Gelling Properties of Heat-Denatured β-Lactoglobulin Aggregates in a High-Salt Buffer

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Thermal denaturation, rheological, and microstructural properties of gels prepared from native β-lactoglobulin (β-LG) and preheated or heat-denatured β-LG (HDLG) aggregates were compared. The HDLG was prepared by heating solutions of 4% β-LG in deionized water, pH 7.0, at 80 °C for 30 min and then diluted to the desired concentration in 0.6 M NaCl and 0.05 M phosphate buffer at pH 6.0, 6.5, and 7.0. When reheated to 71 °C, HDLG formed a gel at a concentration of 2% protein. At pH 7.0, 3% HDLG gelled at 52.5 °C and had a storage modulus (G′) of 2200 Pa after cooling. β-LG (3%) in 0.6 M NaCl and 0.05 M phosphate buffer, pH 7.0, did not gel when heated to 71 °C. The gel point of 3% HDLG decreased by 10.5 °C and the G′ did not change when the pH was decreased to 6.0. The HDLG gel microstructure was composed of strands and clumps of small globular aggregates in contrast to β-LG gels, which contained a particulate network of compacted globules. The HDLG formed a gel at a lower concentration and lower temperature than β-LG in the high-salt buffer, suggesting an application in meat systems or other food products prepared with salt and processed at temperatures of ≤71 °C.

KEYWORDS: β-Lactoglobulin; gelation; microstructure; rheology; denaturation

INTRODUCTION

Whey protein is highly nutritious and is widely used as a functional ingredient in food products. Heat-induced gelation is an important functional property of whey protein. However, commercial whey protein normally gels above 75 °C, which is higher than typical processing temperatures of many food products, such as comminuted meat products.

The ability to form strong gels at temperatures <75 °C broadens the potential use of whey products. Researchers have looked at several ways to lower the gelling temperature of whey protein. Application of hydrostatic pressure to whey protein concentrate (WPC) and whey protein isolate (WPI) promoted sulfhydryl/disulfide interchange reactions, formation of hydrogen bonds, and rupture of hydrophobic interactions to induce gel formation at ambient temperature (*1*). Partially hydrolyzed β-lactoglobulin (β-LG) prepared by limited proteolysis with trypsin had a lower gel point than native β-LG (*2*). Preheating whey protein produced denatured soluble protein aggregates or polymers that formed cold-set gels upon addition of salt or acid

at ambient temperature (*3*−*7*). The preheated whey protein aggregates formed stranded transparent gels with greater strength and water-holding ability than heat-set gels prepared in a single step from native whey protein with the same concentration of salts. Disulfide bonds and hydrophobic interactions were found to be important in the polymerization of whey protein and β-LG (*8*−*10*).

Cold-set gelation of preheated whey protein involves a two-step process. In the first step, heating of whey protein at neutral pH under low salt conditions results in the formation of soluble aggregates of denatured protein. These soluble whey protein aggregates are commonly referred to as preheated protein, heat-denatured protein, or polymerized protein. In the second step, gel network formation is induced by increasing the ionic strength or changing the pH to allow interactions of the soluble aggregates. Ionic strength, pH, protein concentration, time, and temperature were reported to be factors affecting soluble aggregate formation of whey protein during preheat treatment (*7*, *9*−*11*). In general, larger aggregates are formed during the preheating step at higher protein concentrations, pH 6−7, low ionic strength (< 50 mM NaCl), and longer heating times between 70 and 90 °C (*12*).

Heat-denatured whey proteins form gels at lower temperatures than traditional WPC or WPI and may allow increased use of whey proteins in a variety of applications for which protein gelation at low temperature is desirable. Salt-induced cold-set

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gelation of whey protein aggregates could be beneficial when used in food products containing salt and processed at lower temperatures. One such application is in comminuted meat systems that require both salt and heat for optimum quality and yield characteristics. The objective of this experiment was to compare the thermal denaturation, rheological, and microstructural properties of gels prepared from native β-LG and heat-denatured β-lactoglobulin aggregates (HDLG) under buffer conditions (0.6 M NaCl and 0.05 M sodium phosphate, pH 6.0, 6.5, and 7.0) and a heating temperature (71 °C) often used to study meat protein systems.

