Thermal Gelation of β-Lactoglobulin AB Purified from Cheddar Whey. 1. Effect of pH on Association As Observed by Dynamic Light Scattering

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Thermal association of β-lactoglobulin AB (β-Lg) purified from Cheddar whey, a major source of commercial whey ingredients, was studied by dynamic light scattering (DLS). The objective was to observe the effect of the process and/or possible micromolecules in this β-Lg source of commercial relevance. Protein solution (8%, w/v) was heated (25−90 °C) at pH 3.5, 7, and 9.0. DLS data were analyzed according to the method of Cumulants for apparent mean diameter and by the Contin method for percentile size distribution. Data indicated gradual change in mean size that started at 35 °C, much below the reported denaturation temperature of 70 °C. Moreover, the percentile distribution of various micrometer- and submicrometer-sized aggregates differed at the different pH values studied, conceivably indicating conformational alteration limiting spatial freedom for intermolecular association. Monomeric/dimeric form (1−9 nm range) was seen only at pH 3.5 at temperatures below 65 °C. The ubiquitous aggregate size range was 100−599 nm (Agg3), and greatest changes in aggregate size and distribution were detected around 70 °C. Micrometer-sized (>1000 nm) aggregates were formed at this temperature and above, concomitant with disappearance of Agg3. On the basis of polydispersity data and rate kinetics dependent on the disappearance of Agg3, the association tendency between 25 and 70 °C was in the order pH 7.0 > pH 3.5 > pH 9.0.

Keywords: *Thermal; association; gelation; whey; β-lactoglobulin*

INTRODUCTION

Whey is a nutritive byproduct of cheese manufacture. About 17% of all bovine milk produced in the United States in 1993 was used for cheese manufacture, leading to a whey disposal/utilization problem of mammoth proportion (National Cheese Institute, 1994). The most popular cheese produced in the United States is Cheddar (Kosikowski, 1978). Only 60% of whey is used for human consumption (Kosaric and Asher, 1985). Whey proteins constitute about 20% of total milk proteins (Brunner, 1977; Morr, 1985); β-lactoglobulin AB (β-Lg) is the major whey protein. β-Lactoglobulin is the primary gelling protein and presumably contributes to the typical thermal behavior of whey protein concentrates (WPC) (Mulvihill and Kinsella, 1987; Ziegler and Foegeding, 1990). Thermal gelation is an important property of proteins. It impacts the functionality and acceptability of foods (Schmidt, 1981). Proteins tend to self-associate in solution (Adams et al., 1978), and this tendency is markedly enhanced by structural alteration brought about by heating (Whitaker, 1977). Tendency and degree of thermal association of proteins could impact their ability to form gels (Haque and Ansari, 1995).

The association properties of β-Lg have been extensively studied in the highly purified form (Townend and Timasheff, 1958; Townend et al., 1960; Pessen et al., 1985; Papiz et al., 1986), but little or no work has been done with β-Lg purified from its most abundant state in cheese whey. Recently, we looked at thermal association of β-Lg that was purified from Cheddar whey followed by fractionation by preparative gel permeation chromatography (GPC) to remove ultraviolet responsive (280 nm) impurities that were bound to the protein (Sharma et al., 1996). Analytical GPC revealed these to be peptides of small molecular weight (Sharma et al., 1996). In the WPC or whey protein isolate (WPI), this protein will be in the presence of such impurities which are likely to impact association (Haque, 1993).

Although factors affecting gel formation are documented, there is a paucity of studies tracing changes in aggregate size (i.e., building blocks) leading to gel formation. Hence, a study into the "building blocks" or state of aggregation or association prior to gel formation can further understanding of the gelation process and could aid in tailoring desired gel characteristics. Previous studies have used techniques such as sedimentation equilibrium, differential scanning calorimetry (DSC), GPC, high-pressure liquid chromatography (HPLC), and/or electrophoresis. These are all invasive/ perturbing techniques that can introduce artifacts by tending to dissociate facile interactions or by excluding larger aggregates (Harwalkar, 1980; Hegg, 1980; Haque et al., 1987; Mulvihill and Kinsella, 1987; Lapanje and Poklar, 1989; Noh and Richardson, 1989; Laligant et al., 1991).

