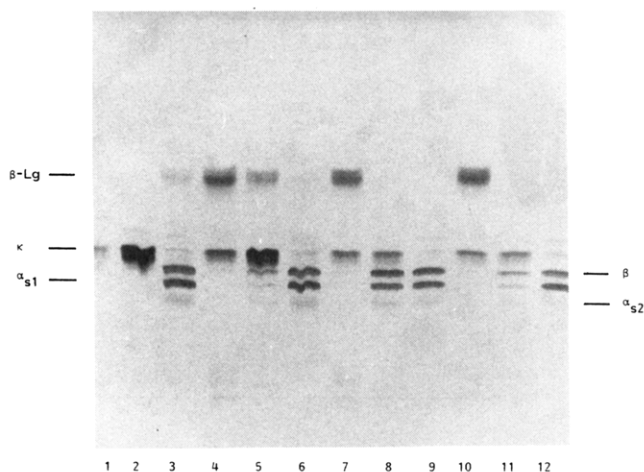

Figure 4. SDS-PAGE analysis of fractions from urea chromatography of milk heated at 85 °C. Tracks 1-3, fractions 1-3 in unheated milk; tracks 4-6, fractions 1-3 in milk heated for 3 min; tracks 7-9, fractions in milk heated for 6 min; tracks 10-12, fractions from milk heated for 9 min. Not visible in the photograph, but detectable in the original gel, are traces of denatured bovine serum albumin in the fraction 1 material.

amounts of denatured β-lactoglobulin on the one hand and α_s- and β-caseins on the other (Figure 4), reflecting the difficulty of separating this component (Yaguchi et al., 1968; Creamer et al., 1978); (3) α_s-, β-, and some less aggregated κ-caseins, with native serum proteins, together with denatured protein material that was soluble in urea and EDTA; (4) nonprotein material as defined in Figure 1, fraction 3. Although fraction 3 in Figure 1 and fraction 4 in Figure 3 contain the same material, the shapes of the peaks in the two chromatograms are different. Since the same column was used in both measurements and only the buffer was changed, the difference does not arise from lack of resolution of the column but rather reflects an effect of urea on the nonprotein material.

Comparison of these two chromatographic analyses allowed a comparison between the denaturation of the protein and the formation of disulfide-linked aggregate to be made. Differences between samples allowed the kinetics to be established.

The elution profiles were analyzed in terms of the contributions defined above by using the integrated areas of the peaks to define the concentrations of the different materials. It was established that fraction 3 in the Ca chromatography and fraction 4 in the urea chromatography, although of different shapes, had the same area and were not affected by any of the heat treatments: they were therefore used as internal standards, and all peak areas quoted are relative to these peaks. Because of the extensive light scattering of the casein micelles, no analysis in terms of area of fraction 1 from Ca chromatography was attempted.

RESULTS

Heating caused differences in the elution profiles of the milk in both chromatographic techniques. The two major changes were that fraction 2 (serum proteins) in the Ca chromatography decreased (Figure 1) and fraction 1 (disulfide-linked aggregate) in the urea chromatography increased (Figure 3). These changes were consistent with the denaturation of serum proteins and the concomitant formation of aggregated material. The behavior of the two individual fractions with time and temperature is shown in Figure 5. It is clear that the disappearance of serum protein (Figure 5a) is almost paralleled by the appearance of aggregated material (Figure 5b) at all of the temperatures studied. Under the conditions used, it appeared that all of the material in fractions 1 and 2 of the urea chromatogram was disulfide linked. Treatment of the material with mercaptoethanol before urea chromatography caused these

