Effect of Disulfide Interactions and Hydrolysis on the Thermal Aggregation of β-Lactoglobulin

Prashant Mudgal, Christopher R. Daubert,* Debra A. Clare, and E. Allen Foegeding

Department of Food, Bioprocessing and Nutrition Sciences, Schaub Hall, Dan Allen Drive, North Carolina State University, Raleigh, North Carolina 27695-7624, United States

ABSTRACT: The roles of sulfhydryl/disulfide interactions and acid/pepsin hydrolysis on β-lactoglobulin (β-lg) thermal aggregation at acidic pH 3.35 and 2 were studied using rheology, sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE), transmission electron microscopy (TEM), and Western blotting. Pepsin promoted additional hydrolysis compared to the acid-hydrolyzed control sample based on a 12% increase in free amino groups. Hydrolysis with pepsin also resulted in an increase in the apparent viscosity by 2 logs upon heating 8% β-lg solutions at pH 3.35. Seemingly, hydrolysis promoted thermal aggregation of β-lg, correlating well with viscosity increases. Large microgels were observed in heated pepsin hydrolysates using TEM, supporting the increased viscosities of these dispersions. During thermal aggregation (85 °C, 3 h) of β-lg at pH 3.35, beyond the existence of limited disulfide interactions, acid hydrolysis and noncovalent interactions more likely play a crucial role in defining the functionality of acidified powdered modified whey ingredients.

KEYWORDS: acid hydrolysis, pepsin, cold thickening, β-lactoglobulin, disulfide, whey protein

■ INTRODUCTION

A cold-thickening, pH-modified whey protein ingredient was developed to impart thickening function upon reconstitution in water, without the addition of salt or heat.[1] Cold-thickening whey ingredients have proven to be a suitable alternative to conventionally used starches for food applications where (1) heat may not be desirable or (2) nutritional benefits are desired.[1,2] Previously, the concentration effects on the cold-thickening mechanism of this ingredient were studied at the pH of the modification procedure, 3.35, using a β-lactoglobulin (β-lg) model system.[3] A critical concentration ($C_c \sim 6.9\%$ w/w solids) was identified for this system, below which no significant thickening function was achieved from modified powders, even when reconstituted at elevated solids concentration (10% w/w solids).[3] During heating (85 °C, 3 h) at pH 3.35, β-lg monomers formed flexible fibrillar networks with ∼5 nm diameter strands and ∼35 nm persistence lengths.[3] β-Lg is the major (∼ 60%) constituent of commercially available whey protein products[4] and is believed to be the only protein responsible for fibril formation in whey products at pH 2.0.[5] β-Lg aggregation mechanisms thus affect the functional properties of commercial whey protein products.

Depending on the pH and ionic strength, β-lg forms different types of aggregates upon heating, leading to the formation of either fine stranded or particulated gels.[4,6] β-Lg forms a particulated network in the pH range of 4−6. Transparent, fine-stranded gels, however, are formed below and above this region of pH and at low ionic strength conditions.[4] This protein is mostly found to exist either as a monomer or dimer, and there is a monomer−dimer equilibrium that is affected by pH, ionic strength, and temperature.[7−9] Upon heating, β-lg denatures, and buried hydrophobic regions and thiol groups become solvent accessible.[10,11] The formation of aggregates during gelation involves covalent (disulfide bonds) or noncovalent interactions, such as hydrophobic, electrostatic, ionic, and van der Waals, where each interaction

is weaker than covalent bonding. Several studies have attempted to define the relative roles played by each type of interaction during whey protein gelation at neutral pH;[6,10,12,13] however, these interactions change with pH. For example, at neutral to alkaline pH, disulfide interactions are favored.[14] Overall, the relative contribution of various interactions remains generally unknown.

At pH 7.0, β-lg aggregation is a result of a combination of various reactions. Upon heating above 70 °C, β-lg denatures, dimers dissociate into monomers, and thiol and hydrophobic groups become solvent accessible. Then, aggregates are formed through thiol−disulfide exchange and also by thiol oxidation and noncovalent interactions.[15] Below pH 7, the reactivity of disulfide bonding is limited; therefore, at low pH, when proteins are heated, other types of bonding become more relevant.[16] At pH 2.0, β-lg is highly charged, carrying a positive charge of +20. At pH 2.0, it was shown that β-lg forms fibrillar aggregates with a diameter of ∼4 nm and a length of 1−10 μm at low ionic strength conditions.[17−23] The length and flexibility of these fibrils vary with pH and ionic strength. Recently, it was observed that at pH 2, acid hydrolysis of β-lg takes place between aspartic acid residues and other amino acids, and peptides between 2000 and 8000 Da were proposed to be the building blocks for fibrils formed at pH 2.[22]