We report here studies on the association/aggregation behavior of β-Lg, purified from fresh Cheddar whey without further GPC fractionation to remove peptides, on stepwise heating from 25 to 90 °C at different pH values (viz., 3.5, 7.0, and 9.0) as monitored by dynamic light scattering (DLS).

MATERIALS AND METHODS

Protein Purification. β-Lactoglobulin was isolated from sweet whey obtained from the Mississippi State University (MSU) dairy plant accroding to the method of Mailliart and

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Figure 1. SDS−PAGE in the presence of 7 M urea of purified β-Lg. Wells M, A, and B were loaded with molecular marker mixture, 10 μg of purified protein, and 20 μg of purified protein, respectively.

Ribadeau-Dumas (1988) as detailed earlier (Haque et al., 1993). β-Lactoglobulin thus obtained was dialyzed against deionized, glass distilled water (× 10000-fold), freeze-dried, and stored in a desiccator at 4 °C until used. It is important to note that unlike our previous experiments (Haque et al., 1993; Sharma et al., 1996), this purified protein sample was not further fractionated by gel permeation or size exclusion chromatography.

Purity was established using vertical slab sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) in the presence of 7 M urea as described earlier (Haque and Mozaffar, 1992; Haque et al., 1993; Sharma et al., 1996). β-Lactoglobulin thus obtained was found to be pure and was a mixture of variants A and B (Figure 1).

Preparation of Buffers. The following buffers representative of the respective pH values were prepared as described by Dawson et al. (1979): (1) imidazole buffer (10 mM), pH 7.0; (2) glycine−HCl buffer (200 mM), pH 3.5; and (3) borate buffer (25 mM), pH 9.0.

Sample Preparation. β-Lactoglobulin was dispersed at its least gelation concentration of 8% (w/v) based on preliminary and related experiments (Lee et al., 1996) in appropriate buffer by vortexing followed by sonication to quickly afford complete solubilization (Sonics and Materials, Model CIA; 600 W, rms; titanium tip diameter 1.2 cm; attenuated to 50% power output). The dispersion was centrifuged (Eppendorf, Model 5415C) at 8000 rpm for 10 min, and the filtrate was filtered to exclude dust by circulating for 10 min using a peristaltic pump (Rainin Co. Inc., Woburn, MA) through a closed sample loop directly into the scatter cell. The closed loop consisted of an inert tubing, a 0.2 μm low protein affinity filter, and a water-jacketed scatter cell (Hellma Model 165). Details were reported earlier (Haque et al., 1993; Sharma et al., 1996).

The sample was filtered into the scatter cell to remove any residual dust particles, and heating was started after the circulating peristaltic pump was stopped. Sample was heated from 25 to 65 °C in steps of 10 °C and thereafter in steps of 5 °C, until 90 °C, and maintained at the respective temperature for 5 min. Water at each of the desired temperatures was circulated through the cell's jacket for exactly 5 min and then turned off. DLS equipment, as described below, was used to obtain autocorrelation functions at each temperature. Data were (described later) analyzed by Cumulant and Contin analysis methods. Experiments were carried out in triplicate at each pH and the results averaged (Haque et al., 1993; Sharma et al., 1996).

