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Influence of Cation Sequestering and pH on Quiescent Thermal Association of β-Lactoglobulin AB from Fresh Cheddar Whey: An Insight into Gelation Mechanism

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Freshly fractionated β-lactoglobulin AB (β-Lg) from Cheddar whey was dispersed at pH 3.5, 7, and 9 buffers containing 20 mM EDTA, and circulated at 25°C through a closed loop containing a 200 nm pore size membrane, to remove traces of dust and large aggregates, and an water jacketed cuvette placed within a Dynamic Light Scattering (DLS) device for real-time data acquisition. Filtered samples were step-wise heated from 25°C to 90°C with continuous data acquisition to study dynamic changes in mean aggregate diameter (MAD). Data were analyzed by cumulant method for apparent MAD and CONTIN for size distribution. Initial MAD (IMAD) of about 200 nm, reflecting the pore size of the filter used, was observed for all experiments prior to heating. Mid-range aggregate, Agg3 (100–599 nm), was ubiquitous in all distributions and Agg1 (monomer-dimer) was only seen at pH 7 and 25°C. Increased temperatures gave rise to larger aggregates (>micron) (Agg4 and 5) with concomitant disappearance of Agg3. The greatest increase in MAD was at pH 3.5 and the lowest was at pH 9. Pre-gelation (<70°C) kinetic rates were 4.29, 1.53, and -0.11×10^{-4} s^{-1} for pH 3.5, 7.0, and 9.0, respectively, giving MADs that were 3.5, 2.2 and 0.8 fold compared to IMAD. Above 70°C (apparent gelation range), relative aggregate enlargement was greatest at pH 3.5 being 55, 21, and 4.2 fold of IMAD, respectively, for pH 3.5, 7, and 9 at 90°C. An apparent gel formed at pH 3.5 and a turbid gel/coagulum formed at pH 7 and none at pH 9. It is conceivable that increased association at pre-gelation temperatures is required for gelation. A mechanism has been postulated.

Keywords: gelation, β-lactoglobulin, chelation, whey, Cheddar, mechanism

β-Lactoglobulin AB (β-Lg), the major protein in whey (Brunner, 1977), is considered to be the primary functional protein and presumed to contribute to the typical physicochemical behavior of whey protein concentrated (WPC) (Morr, 1985). Whey protein concentrates that are dehydrated by various processes are becoming increasingly popular as functional and nutritional ingredients in value-added foods and, more recently, in health foods and drinks. It was recently observed that processing of whey affects the functionality of WPCs (Ji & Haque, 2002) and the quality of food products in which they are used (Haque & Ji, 2002). This is conceivably due to the thermally induced structural alteration of β-Lg which is markedly thermolabile >65°C. Recent data indicate that structural alterations start at temperatures as low as 45°C (Haque, 1995).

We reported on the effect of heat and pH on the association properties of β-Lg fractionated from fresh Cheddar whey without chelation of cations (Haque & Sharma, 1997). In that study and in the present one, exhaustive purification was not carried out to remove trace peptides so as to examine β-Lg in its impurity induced conformational state in which it conceivably exists and functions in food systems. Our data (Haque & Sharma, 1997) indicated a greater degree of association at all stages of heating compared to yet another study using chromatographically pure β-Lg (Sharma *et al.*, 1996). The starting mean aggregate diameter (MAD) prior to heating (25°C) was a reflection of the pore size of the membrane (200 nm) used to remove traces of dust from the sample (Haque & Sharma, 1997). It was also noted that gradual stepwise heating changed the MAD starting at 35°C, much below the reported denaturation temperature of purified β-Lg (carried out at a much lower concentrations) of about 70°C (deWit & Klarenbeek, 1981). Furthermore, percentile distribution of various micron (>1000 nm) and submicron-sized (<1000 nm) aggregates differed at the different pH conditions studied, conceivably reflecting conformational alteration that facilitate or limit spatial freedom for intermolecular association. This previous study in this series showed that the smallest level of association, the monomeric/dimeric form (1–9 nm range) was only seen at temperatures below 65°C at pH 3.5, the ubiquitous aggregate size range was 100–599 nm (Agg3), and greatest changes in aggregate size and distribution were detected around 70°C (Haque & Sharma, 1997). Large, micron-sized aggregates were formed at this temperature and above, concomitant with disappearance of Agg3. Based on rate kinetics dependent on the disappearance of Agg3, association tendency at the pre-gelling range of 25–70°C were 2.45, 3.75, and 2.65×10^{-4} s^{-1} for pH 3.5, 7, and 9, respectively (Haque & Sharma, 1997).