MATERIALS AND METHODS

Preparation of Protein Solutions. Bovine milk β-LG (L0310 lot 114H7055) containing variants A and B was purchased from Sigma Chemical Co. (St. Louis, MO). β-LG was dissolved in 0.6 M NaCl and 0.05 M phosphate buffer at pH 6.0, 6.5, or 7.0 and kept at 4 °C before use. Protein concentration was determined by spectrometric absorption using an extinction coefficient ($E^{1\%}$) of 9.55 at 278 nm (*13*). Heat-denatured β-LG was prepared according to the method of Ju and Kilara (*7*) by dissolving 4% β-LG in deionized water and adjusting the pH to 7.0 with 0.1 N NaOH or HCl. The solutions were placed in 16 × 125 mm glass tubes, heated at 80 °C for 30 min in a water bath, cooled in an ice bath, and kept at 4 °C. The HDLG was diluted with concentrated buffer to prepare solutions of 1.0, 2.0, and 3.0% protein in buffer. These preheated protein solutions were adjusted to the desired concentration immediately before use to avoid gel formation during holding at ambient temperature.

Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) was used to assess the compositions of β-LG and HDLG. Electrophoresis was run in a mini-Protean II dual slab cell (Bio-Rad Laboratories, Hercules, CA) using a tris(hydroxymethyl)-aminomethane glycine electrode buffer, pH 8.3, and 0.1% SDS as described by Laemmli (*14*). The acrylamide concentrations of stacking and resolving gels were 4 and 14%, respectively.

β-LG and HDLG solutions were diluted to 4 mg/mL with sample buffer (0.0625 M Tris, pH 6.8, 2% SDS, 10% glycerol, and 0.2% bromophenol blue) and heated in boiling water for 5 min before use. Sample buffer for reducing conditions also contained 5% of 2-mercaptoethanol. About 5 μL of each protein sample was loaded into a sample well, and the gels were run at 200 V for ∼45 min. Gels were stained for at least 20 min with Coomassie Brilliant Blue R250 solution (0.25% in 9:45:45 v/v/v of acetic acid/methanol/water) and were destained overnight in acetic acid/methanol/water (6:4:7 v/v/v) solution. The molecular mass of the protein bands was determined by comparing relative mobilities to those of standard markers (Bio-Rad Laboratories) under the same electrophoretic conditions (*15*).

Differential Scanning Calorimetry (DSC). The thermal denaturation patterns of 1% β-LG and 1% HDLG in deionized water, pH 7.0, and in 0.6 M NaCl and 0.05 M phosphate buffer, pH 6.0, 6.5, and 7.0, were investigated using an MC-2 differential scanning calorimeter (Microcal Inc., Amherst, MA). The protein and blank solutions (deionized water or buffer) were degassed in a vacuum chamber (Nalgene, Fisher Scientific, Pittsburgh, PA), and 1.24 mL was immediately loaded into the DSC using a syringe. Each experiment was conducted in triplicate at a heating rate of 1 °C/min from 25 to 90 °C. The heat capacity profile was used to define calorimetric enthalpy (ΔH_{cal}) and endothermic peak or melting temperature (T_m) using Microcal DA-2 data acquisition and analysis system software.