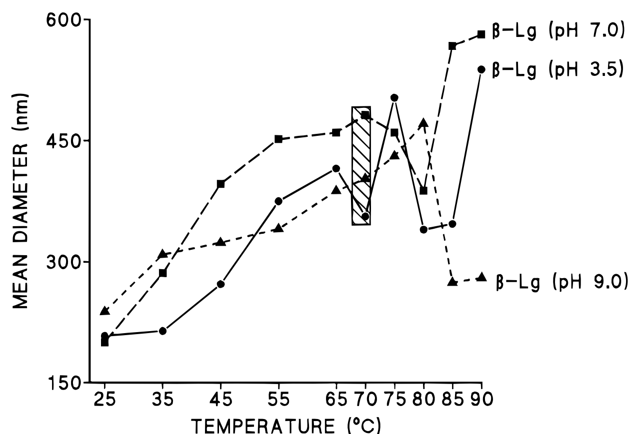

Figure 2. Association tendency of β-Lg at pH 7.0 as seen by DLS. The sample was heated from 25 to 90 °C. Apparent mean diameters from Cumulant analysis are plotted against temperature. Hatched box approximately reflects reported temperature of denaturation. Buffers used were imidazole, 10 mM, pH 7; glycine−HCl, 0.2 M, pH 3.5; and borate, 0.025 M, pH 9.0.

Instrumentation. DLS assembly consisted of a laser source (Jodon He-Ne laser, Model HN-50) with emission wavelength of 632.8 nm. The laser beam was focused on the scatter cell mounted on a goniometer by means of a mirror and system of lenses. Light scattered at an angle of 90° was collected and focused by means of a 10 cm lens onto a photomultiplier tube (Model RCA POF550) through a fiber optic cable. Normalized autocorrelation function was measured by using a digital correlator (BIC 2030AT Brookhaven Instruments Corp., Ronkonkoma, NY) with 72 real time channels and 4 delay channels. Details of the method are given elsewhere (Haque et al., 1993; Sharma et al., 1996).

The correlation data were analyzed by Cumulant analysis for apparent mean diameter (size average) of β-Lg (Chu, 1991). Contin was used to analyze the data for percentile distribution of particle/aggregate sizes (Chu, 1991; Harding et al., 1992). Polydispersity, which is a measure of aggregation, was determined from Cumulant analysis (Harding et al., 1992).

RESULTS

Association at Neutral pH (7.0). At 25 °C, native β-Lg had an apparent mean diameter of 200 nm (Figure 2). On heating, the mean diameter showed a steady increase to 460 nm at 55 °C. It further increased to 475 nm at the reported denaturation temperature of 70 °C (deWit and Swinkels, 1980). At 75 °C, the mean diameter decreased and dipped to 375 nm at 80 °C. It sharply increased thereafter to 560 nm at 85 °C and to 580 nm at 90 °C.

The decrease in mean aggregate diameter at 75−80 °C was conceivably due to sudden unfolding of the molecules resulting from rapid denaturation, which resulted in partial dissociation. Mean diameter at 90 °C did not change even when the sample was subjected to a total heating time of 7 min.

To determine percentile distribution of apparent aggregate sizes, the particle/aggregate sizes as calculated by Contin were split arbitrarily into five aggregate sizes: Agg1 (1−9 nm) (monomer/dimer) < Agg2 (10−99 nm) < Agg3 (100−599 nm) < Agg4 (600−999 nm) < Agg5 (≥1000 nm) (micrometer size). This was done to simplify the results so that trends could be detected.

Monomer/dimer (Agg1) was conspicuously absent even at 25 °C (Figure 3). The larger aggregate 2 was absent at lower temperatures but appeared above 70 °C. Aggregate 3 was ubiquitous at all temperatures. At 25 °C it accounted for ∼95% of the size distribution.