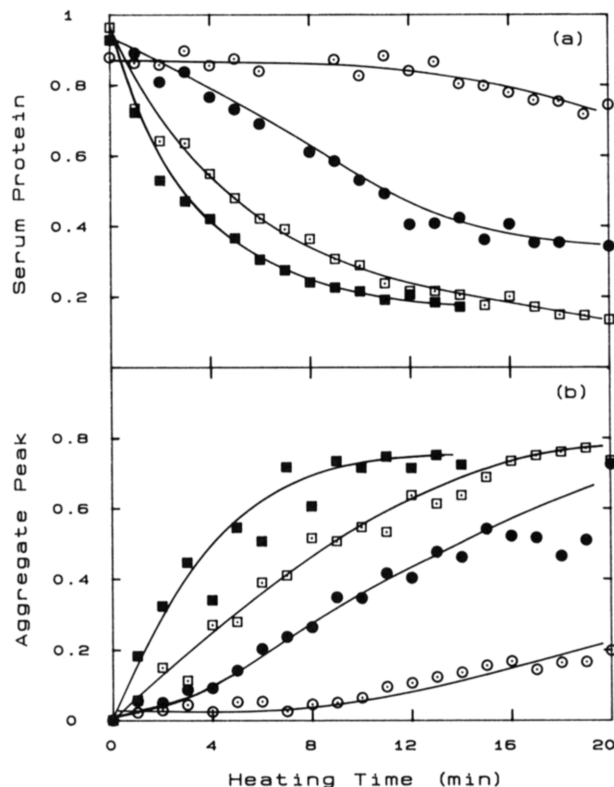

Figure 5. (a) Loss of serum proteins from fraction 2 of the Ca chromatography as functions of heating times at temperatures of 75, 80, 85, and 90 °C. (b) Increase in the size of fraction 1 from urea chromatography at the same times and temperatures. In (a), results are expressed as the area of fraction 2 relative to the area of fraction 3 in the Ca chromatogram, and in (b), results are expressed relative to fraction 4 in the urea chromatogram. ⊙, T = 75 °C; ●, T = 80 °C; ⊡, T = 85 °C; ■, T = 90 °C.

fractions to be lost and incorporated with fraction 3 as the polymeric structures were broken up.

The results at 75 °C confirmed that the denaturation of serum proteins is complex, since both the disappearance of serum protein and the appearance of aggregated material appeared to have an initial slow stage followed by a faster process. There is some evidence for this also at 80 °C, particularly in the growth of the aggregate peak with time, where an initial stage appeared to occur. At higher temperatures, no such stages were apparent: presumably they occurred with sufficient rapidity as to be unobservable on the time scales that we used, i.e., 1-min intervals.

When plotted on a semilogarithmic plot against time, the decreases in serum protein at 85 and 90 °C both showed behavior that could be described by a combination of two straight lines (i.e. simultaneous denaturation of β-lactoglobulin and α-lactalbumin). Subtraction of the first-order plots for lactalbumin denaturation (Lyster, 1970) gave linear semilogarithmic plots, suggesting that the denaturation of β-lactoglobulin under these conditions was a pseudo-first-order process. However, the plot for the results at 75 °C showed a distinct initial stage (whether or not the calculated α-lactalbumin denaturation was subtracted), followed by a more rapid decrease, which could be taken to be first order after the completion of the initial stage. This was also observed for the results at 80 °C, where the initial stage was much shorter and the linear behavior more pronounced (Figure 6). From the slopes of the pseudo-first-order plots, the rate constants for the denaturation reactions could be calculated. The Arrhenius plot of these calculated values showed that, in agreement with other research studies (Dannenberg and

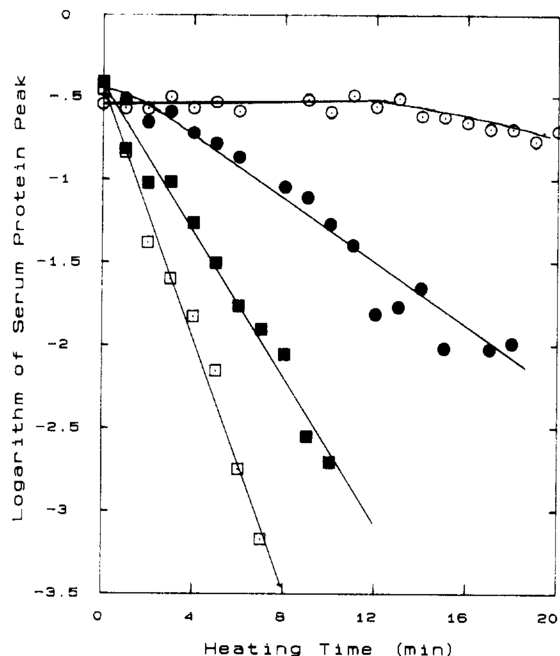

Figure 6. First-order reaction plots for the loss of serum protein from fraction 2 in Ca chromatography, after correction for the loss of α-lactalbumin using the activation energies given by Lyster (1970). ⊙, $T = 75$ °C; ●, $T = 80$ °C; ⊡, $T = 85$ °C; ■, $T = 90$ °C.