Rheological Measurements. Dynamic oscillatory tests of 1−3% HDLG and β-LG in PBS at pH 6.0, 6.5, and 7.0 were performed using a controlled stress rheometer (RS 100, Haake, Karlsruhe, Germany) equipped with a 35 mm diameter stainless steel parallel plate. A circulating water bath was used to control temperature. Storage (G') and loss (G'') moduli were recorded continuously at a fixed frequency of 0.464 Hz using constant stresses (producing strains from 0.1 to 0.3%) within the range of linear viscoelastic behavior determined from stress sweeps performed for each protein preparation at 71 or 90 °C and after cooling to 25 °C. Protein solutions were loaded between the parallel

plate and base with a 1.0−1.1 mm gap. A few drops of maize oil (Mazola, Best Food, CPC International, Inc., Englewood Cliffs, NJ) were used to cover the edge of the plate to prevent evaporation. Solutions were equilibrated at 25 °C for 5 min, heated from 25 to 71 °C at 1 °C/min, held at 71 °C for 60 min, cooled to 25 °C within 10 min, and held at 25 °C for 5 min. In addition, β-LG solutions were also heated to 90 °C, held at 90 °C for 30 min, and cooled to 25 °C within 15 min. The gel point was determined as the temperature at which G' and G'' crossed over in the fixed-frequency test. Frequency sweeps (0.01−100 rad/s) were performed after cooling to confirm gel network formation.

Scanning Electron Microscopy (SEM) of Gels. The β-LG and HDLG gels were prepared at 4 and 2% protein, respectively, in 0.6 M NaCl and 0.05 M phosphate buffer, pH 6.0, 6.5, and 7.0. One milliliter of each protein solution was transferred into a 12 mm × 75 mm glass tube and sealed with Teflon tape. The HDLG were heated in a programmable water bath (model 9510, PolyScience, Niles, IL) from 25 to 71 °C at 1 °C/min, held for 60 min, and cooled to 25 °C in an ice−water bath. β-LG solutions were heated from 25 to 90 °C at 1 °C/min and held for 30 min before cooling. Protein gels were cut into 1 × 2 × 2 mm pieces, fixed in 2.0% gluteraldehyde in 0.1 M sodium phosphate buffer, pH 7.0, for 3 h at 4 °C, and postfixed in 0.1% osmium tetraoxide overnight at 4 °C. Fixed gels were rinsed with 0.1 M sodium phosphate buffer and dehydrated by immersion in a graded ethanol series of 25, 50, 75, and 95% for 20 min per step, followed by three steps in 100% ethanol. Gels were dried using a Balzers critical point dryer (Balzers CPD, FL-9496, Balzers, Liechtenstein) with liquid carbon dioxide as the transitional fluid. Gels were then mounted on metal stubs and coated with a 25−30 nm gold layer in an Emscope sputter coater (Emscope Laboratories Ltd., Ashford, Kent, U.K.). Gel structures were observed with a JEOL scanning microscope (model JSM-6400V, Tokyo, Japan) at a 15 mm working distance using an accelerating voltage of 10−12 kV and 5000× magnification. Gels were prepared in duplicate, and digital images of three samples from each preparation were captured in the SEM. Pore sizes and globule diameters were estimated by comparison with bar size in enlarged images.

Scanning Electron Microscopy of Aggregates. The HDLG aggregates were mixed with an equal volume of 4% gluteraldehyde in 0.1 M sodium phosphate buffer, pH 7.0, for 1 h at 4 °C. After fixation, one drop of the suspension was placed on a coverslip previously coated with poly-L-lysine (Sigma) and allowed to stand for 5 min. The coverslip was then carefully washed with several drops of distilled water, followed by dehydration, critical drying, and coating as previously described except that a 15 nm layer of gold was applied. The HDLG aggregates were examined at an 8 mm working distance using an accelerating voltage of 10 kV and 20000× magnification. The HDLG aggregates were prepared in triplicate, and digital images of three samples from each preparation were captured.