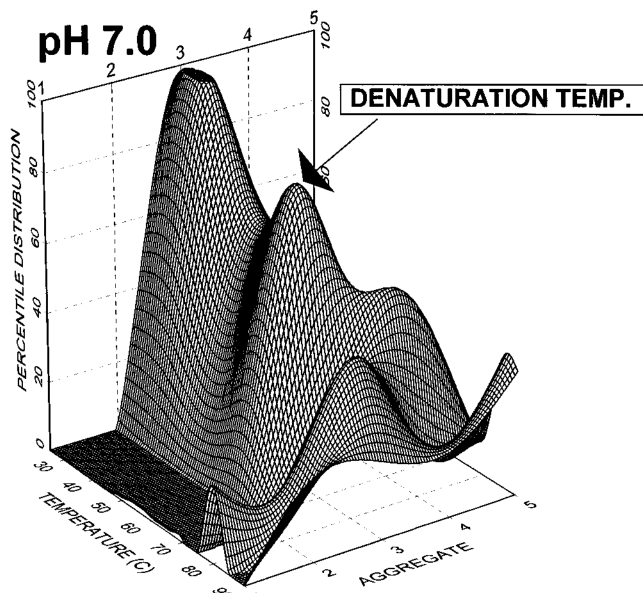

Figure 3. Three-dimensional plot illustrating the effect of heating of β-Lg on percentile distribution of aggregate size at pH 7.0. Aggregates 1, 2, 3, 4, and 5 represent size ranges 1−9, 10−99, 100−599, 600−999, and ≥1000 nm, respectively. These aggregates have been repectively termed Agg1, Agg2, Agg3, Agg4, and Agg5 in the text.

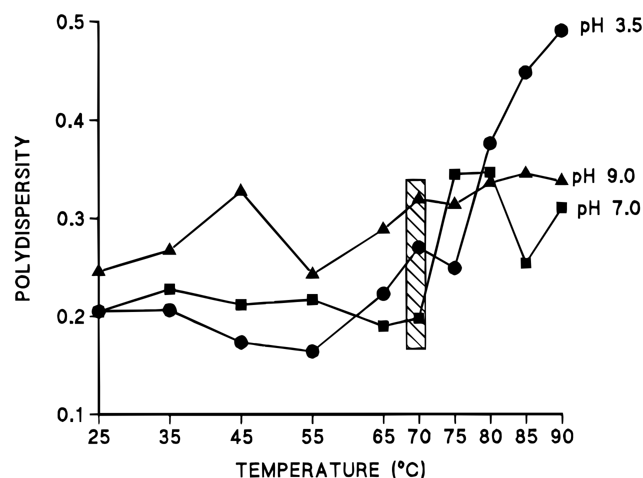

Figure 4. Polydispersity obtained from Cumulant analysis plotted against temperature. Conditions were as in Figure 3. Hatched box approximately reflects reported temperature of denaturation.

On heating to 35 °C, higher aggregates dissociated and the mean diameter of the protein was almost entirely in the Agg3 range (Figure 3). On further heating, aggregates in this size range decreased slowly until 70 °C, when they decreased rapidly with concomitant increases in larger aggregates. Aggregates 4 and 5 appeared above 45 °C, reflecting increased association at increased temperature; aggregate 5 predominated at 90 °C (Figure 3).

Polydispersity, which reflects the degree of association (Haque et al., 1993), was almost constant until 55 °C and decreased thereafter until the denaturation temperature of 70 °C (deWit and Swinkels, 1980) (Figure 4). It increased sharply at 75 °C, dropped at 85 °C, and increased sharply again at 90 °C. Polydispersity thus reflected both number of distribution modes and width of the distribution.

Association at Acidic pH (3.5). At acidic pH the mean size at 25 °C was also 200 nm (Figure 2). It increased with temperature to 420 nm at 65 °C and dropped to 360 nm at 70 °C. It increased to 500 nm at 75 °C, dropped sharply at 80 °C, and increased to 550 nm at the apparent gelation temperature of 90 °C as observed by the formation of gels within the cuvette.