Effects of enzymatic hydrolysis of β-lg on gelation properties and its microstructure have been well documented, especially at pH 7 or 8 using different proteases such as trypsin, Glu and Asp specific protease from *Bacillus licheniformis*, *Bacillus subtilis*,

Alcalase, bromelain, and papain.[24] Limited proteolysis lowered the gel point and improved gelation.[24−30] Fewer studies, however, have focused on the effects of enzymatic hydrolysis on β-lg aggregation and rheology under low pH conditions.[30−32]

The first objective of this study was to investigate the role of disulfide interactions during the thermal aggregation of β-lg to better understand aggregation mechanisms at pH 2, 3.35, and 7 using sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting, electron microscopy, and rheology. The second objective was to determine and compare the role of acid hydrolysis versus pepsin hydrolysis on β-lg thermal aggregation at pH 3.35 using SDS-PAGE, Western blotting, o-phthalaldehyde (OPA) assay, rheology, and electron microscopy, because the modified whey ingredient is manufactured under these conditions.

■ MATERIAL AND METHODS

Protein Material. β-Lg (BioPure, ∼94% pure, total protein 98% dry basis) was donated by Davisco Foods, Inc. The wet basis protein content was determined by micro-Kjeldahl to be 92.81% (AOAC, 1984).

Sample Preparation. β-Lg solutions at desired concentrations (3, 5, 7, 8, and 9% w/w solids) were prepared by dissolving the protein in deionized (DI) water with continuous stirring at room temperature for 1−2 h. Sodium azide (0.02%) was added to all samples to prevent microbial growth. Thereafter, solutions were adjusted to the desired pH of 2, 3.35, or 7 using 6 N HCl or 6 N NaOH. Following pH adjustment, solutions were heated at 85 °C for 3 h in a water bath, cooled, and stored in a 4 °C refrigerator for 24 h prior to further analysis. However, for pepsin hydrolysis, samples were preheated at 85 °C for 15 min, pH 3.35, prior to further processing as described in the next section to render β-lg accessible to pepsin hydrolysis.

Preparation of Pepsin-Hydrolyzed Samples. Native β-lg is resistant to hydrolysis by pepsin because of its compact globular structure.[30,31] Heat treatment above the denaturation temperature for 10−15 min can render it more accessible to pepsin hydrolysis.[30] Therefore, β-lg solutions (8% w/w solids), pH 3.35, were heat denatured at 85 °C for 15 min (less than the critical time for gelation under these conditions). Test solutions were then incubated with pepsin (Sigma Chemicals P-6887, porcine pepsin, 3276 units/mg solids) at 37 °C. The β-lg−pepsin mix was shaken at 130 rpm for 2, 4, 6, 24, and 48 h. Pepsin was added at an enzyme/protein ratio of 1:50, where 3276 units of protease activity was added. Control β-lg solutions, without pepsin treatment, were processed identically for comparison purposes. At least two independent replicates were performed.

Preparation of Modified β-Lg Powders. The next day, resultant sols/gels formed upon heating at pH 3.35 were quickly frozen in 100−200 mL freeze-drying glass bottles by immersion in a mixture of methanol and dry ice. Frozen samples were then freeze-dried using a 4.5 L benchtop freeze-dryer (Labconco 73035, Kansas City, MO), and the dried material was milled manually to a fine powder.

SDS-PAGE. Unheated and heated β-lg solutions were studied at pH 2.0, 3.35, and 7.0. Also, reconstituted modified β-lg powders, prepared at different preliminary protein concentrations (3, 5, 7, and 9% w/w solids), were studied. β-Mercaptoethanol (β-ME) is a denaturant that reduces disulfide bonds, and all aforementioned samples were examined under both reducing (+ 5.0% β-ME) and nonreducing (− β-ME) experimental conditions to examine disulfide bonding patterns. The β-lg samples, (±) pepsin hydrolysis, were electrophoresed under reducing conditions.

A bicinchoninic acid (BCA) assay methodology (developed by Thermo Scientific Inc., Rockford, IL) was performed on all samples prior to electrophoresis to determine the protein content. On the basis of BCA results, β-lg samples were diluted with distilled, deionized (DD) water to a uniform protein concentration of 2 mg/mL. Thereafter,

diluted β-lg samples were mixed with 8% SDS and 0.9 M Tricine−SDS sample buffer [(Invitrogen Inc., Carlsbad, CA), (±) βME, at a 1:1 v/v ratio, and then heated at 100 °C for 10 min prior to loading onto 10−20% Tris−Tricine polyacrylamide gels (Invitrogen Inc.)], similar to the procedure of Truong et al.[33] After electrophoresis, gels were stained using a colloidal blue staining reagent (Invitrogen Inc.).