Experimental Design and Statistical Analysis. Rheological and DSC experiments were conducted using triplicate protein preparations. Differences in gel point, G', and tangent delta (tan δ) of gels due to treatments (protein concentration and pH) were statistically analyzed using one-way analysis of variance. Means were compared using the Tukey−Kramer HSD test with a mean square error at the 5% level of probability (JMP software, version 3.2.2, SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Electrophoretic Patterns. The electrophoretic patterns of β-LG and HDLG solutions were resolved by SDS-PAGE under nonreducing and reducing conditions (**Figure 1**). For β-LG, a dark band corresponding to the β-LG monomer (18.4 kDa) and a lighter band corresponding to the dimer (37 kDa) were observed under both reducing and nonreducing conditions. SDS-PAGE of HDLG under reducing conditions revealed a dark band at 18.4 kDa identified as the monomeric form of LG. Only a small amount of the monomer was observed under nonreducing conditions, suggesting that only a small amount of monomeric β-LG was present in HDLG preparations. HDLG preparations contained a band of protein too large to pass through the stacking

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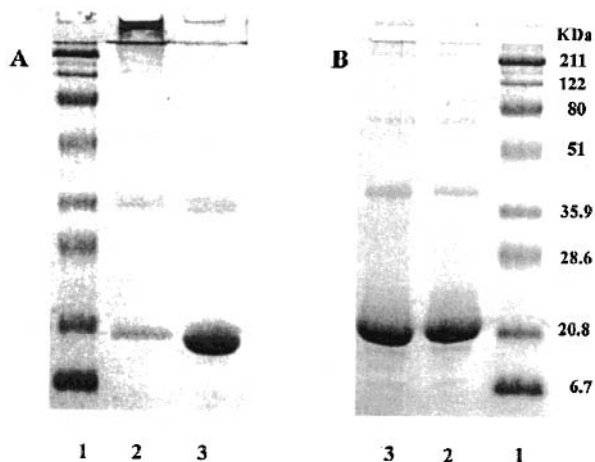
Figure 1. Electrophoregram of (A) nonreduced and (B) reduced native and heat-denatured β-LG: (lane 1) molecular weight markers; (lane 2) heat-denatured β-LG; (lane 3) β-LG.
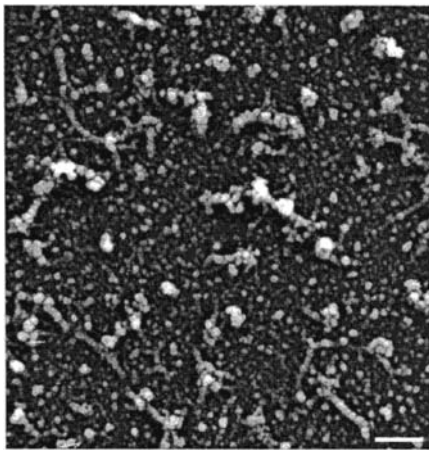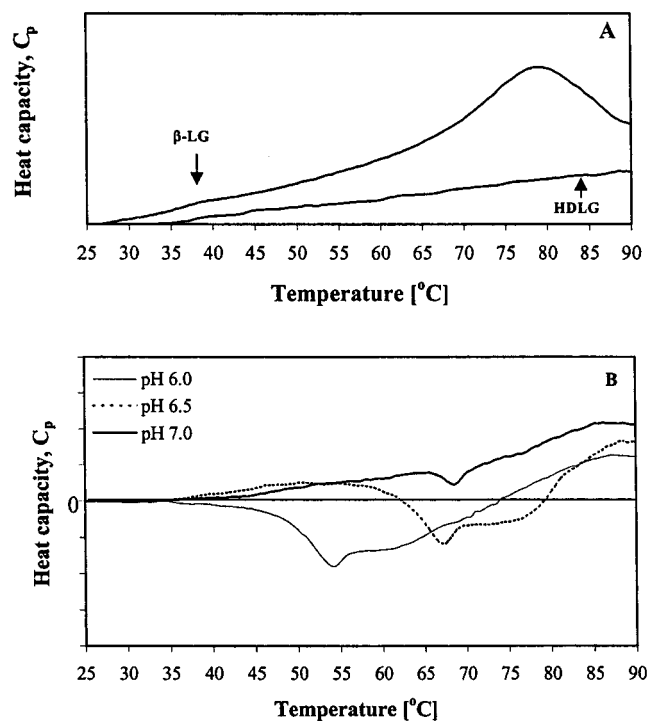

Figure 2. SEM image of heat-denatured β-LG aggregates at 20000× magnification. Bar = 1.0 μm. (Figure is reproduced at 75% of its original size.)