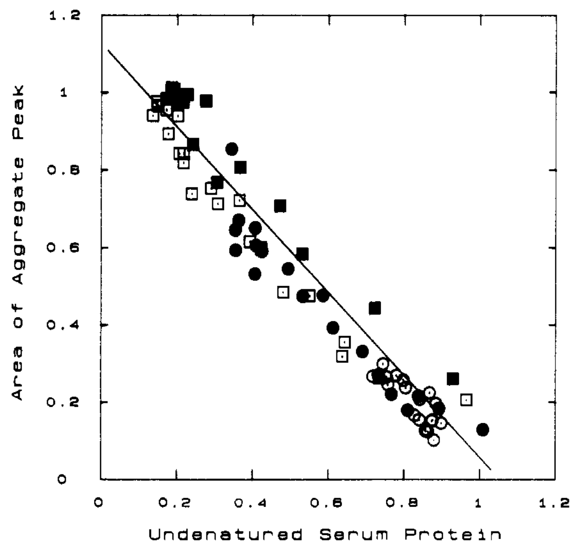

Figure 7. Relation between the extent of aggregate formation (fraction 1 in the urea chromatogram) and the loss of native serum protein (fraction 2 in the Ca chromatogram) in the samples, at all temperatures and heating times. ⊙, $T = 75$ °C; ●, $T = 80$ °C; ⊡, $T = 85$ °C; ■, $T = 90$ °C.

Kessler, 1988a,b; Lyster, 1970; de Wit and Swinkels, 1980; Luf, 1988; Manji and Kakuda, 1986), a linear plot was found for the four temperatures, giving an apparent activation energy of 250 kJ/mol. The temperatures employed in this study were below those at which the lower activation energy denaturation of β-lactoglobulin occurs (Dannenberg and Kessler, 1988; Lyster, 1970).

It was clear that, at all temperatures, there was an almost exact correspondence between the amount of disulfide-linked aggregate formed and the amount of serum protein lost. Figure 7 shows that the slope of the plot between the amount of aggregate formed and the denaturation of serum protein was close to 1 at all temperatures and that, within experimental error, the results at the different temperatures were very similar. There was no suggestion

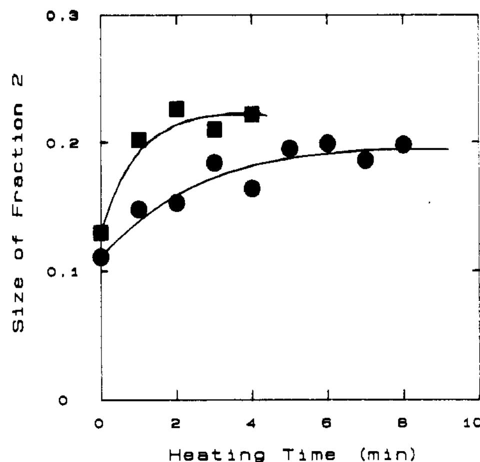

Figure 8. Increase in the area of fraction 2 in the urea chromatograms as a function of heating time at temperatures of 75 (●) and 80 °C (■).

from the chromatographic separations that denatured serum protein appeared at any other point apart from as aggregate in fraction 1 of the urea chromatograms. Thus, there was no indication that small or medium-sized aggregates of denatured serum proteins were being formed. Only relatively large aggregates were formed even when the extent of denaturation was relatively small.