Western Blotting. To confirm the identity of higher and lower molecular weight β-lg bands, Western blotting was performed. After transfer, the membranes were blocked overnight at room temperature using 3% gelatin (Bio-Rad, Inc.) prepared with phosphate-buffered saline containing 0.02% Tween (PBS/Tween). Polyclonal rabbit antibodies, directed against β-lg (Bethyl Laboratories, Montgomery, TX), were diluted 1:2000 with PBS/Tween containing 1% gelatin and incubated with the membrane for a minimum of 2−3 h at room temperature. After three washings with PBS/Tween, the membrane was incubated with the detection antibody solution. In this case, a goat anti-rabbit IgG horseradish peroxidase (HRP) conjugate (Pierce, Rockford, IL) was diluted 1:2000 with PBS/Tween/1% gelatin and incubated for an additional 2−3 h at room temperature. Again, after three rinsings with PBS/Tween, positive β-lg protein bands were visualized after the addition of the HRP substrates, diaminobenzidine and hydrogen peroxide (Pierce Inc.).

OPA Analysis. Hydrolysis of β-lg causes the formation of peptides, thus producing more reactive amino groups. A degree of hydrolysis can be linearly correlated with an increase in the concentration of reactive amino groups and quantitatively measured using an OPA assay. All samples were microcentrifuged (13600g, 5 min, room temperature) prior to analysis, and the soluble supernatant was diluted appropriately. Then, 25 μL of the sample volume was added to 2.0 mL of the OPA reagent and incubated for 5 min at room temperature, and the absorbance was read at $A_{340\ nm}$ using a Gilford 2600 spectrophotometer (Oberlin, OH). All final readings fell within an absorbency range from 0.1−1.5 at $A_{340\ nm}$, and each data point was made in triplicate. This procedure was adapted from the method of Church and co-workers.[34]

Transmission Electron Microscopy (TEM). Heated β-lg solutions (8% w/w) were prepared at pH 3.35 and 7. All samples were diluted with deionized water to a concentration of 0.04%, and the pH was adjusted to 3.35 using 6 N HCl prior to TEM analysis. TEM samples were prepared by negative staining, and a drop of diluted sample was deposited on a copper grid (Formvar coated and further coated with evaporated carbon). Excess sample was removed using filter paper. A drop of 2% uranyl acetate or phosphotungstic acid (PTA), pH 3.5, was added for 60 s and excess reagent removed. Digital electron micrographs were obtained using a FEI/Phillips EM 208S transmission electron microscope (made by Phillips, Czech Republic).

Shear Rate Sweeps. Shear rate sweeps were performed at 25 °C using a stress-controlled rheometer (ATS Rheosystems, Bordentown, NJ) to characterize flow behavior and measure the apparent viscosity of heated β-lg solutions at different pH values. Two concentrations (7 and 8% w/w solids), prepared above the identified β-lg critical concentration for the modification process (C_c ∼ 6.9% w/w solids) were selected. A smooth, 25 mm concentric cylindrical geometry was used, and shear rates were varied from 1 to 100 s^{-1} over a period of 600−900 s using a constant rate program to minimize inertial effects. Hysteresis was identified with two consecutive runs of increasing and decreasing shear rates on β-lg solutions. A preshear condition (15 s^{-1} for 30 s) was applied to all test solutions to obtain uniformity prior to measurement. Furthermore, a thin film of mineral oil was applied to the sample surface to minimize sample dehydration. To characterize flow behavior, power law model parameters were determined according to eq 1, where η is the apparent viscosity, K is the consistency coefficient, n is the power law index, and $\dot{\gamma}$ is the shear rate.

$$\eta = K\dot{\gamma}^{n-1} \tag{1}$$

At least, two independent replicates were performed for all test solutions.

1492

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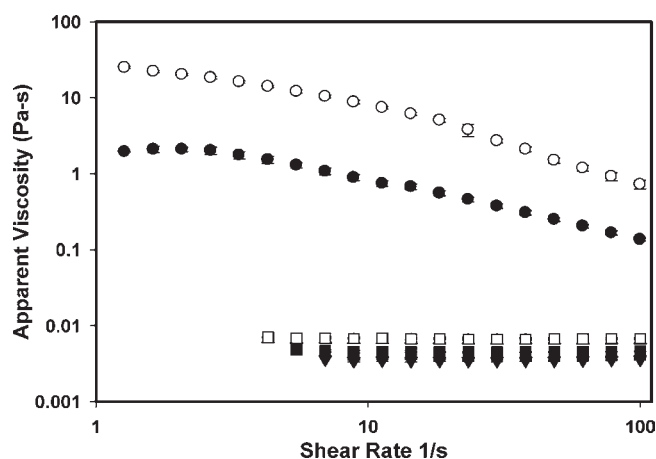
Figure 1. Apparent viscosity of heated β-lg dispersions at pH 2 ($\bullet$, $\bigcirc$), 3.35 ($\blacktriangledown$, $\triangle$), and 7 ($\blacksquare$, $\square$). Solid symbols represent 7% w/w, whereas open symbols represent 8% w/w concentration. Error bars denote one standard deviation.