Calcium affects gelation properties of WPC (Mei *et al.*, 1996; Varunsatian *et al.*, 1983) and β-Lg (Mulvihill & Kinsella, 1988). Exact nature/role of calcium/β-lactoglobulin interaction is not clear though significant interaction between calcium and free sulfhydryl groups have been reported in whey proteins (Schmidt *et al.*, 1984). Haque and Kinsella (1988) used a calcium ionophore and observed rapid exposure of Ca^{2+} binding sites in β-Lg

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around 70°C; limited exposure started around 50°C. β-Lactoglobulin has one free sulfhydryl group at position 121, which lies buried at the sheet-helix interface (Papiz *et al.*, 1986). It is speculated that this sulfhydryl group might interact with cations, *e.g.*, calcium.

It is conceivable that in the absence of calcium these binding sites could participate in inter- and intra-molecular electrostatic interactions. Though work has been done related to the thermal association of β-Lg, not much is available with sequestered β-Lg where the influence of calcium is eliminated. Sequestering with EDTA removes divalent cations, principally calcium. In continuation of our study on initial molecular associations of step-wise heated β-Lg (25°C to 90°C) (Haque & Sharma, 1997), we report here the effect of heat on the cation sequestered protein under the same conditions of pH and temperature.

Materials and Methods

Fresh Cheddar whey manufactured from mixed herd milk (16.7% Jersey and 83.3% Holstein, Mississippi State University South Dairy Farm, Starkville, MS) was obtained from the Mississippi State University dairy plant, flash frozen using liquid nitrogen, and lyophilized to obtain non-heat treated Cheddar whey powder. Method of Cheddar manufacture and the starter cultures used are reported elsewhere (Ji & Haque, 2002). Bio-Rad protein assay dye kit was obtained from Bio-Rad Chem., Richmond, CA. Urea, imidazole, ethylene diamine-*N,N*-tetraacetic acid (EDTA), 2-mercaptoethanol (2-ME) were obtained from Sigma Chem. Co., St. Louis, MO. All other reagents were analytical grade.

Preparation of DLS grade water Deionized water was double-distilled using a glass distillation unit and filtered twice through a 0.2 nm filter (Type HA, Millipore) under vacuum suction. All protein solutions, buffer preparation and final instrument and cell cleaning/rinsings were done with this freshly prepared DLS grade water.

Protein purification β-Lactoglobulin AB (β-Lg) was fractionated from the whey powder according to a slight modification of the method of Mailliart and Ribadeau-Dumas (1988) as described earlier (Haque & Sharma, 1997). The fraction thus prepared was apparently pure by alkaline-urea-polyacrylamide gel electrophoresis (Haque & Sharma, 1997) and was not subjected to gel permeation or size exclusion chromatography for further purification as was done in an earlier experiment to remove tenaciously bound trace impurities that absorb at 280 nm (Sharma *et al.*, 1996). In the present experiment, these tenaciously bound proteineous materials were not excluded to preserve the native associated state of the β-Lg fraction that is in synergy with trace impurities as it exists in food systems (Haque & Sharma, 1997). The β-Lg fraction was exhaustively dialyzed (×10,000 fold) against DLS grade water, flash-frozen with liquid nitrogen, lyophilized, and stored in desiccators at 4°C until used. Purity was established using vertical slab polyacrylamide gel electrophoresis in the presence of 7 M urea and SDS as described earlier (Haque & Sharma, 1997).