Figure 3. Heat capacity profiles of (A) 1.0% β-LG and 1.0% heat-denatured β-LG (HDLG) in deionized water, pH 7.0, and (B) 1.0% HDLG in 0.6 M NaCl and 0.05 M sodium phosphate buffer, pH 6.0, 6.5, and 7.0.

gel using nonreducing SDS-PAGE, suggesting polymers >1 million in molecular mass (*16*). This band was not observed when HDLG was resolved under reducing conditions, verifying that disulfide bonds contributed to soluble aggregate formation at neutral pH as previously reported (*8, 17, 18*).

Microstructure of Heat-Denatured Aggregates. The HDLG was composed primarily of small globular aggregates of 30−50 nm, although some of the globules appeared to associate into larger clumps and long strands (**Figure 2**). Native β-LG has a hydrodynamic diameter of 3−5 nm (*19*), suggesting HDLG aggregates were formed by several β-LG molecules. The HDLG aggregates were similar in size to those of whey protein aggregates (21−61 nm) prepared by heating WPI at 80 °C for 30 min (*7, 20*) and HDLG aggregates (38 nm) heated at 80 °C at pH 7.0 and 0.1 M ionic strength (*21*). The size and shape of protein polymers greatly affect the network formation of gels (*22*). Systematic studies are needed to determine how changes in preheating conditions of β-LG alter polymer composition and subsequently influence the properties of gels made from these polymers.

Thermal Denaturation of β-LG and HDLG. The thermograms of 1% β-LG and 1% HDLG in deionized water, pH 7.0, were different (**Figure 3A**). β-LG melted at 79.0 °C. No thermal transitions were found for HDLG, demonstrating that preheating of β-LG at 80 °C for 30 min resulted in irreversible denaturation

of the protein. The denaturation profiles of 1% HDLG in 0.6 M NaCl and 0.05 M phosphate buffer, pH 6.0, 6.5, and 7.0 showed small exothermic peaks at about 56, 67, and 71 °C, respectively (**Figure 3B**). The exothermic peaks were possibly caused by additional aggregation of HDLG during heating in the DSC (*23*). The isoelectric point of β-LG is ~5.2; thus, the aggregation temperature may have increased with pH due to a greater net negative charge on HDLG that inhibited aggregation of the protein.

Rheological Properties of β-LG and HDLG. The gel points (as defined by the crossover of G' and G'' on the rheogram) of β-LG in 0.6 M NaCl and 0.05 M sodium phosphate buffer, pH 7.0, decreased from 79 °C at 1.0% protein to 73 °C at 3.0% protein. The rheological properties of β-LG could not be measured accurately during cooling due to precipitation and shrinkage of the gels, which led to rapid fluctuations of G' (**Figure 4**).

At 1−3% protein, β-LG did not form a gel when heated to 71 °C in 0.6 M NaCl and 0.05 M phosphate buffer, pH 7.0. The G' after holding at 71 °C for 60 min was low (4−9 Pa), and no crossover point was observed on the rheogram. In contrast, HDLG formed a gel network at pH 7.0 when heated to 71 °C (**Figure 5**). Gels formed using 1% HDLG were weak at pH 7.0 (16 Pa). As protein concentration was increased from 1 to 3% at pH 7.0, the gel point of HDLG decreased by 19 °C and G' after cooling increased 137-fold (**Table 1**). Three percent HDLG at pH 7.0 had a gel point at 52.5 °C and a G' of 2200 Pa after cooling.