■ RESULTS AND DISCUSSION

pH Effects on the Apparent Viscosity of Heated β-Lg Dispersions. To determine pH effects on the rheology of heated β-lg dispersions, shear rate sweeps were performed. The apparent viscosity of heated β-lg dispersions (85 °C, 3 h) at 7 and 8% w/w solids (above $C_c \sim 6.9\%$ w/w, pH 3.35) at pH 2, 3.35, and 7 were measured, and power law indices were calculated. A power law index (n) closer to 1 represents Newtonian flow behavior, whereas lower n values denote pseudoplasticity. Dispersions at pH 3.35 and 7 displayed more Newtonian-like flow behavior ($n > 0.98$) at both 7 and 8% (w/w) concentrations with comparable viscosities (Figure 1). On the other hand, heated dispersions at pH 2 were very viscous (2–3 logs higher than at pH 3.35), exhibiting pseudoplastic behavior ($n \sim 0.33$ at 7% w/w and $n \sim 0.17$ at 8% w/w), and formed a solid-like gel at 8% solids concentration. The apparent viscosity increased with concentration (7–8% w/w) at all pH values, with a significant increase at pH 2 ($\sim$1 log). The viscosity of a solution is affected by the shape, size of aggregates, and interactions among these aggregates. β-Lg is known to form long, linear fibrils at pH 2, which increase viscosity on a weight-efficient basis because of the large hydrodynamic volume, resulting in a reduced critical concentration for gelation.[22,23]

Role of Disulfide Interactions during Modification Process at pH 3.35 and 2 ($\pm$) βME. Disulfide interactions are favored at neutral to alkaline pH and play an important role in β-lg aggregation at pH 7.[6,10,13,14] To determine whether disulfide bonds result from the modification process at pH 3.35, SDS-PAGE was performed under reducing ($+\beta$ME) and non-reducing conditions ($-\beta$ME). Monomers, dimers, and some distinct oligomeric bands were observed in nonheated solutions at pH 3.35 (Figure 2A, lane 1). Western blotting confirmed that these high molecular weight (HMW) bands were indeed β-lg oligomers (Figure 5B). These oligomers were present in the unheated samples and could not be reduced to monomers in the presence of β-ME. This finding suggested that either β-ME/protein ratios were low or isopeptide bond formation had occurred. Increasing β-ME/protein ratios did not result in further reduction of these dimers and oligomers (data not shown), suggesting that they were linked together through isopeptide bonds. Isopeptide cross-links
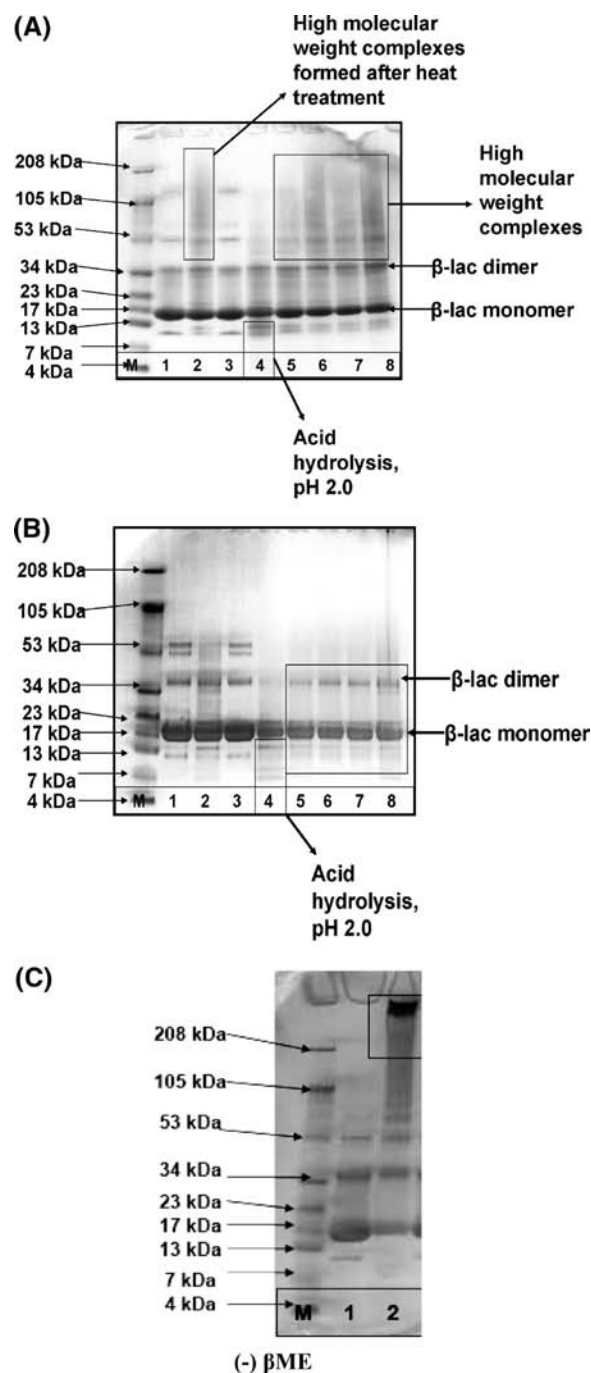
Figure 2. SDS-PAGE banding profile of unheated and heated β-lg at pH 2 and 3.35, prepared at 7% w/w (solids) and rehydrated modified β-lg powders prepared from 3, 5, 7, and 9% (w/w) β-lg in the absence of β-ME (**A**) and in the presence of β-ME (**B**). Lanes: M, marker; 1, pH 3.35 (unheated); 2, pH 3.35 (heated); 3, pH 2 (unheated); 4, pH 2 (heated); 5, mβ-lg (3% w/w); 6, mβ-lg (5% w/w); 7, mβ-lg (7% w/w); 8, mβ-lg (9% w/w). All rehydrated mβ-lg samples in lanes 5–8 had been heated at pH 3.35 during the modification process. (**C**) SDS-PAGE banding profile of unheated and heated β-lg at pH 7. Lanes: M, marker; 1, β-lg, unheated, pH 7, ($-$) βME; 2, β-lg, heated, pH 7, ($-$) βME.