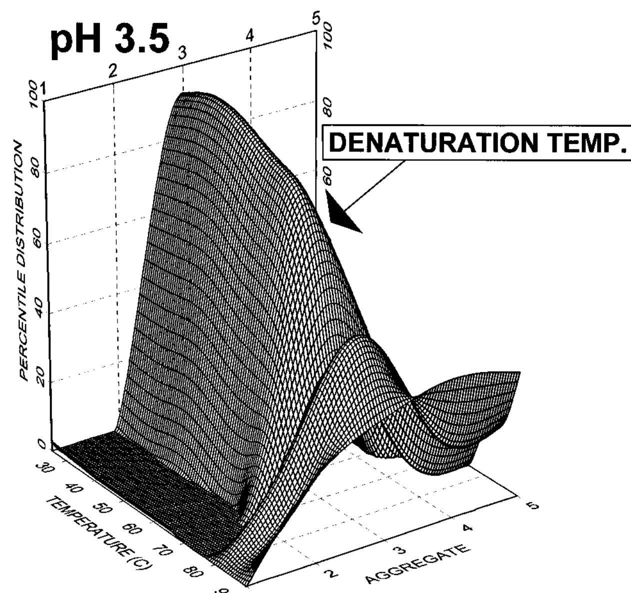

Figure 5. Three-dimensional plot illustrating the effect of heating of β-Lg on percentile distribution of aggregate size at pH 3.5. Aggregate sizes are as described in Figure 3.

The predominant aggregate at 25 °C was Agg3, and on heating it decreased steadily to 35% at 90 °C. As at pH 7, the amount of Agg4 and Agg5 increased above 45 °C. Here, too, Agg2 increased above 65 °C to significant levels (22%) at 90 °C (Figure 5).

Polydispersity values increased steadily up to 70 °C, dropped at 75 °C, and increased thereafter (Figure 4). The drop at 75 °C perhaps reflected a sudden drop in association due to sudden unfolding of β-Lg.

Association at Alkaline pH (9.0). At the alkaline pH, the starting mean diameter at 25 °C was slightly higher (234 nm), possibly indicating a change in the initial conformation (Figure 2). Upon heating, this increased until it reached a maximum of 460 nm at 80 °C. On further heating mean diameter decreased to 274 nm at 85 °C and increased slightly to 280 nm at 90 °C. Data indicated that the mean aggregate size at this temperature was much smaller compared to that at neutral and acidic pH.

The predominant aggregate size at 25 °C, as in the other cases, was Agg3 (Figure 6). Small amounts of Agg2 and Agg4 were also seen. Again, as at pH 3.5 and 7, the amount of Agg3 decreased with heating, being sharpest at the reported denaturation temperature of 70 °C (deWit and Swinkels, 1980). The largest aggregate, Agg5, was seen at high amounts at 70 °C, and its relative proportion was much higher (31.9%) than it was at pH 3.5 (2%) and pH 7 (2%).

The polydispersity value at 25 °C was highest (0.25) with very little further increase on heating to 90 °C (Figure 4) compared to that at acidic and neutral pH, indicating conformational change. This reflected a reduced association tendency at alkaline pH. If gelation is influenced by the association tendency, then data indicated that gelation could not occur at pH 9.0. Formation of gels and the viscoelasticity aspects have been studied and will be published later in this series.

Kinetics of Association in β-Lactoglobulin. Since Agg3 was the dominant species with little or no Agg1