Preparation of buffers The following buffers containing 20 mM EDTA were prepared as described by Dawson *et al.*, (1979) using DLS grade water; 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 in appropriate buffers by vortexing followed by sonication (Sonics and Materials, Model CIA; 600 W, RMS; titanium tip diameter 1.2 cm; attenuated to 50% power output) (22°C) at its least gelation concentration of 8% (w/v) based on preliminary and related experiments (Lee *et al.*, 1998). To remove dust that would interfere with dynamic light scattering (DLS), dispersions were centrifuged (Eppendorf, Model 5415C) at 8000 rpm for 10 min and the filtrate was filtered by circulating for 10 min using a peristaltic pump (RAININ Co. Inc., Woburn, MA) through a closed sample loop that consisted of inert tubing, low protein affinity filter with mean pore size of 200 nm and a water-jacketed scatter cell (Hellma Model #165) that was placed within the DLS device. Details were reported earlier (Haque *et al.*, 1993; Haque & Sharma, 1997; Sharma *et al.*, 1996). The process of filtration not only removed traces of dust from the various buffers but also resulted in a mean Initial Mean Aggregate Diameter (IMAD) reflecting the filter pore size of for all experiments as shown earlier (Haque & Sharma, 1997).

Once filtered, the circulating peristaltic pump was stopped and the cell 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. The heating rate was 60°C/min. Water at the desired temperature was circulated through the cell's jacket for exactly 5 min and then turned off to immediately obtain DLS data. The DLS equipment, as described below, was used to gather auto-correlation functions at each temperature. Experiments were carried out in triplicate at each pH and the results averaged as described earlier (Haque *et al*., 1993; Sharma *et al*., 1996). Data were analyzed by cumulant and CONTIN analysis methods as described earlier (Sharma *et al*., 1996).

Instrumentation The DLS assembly, as described earlier (Haque *et al*., 1993; Haque & Sharma, 1997), 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 photo multiplier tube (Model RCA POF550) through a fiber optic cable. Normalized auto-correlation function was measured by using a digital correlator (BIC 2030AT Brookhaven Instruments Corp., Ronkonkoma, NY) with 72 real time channels and 4 delay channels.

The correlation data was 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 were based on methods of Chu (1991) and Harding *et al.* (1992) as described earlier (Haque *et al.* 1993).

Polydispersity, which is a measure of the degree of aggregation, was determined from cumulant analysis and is given by K_2/K_1^2 (second moment)/(first moment)^2 (Harding *et al*., 1992).

Classification of aggregates The calculated aggregate sizes (by CONTIN) were split into five aggregate size ranges: Aggregate 1 (Agg1) (1–9 nm) (monomer/dimer) < Aggregate 2 (Agg2) (10–99 nm) < Aggregate 3 (Agg3) (100–599 nm) < Aggregate 4 (Agg4) (600–999 nm) < Aggregate 5 (Agg5) (1000 nm) (micron size). This was done to simplify the results so that trends could be detected and quantified.

Results

Association and aggregation effects at acidic pH (3.5) The IMAD of β-Lg at 25°C was 212 nm and this increased with temperature to a MAD of 626 nm at 70°C (Fig. 1). At the denaturation temperature around 70°C (deWit & Klarenbeek, 1981), MAD increased to micron size (~1240 nm), increased further to 2400 nm at 80°C, and at 85°C, the sample gelled within the cuvette to give an opaque gel within one minute of hot water circulation. Auto-correlation function gave a MAD at 80°C of 2421 nm.

Polydispersity, which is a reflection of the degree of association, showed little change until 55°C above which it increased gradually, and above 75°C the increase became sharper (Fig. 1, Inset). Data above 80°C at pH 3.5 is not shown because of excessive aggregation and formation of a gel in the cuvette.