are not broken with β-ME and can form between the side-chain amine of lysine and the side-chain carboxyl group of either glutamate or aspartate.[35] It has been reported that about 1–2% of the lysine in milk proteins is bound in isopeptide cross-links.[36]

As whey proteins undergo some degree of heat treatment during purification, the amount of isopeptide bonding likely increases, which may explain dimer and oligomer formation in β-lg starting materials. Formation of β-lg isopeptide bonds upon heating was previously reported as well.[37] A HMW smearing pattern (Figure 2A, lane 2) was also seen in the heated sample at pH 3.35, which disappeared in the presence of β-ME (Figure 2B, lane 2), suggesting that these complexes were most likely linked through disulfide bonds. These results confirmed that disulfide-linked aggregates were formed during the modification process (85 °C, 3 h of heating, pH 3.35). Smearing between β-lg dimers and trimers was also seen indicative of the formation of intermediate-sized proteins based on limited acid hydrolysis of the protein under these conditions.

In the nonreducing gel, dimers and some distinct oligomeric bands were also observed in unheated β-lg at pH 2 (Figure 2A, lane 3), further supporting the hypothesis that oligomers were present in the starting material, whereas in heated samples at pH 2 (Figure 2A, lane 4), no continuous smearing of high molecular weight complexes was observed, suggesting no disulfide aggregate formation. For heated samples at pH 2.0, oligomers disappeared under both reducing and nonreducing conditions (Figure 2A, lane 4), with some loss of dimers under reducing conditions (Figure 2B, lane 4). Several distinct bands of low molecular weight were also seen, likely caused by acid hydrolysis during extensive heating of samples at pH 2 (85 °C, 3 h) (Figure 2, lane 4). A few distinct lower molecular weight bands were also observed at pH 3.35, further suggesting that some degree of acid hydrolysis took place under these experimental conditions (Figure 2B, lane 2). Although acid hydrolysis occurred at both pH 3.35 and 2 as expected, it appeared to be more effective at pH 2. Seemingly, the disappearance of dimers and oligomers in the presence of β-ME is a combination of two reaction processes, that is, (1) disulfide bond reduction and (2) acid hydrolysis. Because some dimeric and oligomeric bands were present after heating under nonreducing conditions (Figure 2A, lanes 2 and 4), they did disappear to some extent in the presence of βME (Figure 2B, lanes 2 and 4), and it is apparent that the dimers and oligomers were held by both disulfide and isopeptide linkages. There is recent additional evidence of acid hydrolysis and its role in fibril formation at pH 2 by independent investigators.[21,22] Moreover, it was reported that peptides resulting from acid hydrolysis at pH 2 participated in β-lg fibril formation.[32]