Although fraction 2 of the urea chromatogram is rather poorly defined, it was possible to identify small changes that occurred in this fraction, which could be detected by repeated experiments, all of which showed the same trend. In unheated milk, the fraction was found to contain only κ-casein, and it was separated from the other caseins because it contains polymeric, disulfide-linked, material (Yaguchi et al., 1968). In the earliest stages of the heating, at the lower temperatures (75 °C for 1–10 min, 80 °C for 1–4 min), where there was little denaturation of the serum proteins, this fraction increased in size by a factor of about 2 (Figure 8). SDS–PAGE analysis showed that no other proteins were present in the fraction at this stage, and the reason for the increase in the size of the fraction appeared to be an increased polymerization of κ-casein and, therefore, a transfer of κ-casein from fraction 3 to fraction 2 of the chromatogram. At these times at 75 and 80 °C little or no denaturation of serum protein appeared to occur, as gauged by the gel filtration chromatography profiles. It is therefore possible that the micellar κ-casein was rearranging during the early stages of heating. At higher temperatures, and at longer times at the lower temperatures, the growth of fraction 1 was so rapid and extensive that it obscured any changes in fraction 2, so the phenomenon could not be described at higher temperatures.

DISCUSSION

There has been no general agreement on the order of the denaturation reaction of β-lactoglobulin. De Wit and Swinkels (1980) and Luf (1988) found a first-order reaction. On the other hand, Lyster (1970), Hillier and Lyster (1979), and Manji and Kakuda (1986) found the reaction to be second order. Dannenberg and Kessler (1988a,b) found their results were best described by a reaction of order 1.5. Our results indicate a pseudo-first-order reaction at the higher temperatures but suggest that at temperatures below 85 °C there is an initial stage to the reaction, which precedes any more rapid denaturation. This stage may well be present at the higher temperatures also but be so short as to be undetectable. The results at the lower temperatures suggest that a buildup of some possibly

catalytic units is required before the full denaturation reaction can occur. Despite the differences in proposed mechanism, our estimated activation energy for the pseudo-first-order stages of the denaturation is in good agreement with those of other authors.

In our experiments, the parallel behavior of the loss of serum proteins in the Ca chromatography and the increase in the aggregated material in the urea chromatography strongly suggest that all of the denaturated serum protein is rapidly and efficiently aggregated, either with itself or with the κ-casein, and that no small intermediate aggregates (e.g., dimers, trimers) of free β-lactoglobulin are formed permanently. Such particles would be especially obvious at the lowest temperatures, where denaturation was slow, but aggregate was only found in fraction 1 of the urea chromatography and not in fraction 2.

Neither of the chromatography systems was capable of detecting protein that was denatured but not aggregated. Therefore, it is not possible to rule out any denaturation reactions in which a simple conformational change occurs. However, since the urea chromatography in these experiments detects only disulfide-linked aggregates and the Ca chromatography will show decreases in the serum protein if any type of aggregate is formed, the results do not provide any evidence to support the view that stable aggregates, not linked by disulfide bonds (Smits and van Brouwershaven, 1981; Haque and Kinsella, 1988), are formed at any stage in the heating process. This may reflect a difference between model systems and milk.

The disulfide-linked fractions found in the urea chromatography contain both κ-casein and β-lactoglobulin and, at higher temperatures and longer times, some α-lactalbumin and bovine serum albumin. It has been shown by their position in the chromatogram that the fractions contain material of large molecular weight. This is explained by the binding of individual denatured β-lactoglobulin molecules or aggregates to the already partly aggregated κ-casein to form large complexes (Haque et al., 1987) and is undoubtedly the reason no intermediate sizes of β-lactoglobulin aggregates were found in the experiments. The β-lactoglobulin/κ-casein complexes are clearly limited in size, since they do not become large enough to cause appreciable light scattering, which would be evident as an upward curvature in the plot in Figure 7 as the extent of protein denaturation, and hence the sizes of the protein aggregates, increased. It is therefore clear that the particles formed from denatured serum protein do not form infinite aggregates but ones whose size is limited, presumably by the amount of κ-casein present. The fact that the aggregates are limited in this way at all temperatures (since the graphs in Figure 5b level off when all of the β-lactoglobulin is denatured and all of the temperature effects overlap in Figure 7) can be taken as a confirmation that β-lactoglobulin/κ-casein complexes can be formed at all temperatures from 75 °C upward. Some of these complexes are altered by changing the pH (Banks et al., 1987; Singh et al., 1988), since it is possible to incorporate whey protein from heated milk into cheese curd by altering the pH at which renneting is carried out. The reasons for this, however, have not been established, since they do not appear to involve dissociation of the micelle, and further studies of this phenomenon are required.

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