The ubiquitous aggregate range, Agg3, (100–599 nm) predominated at 25°C (Fig. 2). Smaller-sized aggregates, *i.e.*, Agg1 and Agg2, were negligible to non-existent under this condition. There was a sharp decrease in Agg3 above 45°C with concomitant increase in Agg4 indicating increased association tendency. At 65°C, the trend became stronger and there was a sharp increase in the amount of the largest aggregate distribution range (micron-sized aggregates) (Agg5). At 70°C, the shift to larger size was clearly shown by the fact that Agg4 and Agg5 made up 60% of the aggregate distribution. Above the denaturation temperature at 75°C, percentage of Agg5 alone had already constituted ~70% of the distribution. With further increase in temperature, Agg5 became the dominant aggregate range.

Association and aggregation effects at neutral pH (7.0) The MAD increased gradually to ~300 nm at 55°C and above the denaturation temperature of 70°C, MAD actually decreased back to the IMAD range (~200 nm) (Fig. 1). On further heating, it increased to 628 nm at 80°C after which point a soft turbid gel (coagulum) became visible. However, increase in diameter was less compared than at pH 3.5 at both pre-gelation (<70°C) and gelation (>70°C) stage.

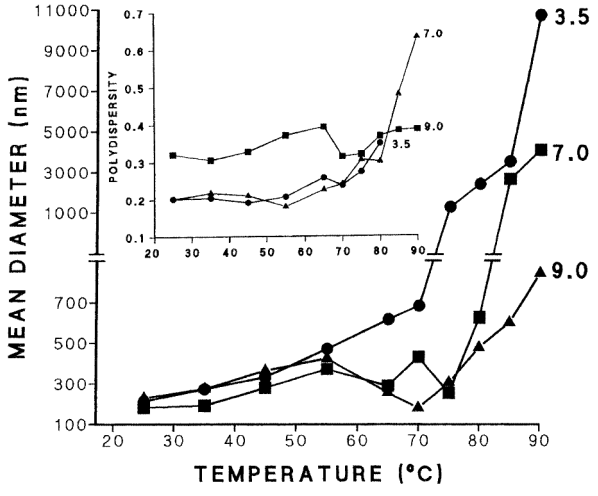

Fig. 1. Heat-induced change in diameter of β-lactoglobulin AB aggregates in the presence of EDTA at pH 3.5, 7, and 9. Data were obtained by cumulant analysis of Dynamic Light Scattering (DLS) data obtained during thermal treatment. The y-axis represents Mean Aggregate Diameter (MAD) on step-wise heating between 25–90°C. The inset shows polydispersity (y-axis) under the same conditions of pH and temperature (x-axis). Data were derived by cumulant analysis of the same DLS data.

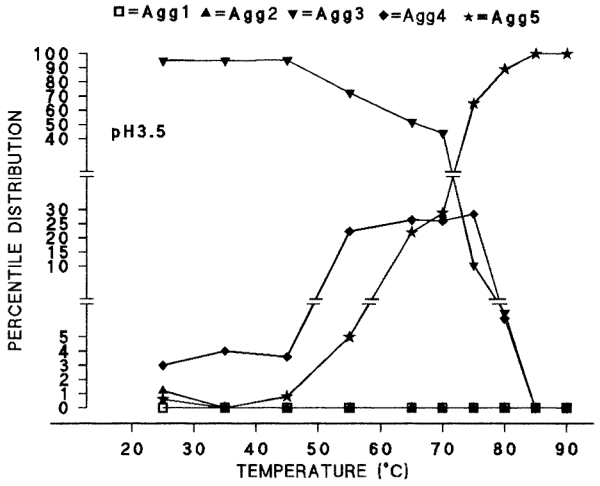

Fig. 2. Effect of heating of β-lactoglobulin AB in the presence of EDTA at pH 3.5 on percentile distribution of aggregates of different sizes. Data were obtained by CONTIN analysis of Dynamic Light Scattering data. Aggregates Agg1, Agg2, Agg3, Agg4, and Agg5, as so denoted in the text represent aggregate size ranges, 1–9, 10–99, 100–599, 600–999, and >=1000 nm, respectively