In an earlier study, a critical concentration (C_c) was identified as 6.9% w/w solids (using a β-lg model system, pH 3.35).[3] No significant thickening function was achieved using modified powders prepared at starting concentrations below the C_c, regardless of reconstituting concentration.[3] To understand these concentration-dependent differences, SDS-PAGE was performed on modified β-lg powders prepared at two solids concentrations below (3 and 5% w/w) and two above (7 and 9% w/w) the C_c. HMW complexes were observed in all modified β-lg samples at different starting solids concentrations, as evidenced by the banding patterns seen in the nonreducing gel (Figure 2A, lanes 5−8). These complexes were much less prominent in the reducing gel, containing β-ME, following reduction of disulfide bonds. Similar protein band profiles were obtained for all modified samples, irrespective of the initial solids concentration from which they were manufactured. Previously, concentration-dependent differences in thickening functionality were clearly established by Mudgal et al.,[3] in which case, those results combined

with the present observations strongly suggest that in addition to disulfide bonds, noncovalent interactions most likely play a crucial role in defining the functionality of subsequently prepared modified β-lg dried powders as well as modified whey ingredients per se at pH 3.35.
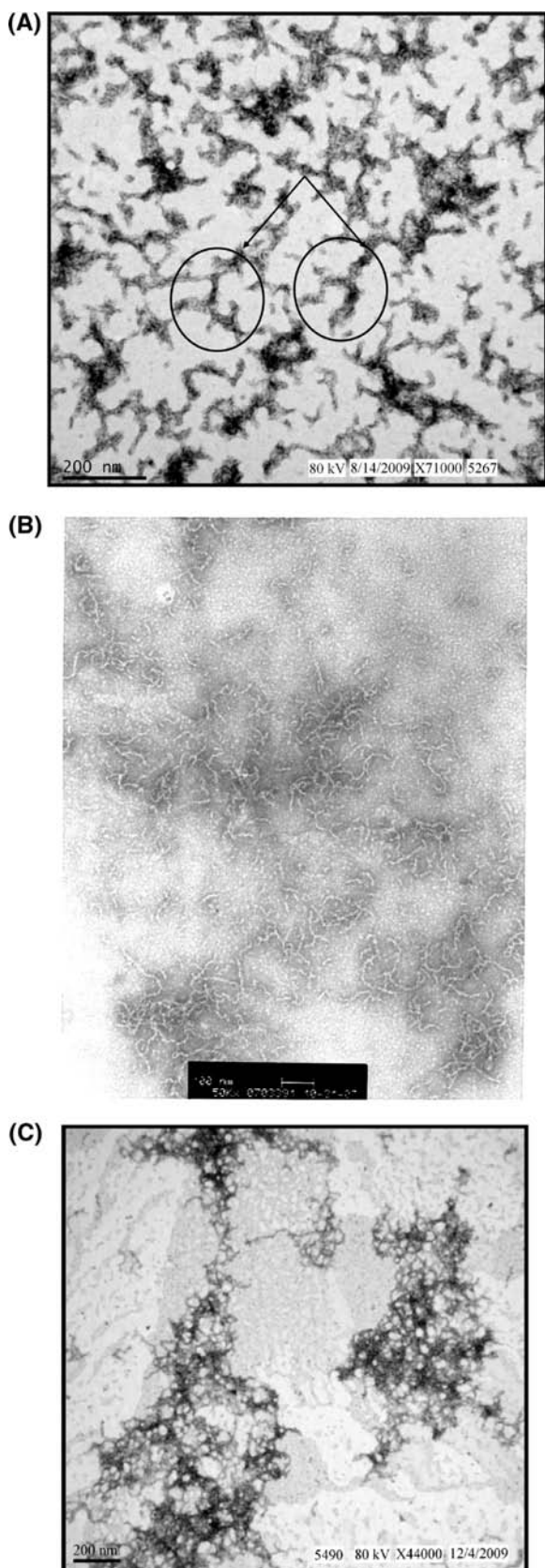
Finally, SDS-PAGE results at low pH (2 and 3.35) were compared to those at pH 7.0 under nonreducing conditions (Figure 2C). These patterns revealed formation of very high molecular weight complexes at pH 7.0, as compared to either pH 3.35 or 2.0, and may be attributed to more favorable disulfide interactions at higher pH as thiol ($-SH-$) groups per se are less stable at alkaline pH (Figure 2C).