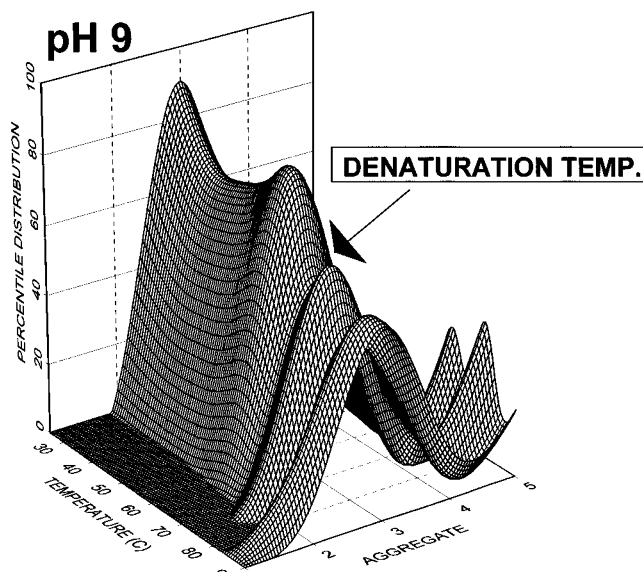

Figure 6. Three-dimensional plot illustrating the effect of heating of β-Lg AB on percentile distribution of aggregate size at pH 9.0. Aggregate sizes are as described in Figure 3.

and Agg2 at the starting temperature at all pH conditions studied (Figures 3, 5, and 6), its disappearance was used to calculate the reaction kinetics assuming first order. The overall association rate prior to denaturation (i.e., from 25 to 70 °C) was calculated. The rates were 3.75×10^{-4} s^{-1} at pH 7.0, 2.45×10^{-4} s^{-1} at pH 3.5, and 2.65×10^{-4} s^{-1} at pH 9.0. These data indicate that association tendency was higher at pH 7.0 than at either pH 3.5 or 9.0. This was in agreement with the polydispersity data (Figure 3).

DISCUSSION

Data showed that at 25 °C, β-Lg had a mean size of ~200 nm regardless of pH. This reflected the maximum cutoff (pore size) of the filtration membrane used to filter the protein dispersion prior to DLS experiments to exclude dust particles. The smallest aggregate (monomer/dimer), Agg1, was absent in all of the samples except at acidic pH between 25 and 65 °C. It is interesting that the same protein, when further fractionated by GPC to remove peptide impurities, gave a molecular masses of 18 300 and, up to 35 °C, was predominantly in the monomeric/dimeric state (<10 nm) (Sharma et al., 1996). Data therefore indicate large differences in association tendency. This is conceivably due to the presence of amphipathic peptides that markedly impact β-Lg functionality (Haque, 1993; Haque and Bohoua, 1997a,b).

The ubiquitous aggregate, Agg3, was seen under all conditions and appeared to be an important size range in the formation of larger (micrometer) aggregation. Its content decreased sharply at temperatures above the denaturation temperature (≥75 °C), leading to the formation of larger aggregates (Agg4 and Agg5; Figures 2, 3, 5, and 6).

The stability of β-Lg in solution at 25 °C is contributed to by entropic forces as a result of ordering of water molecules (first hydration shell) around hydrophobic patches on its surface (Jaenicke, 1987; Wodak et al., 1987; Privalov and Gill, 1988; Dill, 1990; Privalov et al., 1990). It is known that in some proteins even on folding, some hydrophobic residues remain exposed on the surface (Mozhaev et al., 1988). Wishnia et al. (1966) reported the presence of a hydrophobic patch in β-Lg thought to be the binding site for a hydrophobic ligand.

With increase in temperature, there is less ordering of water around the hydrophobic patches. A consequent decrease in the absolute value of the entropy of hydration at higher temperatures is thought to lead to a complex interplay between enthalpic and entropic contributions in hydrophobic hydration. The strength of hydrophobic interactions increases with temperature up to 60–70 °C (Brandts, 1967). Hydrophobic interactions, the main driving force for protein folding, are made up of chain free energy as well as hydration free energy (Oobatake and Ooi, 1993). The increase in hydrophobic effect with increased temperature is attributed to reduced hydrogen bonding and a consequent increase in the packing density of water (Privalov and Gill, 1988; Privalov et al., 1990).

The increase in hydrophobic interactions with temperature favors an increase in free energy associated with decrease in accessible free surface area (or surface area that is accessible to water) when proteins associate (Wodak et al., 1987). This is perhaps the driving force resulting in progressive association, which was seen when β-Lg was heated up to the denaturation temperature of 70 °C under all pH conditions studied as reflected in the data obtained. Data showed a progressive decrease in Agg3 with a concomitant increase in submicrometer- and micrometer-sized Agg4 and Agg5.