In terms of the aggregate size distribution, Agg3, which predominated below 65°C, decreased to small levels (4%) above 85°C (Fig. 3). A biggest aggregate, Agg5, appeared in trace amounts above 55°C and increased dramatically above 70°C and was predominant at 90°C. However, the transition from Agg3 to Agg4 and Agg5 was not as early (45°C for Agg3), or as rapid and clear as it was for pH 3.5 (Fig. 2).

Association and aggregation effects at alkaline pH 9.0 The IMAD (200 nm at 25°C) gradually increased to a MAD of 450 nm at 55°C and dropped back to 170 nm at the denaturation temperature of 70°C (Fig. 1). The MAD increased meagerly to only 480 nm at 80°C and there was no appearance of gelation even after further heating. Similarly, polydispersity also markedly decreased around the denaturation temperature and

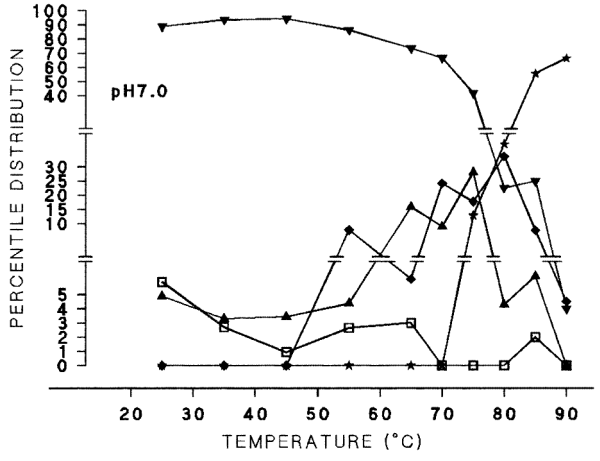

Fig. 3. Effect of heating of β-lactoglobulin AB in the presence of EDTA at pH 7 on percentile distribution of aggregates of different sizes. Data were obtained as described in Fig. 2. The symbols refer to the various aggregates and are the same as explained in the legend of Fig. 2.

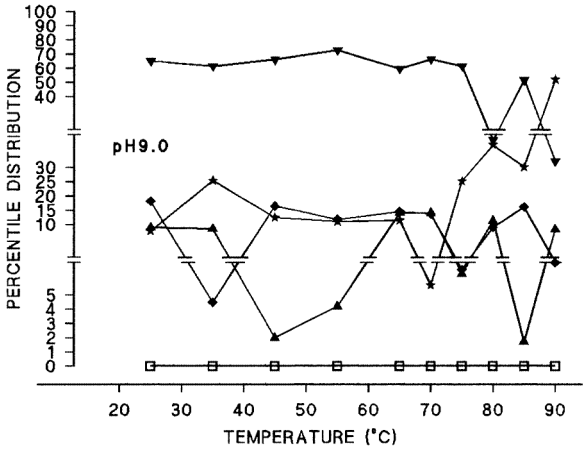

Fig. 4. Effect of heating of β-lactoglobulin AB in the presence of EDTA at pH 9.0 on percentile distribution of aggregates of different sizes. Data were obtained as described in Fig. 2. The symbols refer to the various aggregates and are the same as explained in the legend of Fig. 2.

only increased by 0.05 compared to what it was prior to heating (0.32) (Fig. 1, Inset). This reflected little of increase in the association tendency.