A lower gel point and greater gel rigidity have been previously reported when preheated WPC and WPI were compared to the gels made from the same protein preparations that were not preheated (*3, 4, 6, 7*). In 0.2 M NaCl, pH 7.0, 9% preheated WPI gelled at 48 °C, whereas the unheated WPI formed a gel at ≥77 °C (*4*). Doi (*24*) reported that a two-step heating method formed stronger and more transparent gels from
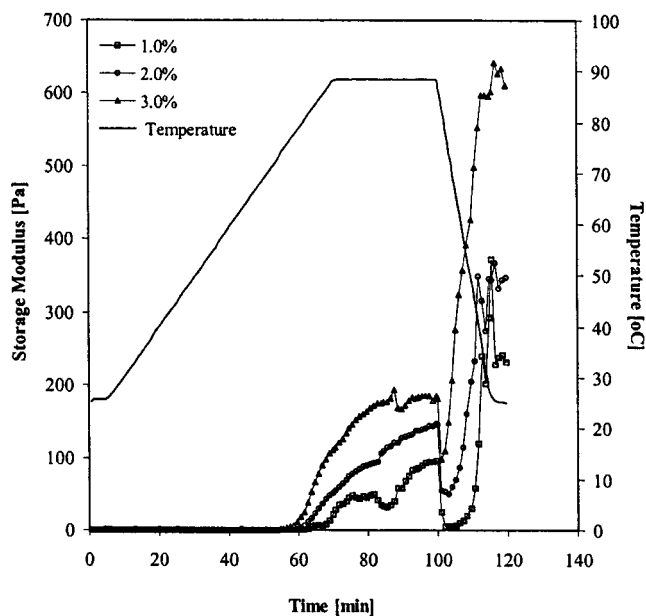
Figure 4. Storage moduli of β-LG in 0.6 M NaCl and 0.05 M sodium phosphate buffer, pH 7.0, during heating at 1 °C/min from 25 to 90 °C, holding at 90 °C for 30 min, and cooling to 25 °C.
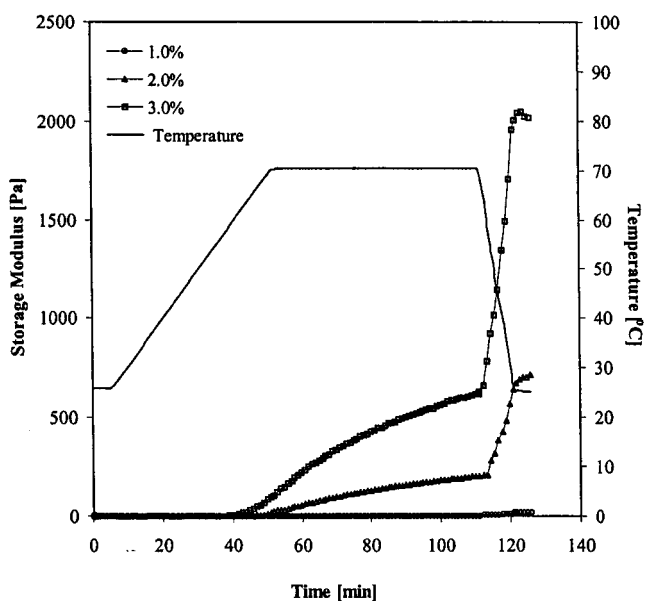

Figure 5. Storage moduli of 1–3% heat-denatured β-LG in 0.6 M NaCl and 0.05 M sodium phosphate buffer, pH 7.0, during heating at 1 °C/min from 25 to 71 °C, holding at 71 °C for 60 min, and cooling to 25 °C.

ovalbumin, bovine serum albumin, and egg lysozyme when compared to those formed by a single-step heating method. All of this work was performed at salt concentrations of <0.6 M.

The effects of pH on the gelling properties of HDLG were compared (**Figure 6**). When the pH was decreased from 7.0 to 6.0, the gel point of 3% HDLG decreased from 52.5 to 42 °C in 0.6 M NaCl and 0.05 M phosphate buffer (**Table 1**). The exothermic peak temperatures recorded by DSC indicating HDLG aggregation also decreased as the pH decreased (**Figure 3**). Aggregation may occur more readily at pH 6.0 as there was less net negative charge on the protein than at pH 7.0. The G' of HDLG after cooling was not affected ($p > 0.05$) by pH. Tan δ values after cooling of 2.0–3.0% HDLG gels were $\leq$0.1 at pH 6.0, 6.5, and 7.0, indicating a typical viscoelastic gel (*25*). These results suggest that electrostatic interactions caused by