There was a marked difference in the extent of association at different pH conditions studied. Association tendency at pH 9.0 was least, perhaps due to a reversible transition in molecular conformation that takes place around pH 7.5. Following this transition, increased thiol activity has been observed (Swaisgood, 1986). Such activity could conceivably make the molecule more rigid and unable to partake in spatial arrangement to accomplish optimum intermolecular contact to minimize the hydrocarbon/aqueous interface. A decrease in hydrocarbon/aqueous interface is a driving force in hydrophobic interaction (Tanford, 1980) that is enhanced by heating (Haque et al., 1987; Haque and Kinsella, 1988).

Around 70 °C, β-Lg unfolds and undergoes irreversible denaturation (deWit and Swinkels, 1980). Unfolding is partial because the gain in conformational freedom of chain (or chain entropy) is more advantageous than the gain of interactions between nonpolar contacts (Jaenicke, 1987). The presence/persistence of Agg3, at temperatures above 70 °C, may be the result of partial unfolding.

In their calorimetric studies on thermal denaturation of β-Lg, DeWit and Klarenbeek (1981) observed a second enthalpic peak at 140 °C in addition to the regular denaturation peak. This was attributed to a partial unfolding at 70 °C. Such a partially unfolded state, also seen in α-lactalbumin, is termed "compact intermediate" (Jaenicke, 1987; Creighton, 1990). It is believed to arise from a compensation between gain in entropy by the native structure as a result of released side chains resulting in increased molecular volume and a loss in van der Waal and hydrophobic interactions due to loosening in structure (Rashin, 1993). This may explain the persistence of aggregate 3 without further enlargement as temperature increased to 90 °C.

Aggregation that is consequent to denaturation proceeds through hydrophobic interactions and/or SH/SS interchange reactions (Laligant et al., 1991). Site-directed mutagenesis studies in β-Lg have substantiated

the role of SH/SS interchange reactions in thermal aggregation, which is a time-dependent process (Lee et al., 1992). Using fluorescein mercuric acetate as a probe, Haque and Kinsella (1988) observed noticeable SH/S−S activity in β-Lg only after about 30 min of continuous exposure to denaturation temperature. In the present investigation, the time of exposure to (post)-denaturation temperature was <20 min. It is therefore likely that large aggregates may not have formed through S−S stabilized bonds. This should particularly be true at acidic and neutral pH values when the reactive thiol groups are not deprotonated (Lehninger, 1971).

At 70 °C the partial unfolding of β-Lg on denaturation may have resulted in the partial dissociation of oligomers into smaller ones (in the 10−99 nm range). At pH 9.0 reversible transition accompanied by a change in molecular conformation may have resulted in aggregates with smaller sizes.

In conclusion, under quiescent conditions, purified native β-Lg that had not been fractionated by size exclusion chromatography predominantly existed as large micellar clusters with size range of 100−599 nm (Agg3) regardless of pH. Heating increased aggregation, and this tendency was higher at pH 7.0, resulting in larger aggregates than at either pH 3.5 or pH 9.0. The pH also caused changes in initial distribution of different aggregates at low temperatures (25−65 °C), much below the reported denaturation temperature of about 70 °C (deWit and Swinkels, 1980). Further work related to the effect of chelating agents was looked into (Sharma and Haque, 1997).

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Received for review October 15, 1996. Revised manuscript received May 19, 1997. Accepted May 21, 1997.⊗ Approved for publication as Journal Article J-8984 of the Mississippi Agriculture and Forestry Experment Station, Mississippi State University. Research was funded as part of the Mississippi Agricultural and Forestry Experiment Station, Project 3144.

JF960794U

⊗ Abstract published in *Advance ACS Abstracts,* July 1, 1997.