Unlike at pH 3.5, there was no marked change in the distribution of aggregates until the denaturation temperature of 75°C was reached. The least associated form, Agg1, was absent throughout the temperature range studied. Although Agg3 predominated at this pH, large amounts of Agg4 and Agg5 were also seen (Fig. 4). At 75°C, Agg3 started decreasing with concomitant sharp increase in Agg5 to 30%. At 90°C, Agg 3 and Agg 5 accounted for 55% and 35%, respectively. Though, Agg 4 was observed up to maximum of 20%, a clear transition from it to Agg 5 was not seen as was the case at pH 3.5.

Discussion

Data showing large IMAD (~200nm) (reflecting the pore size of the filter used) under all pH conditions (Fig. 1) compared to IMAD of chromatographically pure β-Lg (Sharma *et al*., 1996) is substantiated by earlier observations regarding the influence of casein derived peptides and zwitterionic amphiphiles on aggregate enlargement under quiescent conditions (Haque *et al.,* 1993). Casein derived peptides, that normally exist in whey, dramatically increase the hydrodynamic radius (R_i) of unheated β-Lg aggregates at pH 7, 25°C. This effect was influenced by the size of the peptides; larger (undialyzable) peptides increased R_i and the poly, which is a measure of the width of the distribution, more than did the smaller dialyzable ones (Haque *et al*., 1993). Recent observations indicate increased structural stability of β-Lg as a result of milk peptides (Barbeau *et al*., 1996).

Data showed gradual enlargement of β-Lg aggregates with temperature as reflected by cumulant analyses (Fig. 1). This trend was substantiated by CONTIN measurements as progressive decrease in Agg3 with concomitant increase in Agg4 and Agg5 (Figs. 2–4).

The overall association rates (k_1 s^{-1}) (assuming first order kinetics) from 25 to 70°C, *i.e*., the pre-gelation range (A) (Fig. 5), calculated based on the rate of disappearance of Agg3, the predominant aggregate at 25°C (reactant), and formation of Agg4 and Agg5 (products) (Atkins, 1990), were 4.29, 1.53, and -0.11×10^{-4} s^{-1} for pH 3.5, 7.0, and 9.0, respectively. These rates (k_1) are shown between 25 and 70°C in a schematic visualization of the empirical stages of thermal association (Fig. 5).

In temperature range A, hydrophobic interaction is the primary force that causes intermolecular association. Such forces reach a peak at 60–70°C (Brandts, 1967). In a related study, we have seen a dramatic increase in surface hydrophobicity of β-Lg at around 70°C making it the driving force to intermolecular interactions (Haque & Kinsella, 1987). On the other hand, Brownian movement conceivably has greater impact on gelation at temperature range (B) (>70°C) (Boulet *et al*., 2000).

Purified native β-Lg is reportedly dimeric at 25°C at the physiological pH of 6.7 (Brunner, 1977). However, it is important to note that most reported experiments were conducted at very low dilutions compared to our "pragmatic" (from a food system point of view) protein concentration of 80 mg/ml. Moreover, our protein preparation contained trace amounts of impurities that absorb at 280 nm. Chromatographic removal of these resulted in a completely different association tendency as reported earlier (Sharma *et al.*, 1996).

On being exposed to the various pH at 25°C, the size distribution changed (Figs. 2–4). At pH 3.5, the least aggregated state, Agg1, was not detected; 95% of β-Lg was Agg3 and the rest were Agg4 and Agg5 indicating a pre-existing (*i.e.*, prior to heating) tendency for association. At this acidic pH, β-Lg is known to undergo polymerization at low temperatures and high concentrations (Brunner, 1977). The schematic therefore visualizes the starting point at 25°C as a polymer (octamer) (Fig. 5). In range A, association rate was the highest at pH 3.5, resulting in a 3.5 fold increase in MAD at 70°C. This almost quadrupled to 13 between 70 and 80°C and increased approximately another five-fold be-tween 80 and 90°C to 55 (Fig. 5). Such enormous and rapid en-largement without precipitation conceivably indicated a high level of hydration or water entrapment as visualized in Fig. 5. This premise is substantiated by juxtaposing MAD and polydispersity data (Figs. 1 and Fig. 1, Inset). The fact that increase in polydispersity, which reflects molecular association (Haque &