Table 1. Gel Point, Storage Moduli (G'), and Tangent Delta (tan δ) after Cooling of 1–3% Heat-Denatured β-Lactoglobulin (HDLG) Heated to 71 °C in 0.6 M NaCl and 0.05 M Sodium Phosphate Buffer, pH 6.0, 6.5, and 7.0[a]

parameter	protein concn (%)	pH 6.0	pH 6.5	pH 7.0
gel point (°C)	1	63.0 ± 0.5[ax]	71[ay]	71[ay]
	2	49.0 ± 0.9[bx]	58.5 ± 0.5[by]	62.0 ± 0.5[bz]
	3	42.0 ± 0.5[cx]	50.0 ± 1.0[cy]	52.5 ± 1.0[cz]
G' (Pa)	1	100 ± 14[ax]	40 ± 11[ay]	16 ± 2[ay]
	2	860 ± 50[bx]	700 ± 108[bx]	585 ± 58[bx]
	3	3100 ± 57[cx]	2700 ± 150[cx]	2200 ± 140[cx]
tan δ	1	0.12 ± 0.01[ax]	0.14 ± 0.02[ax]	0.13 ± 0.01[ax]
	2	0.10 ± 0.02[bx]	0.10 ± 0.00[bx]	0.10 ± 0.01[bx]
	3	0.10 ± 0.00[bx]	0.09 ± 0.01[bx]	0.09 ± 0.00[bx]

[a] Values are means of three observations ± standard deviation. Means with different superscripts (a–c) in each column are significantly different ($p < 0.05$). Means with different superscripts (x–z) in each row are significantly different ($p < 0.05$).
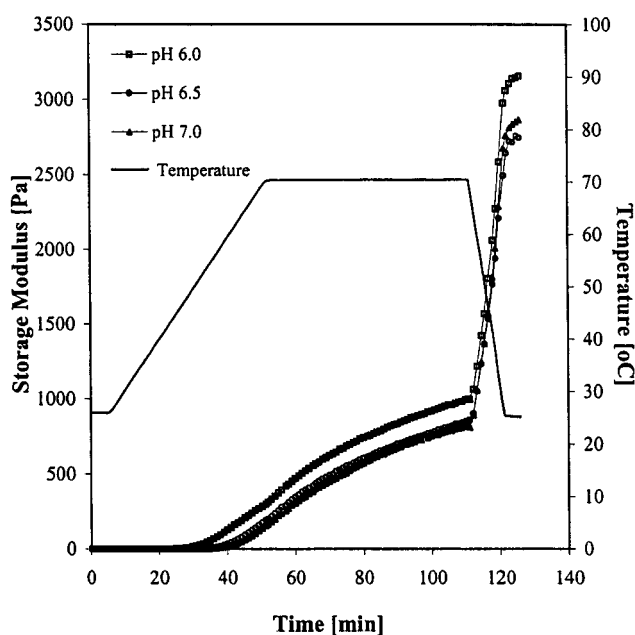

Figure 6. Storage moduli of 3% heat-denatured β-LG in 0.6 M NaCl and 0.05 M sodium phosphate buffer, pH 6.0, 6.5, and 7.0, during heating at 1 °C/min from 25 to 71 °C, holding at 71 °C for 60 min, and cooling to 25 °C.

pH changes affected the aggregation temperature, whereas hydrophobic interactions during heating contributed to the formation and strengthening of the HDLG gel network.

Gel Microstructure. Microstructures of 4% β-LG gels prepared at 90 °C for 30 min and those of 2% HDLG gels prepared by heating at 71 °C for 60 min were compared (**Figure 7**). These concentrations and heating conditions allowed for the formation of free-standing gels that could be manipulated during preparation for viewing in the SEM. β-LG formed particulate gels composed of grape-like compacted globular aggregates. Globule diameter decreased from 1–3 μm at pH 6.0 to 0.2–0.4 μm at pH 7.0. These globule sizes were similar to those of β-LG gels prepared by heating 0.9% β-LG in 0.5 M NaCl, pH 7.0, at 68.5 °C for 1.5 h (*26*).