Structural Differences at pH 7 and 3.35 As Observed through TEM. TEM was accomplished to compare network characteristics of heated β-lg dispersions at pH 7 and 3.35, as these systems displayed similar viscosities versus those at pH 2 (Figure 3A,B). At pH 7, repeating structural units were connected, and β-lg strands were visible within these structures (Figure 3A), whereas fine-stranded flexible fibrillar structures were observed at pH 3.35, 8% w/w (Figure 3B). Langton and Hermansson[4] reported differences in strand characteristics above pH 6 from those below pH 4.

Network characteristics of heated β-lg pepsin hydrolysates (24 h) were also investigated using TEM. As observed in Figure 3C, large fine-stranded aggregates composed of flexible fibrils were formed. Presumably, the formation of these larger microgels caused an increase in the apparent viscosity of heated pepsin hydrolysates due to an increased size of the aggregates, together with stronger interparticle interactions as discussed in the next section.

Pepsin Hydrolysis of β-Lg and the Effect on Apparent Viscosity of Heated β-Lg Dispersions at pH 3.35. In recent studies, it was shown that acid hydrolysis of β-lg at pH 2 played an important role in fibril formation.[22,32] In the present work, acid hydrolysis was detected at pH 2, with lesser hydrolysis at pH 3.35, based on SDS-PAGE results. Using this information, together with viscosity data, acid hydrolysis of β-lg at low pH was hypothesized to promote thermal aggregation, which in turn raised the viscosity of heated protein dispersions. To further address this idea, β-lg was hydrolyzed with pepsin at pH 3.35 to determine the effects of additional enzymatic cleavage on the flow properties of heated β-lg solutions.

Preheat treatment at 85 °C for 15 min rendered β-lg more susceptible to pepsin cleavage with no significant increase in the viscosity at pH 3.35. Pepsin-treated β-lg and the control, both held at 37 °C for 24 h, exhibited similar flow behaviors and displayed Newtonian flow. Because the viscosities of native β-lg, preheated β-lg (85 °C,15 min), and pepsin-hydrolyzed β-lg (37 °C, 24 h) were very similar, only the data for pepsin-hydrolyzed β-lg samples is shown in Figure 4. After 24 h of incubation with the enzyme, an increase of approximately 12% in the concentration of free amino groups was measured, compared to that of the control samples, based on OPA assay measurements (data not shown). No further increase was seen after 48 h; however, the viscosity of pepsin$-\beta$-lg was higher than that of the 24 h samples (Figure 4). This outcome might be attributed to the formation of additional aggregates, generated during hydrolysis of β-lg for longer periods. The 48 h pepsin-treated sample also displayed pseudoplasticity ($n \sim 0.88$) as explained earlier. The apparent viscosity of pepsin-hydrolyzed β-lg solutions (24 and 48 h) increased significantly ($\sim$2 logs) after heating as compared to control samples at pH 3.35 and exhibited similar flow parameters after heating (Figure 4). Pepsin-treated β-lg also displayed a
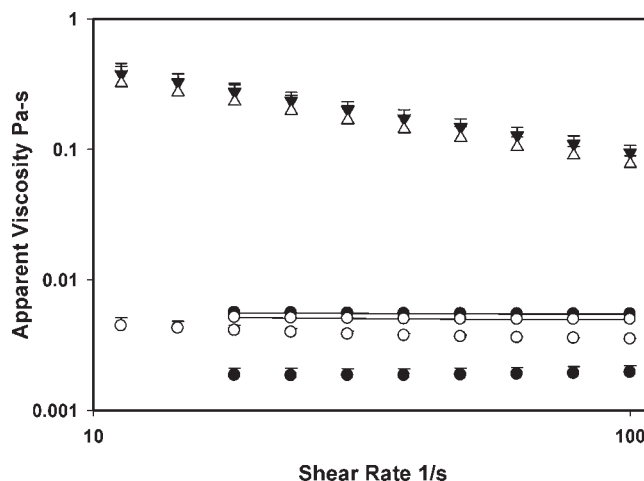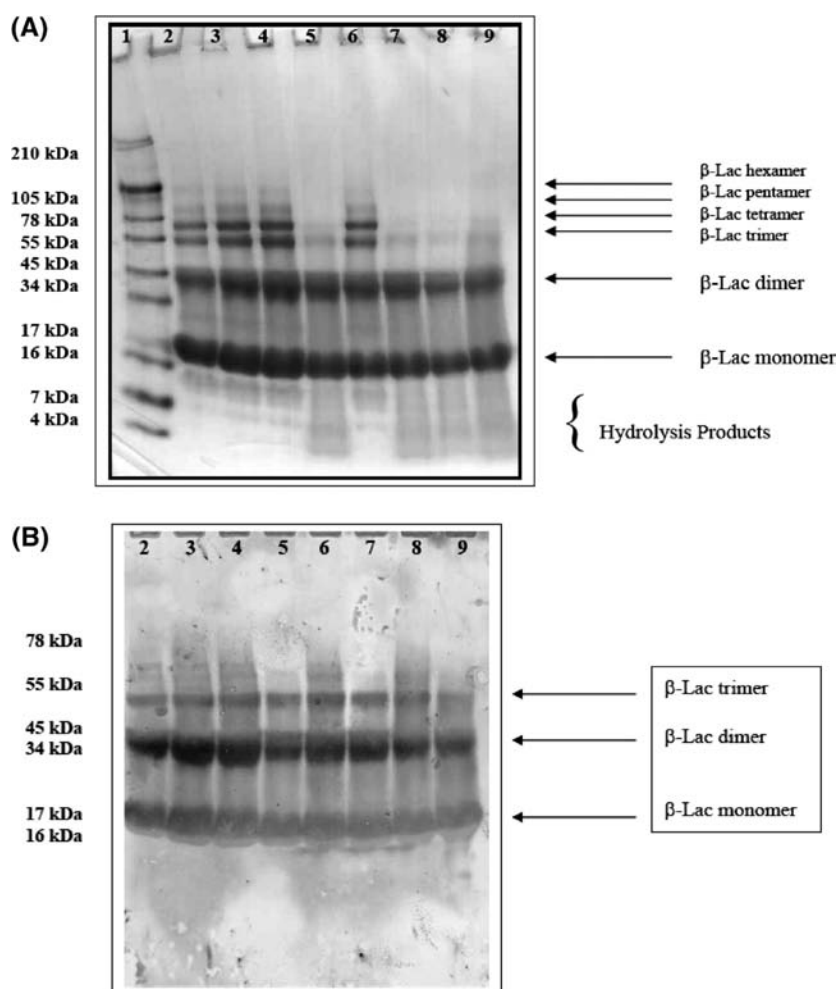
Figure 4. Apparent viscosity of pepsin-hydrolyzed β-lg, compared with control ($\pm$) extended heating (85 °C, 3 h, pH 3.35): ($\bullet$) pepsin-hydrolyzed β-lg (24 h); ($\bigcirc$) pepsin-hydrolyzed β-lg (48 h); ($\blacktriangledown$) heated pepsin-hydrolyzed β-lg (24 h); ($\triangle$) heated pepsin-hydrolyzed β-lg (48 h); ($\bullet$ with lines) heated β-lg (pepsin negative, 24 h); ($\bigcirc$ with lines) heated β-lg (pepsin negative, 48 h). β-Lg solids concentration was 8% w/w for all samples. All β-lg samples ($\pm$ pepsin) were incubated at 37 °C for either 24 or 48 h prior to the extended heating. "Heated" refers to extended heating at 85 °C for 3 h. Error bars denote one standard deviation.