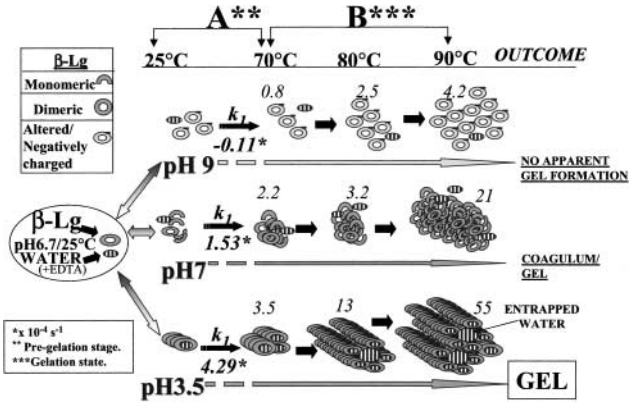

Fig. 5. Postulated schematic visualization (not to scale) of the thermal association of cation sequestered β-lactoglobulin AB at various pH conditions. Temperature range 25 to 70°C (A) represents the pre-gelation range and 70–90°C (B) represents the gelation range. Numbers in italic above each aggregate represents the number of fold increase in mean aggregate diameter (MAD) compared to initial MAD for that pH. The numbers in bold italics below the arrow in range A give the reaction rate for the pre-gelation thermal association tendency at that given pH.

Sharma, 1997; Koppel, 1972; Vaidya *et al.*, 1987), was relatively low even though MAD was dramatically high, implied a high degree of hydration.

Stability of β-Lg is contributed to by entropic forces due to ordering of water molecules around exposed hydrophobic patches on its surface (Jaenicke, 1987; Wodak *et al.*, 1987). Hydrophobic interactions, the main driving force for protein folding, are made up of chain-free energy as well as hydration-free energy (Oobatake & Ooi, 1993). These interactions and resulting association causes an overall decrease in surface hydrophobicity of proteins due to overlapping (Haque, 1989; Haque & Kinsella, 1988). The β-Lg molecules had already interacted to different degrees based on pH at the pre-gelation stage (<70˚C) (Fig. 1 and 5). This caused decreased surface hydrophobicity due to overlapping, and thus, the thermodynamic drive to decrease hydrocarbon-aqueous interface had proportionately diminished. The MAD of β-Lg at pH 3.5 increased from 212 nm at 25˚C to 685 nm at 70˚C, temperature at which strength of hydrophobic interactions is at a maximum (Brandts, 1967). In contrast, MAD at 70˚C for pH 7 and 9 were 433 nm and 180 nm, respectively.

The initial association profile at pH 7 was markedly different from that of pH 3.5. The least associated state, Agg1, was detected only at this pH at 25˚C (Fig. 3) where ~11% of the β-Lg existed as Agg1 or Agg2 and the remainder was Agg3 without larger aggregates (Fig. 3). This meant that total number of particles with potential for lasting contact was at a maximum at this condition. Data also showed a decrease in MAD from 373 nm at 55˚C to 290 nm at 65˚C. Interestingly, the relative association, as reflected by polydispersity, markedly increased under the same conditions from 0.18 at 55˚C to 0.225 at 65˚C (Fig. 1. Inset). This reflected tightening of the aggregates that could conceivably expel "entrapped" water resulting in a coagulum or precipitate rather than a gel as postulated and visualized in Fig. 5. The scheme therefore proposes molecular interaction/orientation that resulted in compacter and tighter aggregates due to "wedging" where smaller particles filled spaces that were inaccessible to larger ones. Heating range A caused an enlargement of MAD by 2-fold compared to IMAD at 70˚C, followed by dramatic increase to 22-fold at 90˚C (Fig. 5).