HDLG formed a protein network comprising small globules of 30–70 nm diameter arranged in clumps and strands. On the basis of the size and shape of the HDLG soluble aggregates, the HDLG gel networks appeared to form via cross-linking of

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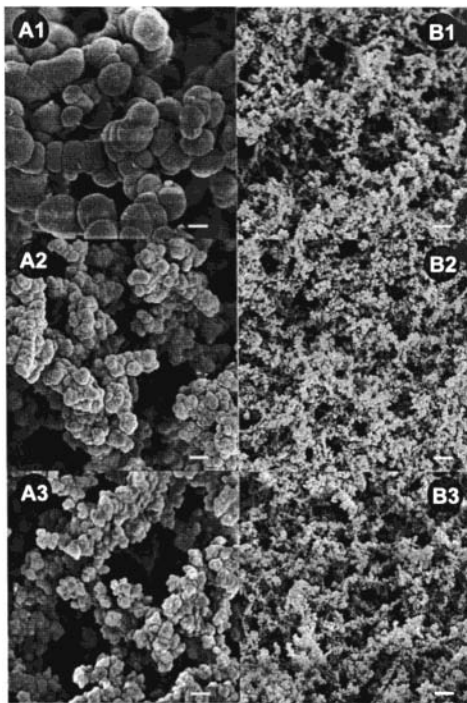
Figure 7. SEM images at 5000× magnification of (A) 4% β-LG gels heated to 90 °C for 30 min and (B) heat-denatured β-LG gels heated to 71 °C for 60 min in 0.6 M NaCl and 0.05 M sodium phosphate buffer: (1) pH 6.0; (2) 6.5; (3) 7.0. Bar = 1.0 μm. (Figure is reproduced at 75% of its original size.)

the primary soluble aggregates. When compared to β-LG gels, the HDLG gel structure was much finer with smaller void spaces.

The pore sizes of HDLG gels at pH 6.0 were larger than those at pH 6.5 and 7.0, although the *G′* of HDLG gels after cooling did not change with pH. The β-LG gel networks were composed of 50-fold bigger aggregates than those of HDLG gels and were visually more opaque. Cold-set gels with larger aggregates and bigger pore sizes were more opaque than gels with finer structure (*6*) due to increased light scattering (*3, 27*).

In summary, preheating of β-LG at 80 °C for 30 min in distilled water at pH 7.0 led to the formation of soluble aggregates from denatured molecules via disulfide bonding. When reheated at 71 °C in 0.6 M NaCl, HDLG gels were formed through the secondary association of the primary aggregates. The two-step aggregation process allowed β-LG to form a finer gel structure at a lower protein concentration and lower temperature when compared to a one-step aggregation process using untreated β-LG.

These experiments were carried out at temperature and pH conditions favorable for meat protein gelation, confirming the potential use of whey protein aggregates in meat systems. The HDLG gel points between 42 and 71 °C are within the typical range of processing temperatures used in the meat industry. Current U.S. Department of Agriculture safe harbor guidelines require that roast beef and poultry products be cooked to ≥54 °C for specific holding times, whereas beef patties should be cooked to ≥66 °C for specific holding times (*28*). Thus, if added to meat products, preheated whey proteins could be expected to form gels during cooking, unlike untreated whey proteins that have much higher gelling temperatures (*29*).

More experiments are needed to understand interactions between HDLG and myosin in mixed systems. An understanding of factors influencing the characteristics of the primary ag-

gregates, which ultimately govern the textural properties of the final gels, will provide direction for achieving desirable functional properties of whey products. Preaggregation by heating could be used to optimize properties of whey products needed by meat processors and may contribute to broader applications of whey when protein gelation at lower temperatures is preferred.

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