According to Smoluchovski's equation for equal-sized spheres, the number J of encounters/unit volume and time is give by,

$$J_B = 4/3\ kT\ N^2/\eta, \qquad (1)$$

where, N is the number of particles per unit volume and η is the viscosity of the continuous phase (Walstra & Jenness, 1984). When the encounter leads to a lasting contact, there is enlargement of the aggregates or "floccules" and an overall reduction in the number of particles. As the floccules become larger, the tendency for settling out of dispersion or "precipitation" is increased as dictated by Stokes' law. At pH 7, the aggregate distribution at 80˚C was 4.4%, 22.9%, 34.2% and 38.5% of Agg2, Agg3, Agg4, and Agg5, respectively. On the other hand, at pH 3.5, the distribution at the same temperature was 0.0%, 6.5%, 6.2%, and 87.3% of the same aggregates. The total number of particles (N) was markedly higher at pH 7 resulting in rapid aggregation as illustrated by Equation 1. Moreover, viscosity of water, which is inversely related to J in Smoluchovski's equation (Eq. 1), decreases from 404 at 70˚C to 314.5 Pa s at 90˚C enhancing the Brownian motion induced collisions required for aggregation (Lide & Frederikse, 1993).

At pH 9, the association profile represented by CONTIN size distribution was completely different, indicating structural alteration (Fig. 4). The size distribution of aggregates at pH 9 was much broader with no mono- or dimeric form (Agg1), ~65% of β-Lg being Agg3 and the rest, Agg2, Agg4 and Agg5 (Fig. 4). The thermal association at this pH was also minimal. This was perhaps due to the reported existence of a reversible transition involving change in molecular conformation at about pH 7.5 (Mailliart & Ribadeau-Dumas, 1988; Swaisgood, 1986). Furthermore, at pH 9, all surface nucleophiles, *i.e.*, -SH, both *N*-terminal and ε-NH_3, alcoholic and phenolic-OH, become deprotonated and thus potentially negatively charged in the absence of counter ions (due to EDTA) (Lehninger, 1982). This would repel association due to charge repulsion. Therefore, there was a reduction of MAD in range A and little increase in range B (Figs. 1, 4, 5).

Based on Coulomb's law, both force and energy of interaction between charged particles are inversely related to the dielectric constant (DE) of the dispersing medium and this parameter decreases as temperature increases. The DE for water at 25˚C is 78.6 and 58.12 at 90˚C (Lide & Frederikse, 1993) resulting in increased potential for repulsion between negatively charged aggregates as postulated in the visualization in Fig. 5. Based on recent work using various DE altering solvents, Narizhneva and Uversky (1998) have shown that β-Lg undergoes changes without complete unfolding as DE was decreased. The presence/persistence of Agg3, at temperatures above 70˚C, could be the result of partial unfolding and persistence of residual structure. 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 attributed to a partial unfolding at 70˚C. Recent data indicate that the β-Lg structure exists in different levels of energy minima on thermal alteration; a phenomenon referred to as molten globular state (Dufour *et al*., 1994; Narizhneva & Uversky, 1998).

Conclusions

Effect of pH lowering in the presence of EDTA, that sequestered cations, had dramatic influence on association tendency. At pH 3.5, β-Lg had the highest association tendency both at the pre-gelation (A) and gelation stage (B) (Fig. 5). The apparent gelation temperature was decreased to 85˚C and a gel was formed. At neutral pH of 7, the least aggregated state, Agg1, was detected; association tendency was lower at the pre-gelation stage but dramatic at the gelation stage resulting in a soft gel. At pH 9.0, β-Lg did not associate at the pre-gelation stage and showed little aggregation at the gelation stage giving a turbid solution without gel formation. Differences in the association tendency at the different pH, and whether there was resulting gelation, are postulated to be due to charge repulsion in the absence of counter ions. Subsequent studies in this series deal with the conformational changes of β-Lg that is brought about by this heating regimen with and without EDTA, NaCl, and 2-ME.

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