higher degree of pseudoplasticity ($n \sim 0.42$) upon heating, indicating stronger interactions and possibly the formation of very large aggregates. Aggregate formation after enzymatic hydrolysis at pH 7 was reported in past research,[24,25] and improved gelation of β-lg protein solutions after proteolysis was previously reported at neutral pH as well.[24,26,27]

Analysis of the Molecular Weight Distribution of Aggregates Formed during Heating of Pepsin Hydrolysates Using SDS-PAGE. To confirm that additional hydrolysis of β-lg occurred with pepsin, SDS-PAGE experiments were performed. Pepsin-treated β-lg (24 h, 37 °C) revealed the generation of a new protein band exhibiting a molecular weight of <4 kDa (Figure 5A). Furthermore, larger forms of the protein substrate (tri-, tetra-, and oligomers) significantly diminished after pepsin digestion (Figure 5A), and it appeared that pepsin was more effective in cleaving these larger complexes versus monomeric or dimeric forms.

Continued incubation with pepsin for a total of 48 h at 37 °C revealed a protein staining pattern that was essentially identical to the one seen with the 24 h pepsin-treated β-lg. These results suggested no further hydrolysis as confirmed using the OPA assay method (data not shown). Subsequent heating of the control sample (24 h, 37 °C) at 85 °C for 3 h also produced a similar banding pattern and revealed the generation of smaller sized β-lg fragments; however, in this case, these products were generated via acid hydrolysis (Figure 5A). Again, a concomitant loss of higher molecular sized forms of β-lg was noted. Taken together, the hydrolytic end products that were generated after pepsin digestion represented a combination of acid/protease -released fragments which exhibited a significantly higher apparent viscosity compared to the effects that occur on the basis of acid hydrolysis alone.

Confirmation of β-Lg Banding Patterns Using Western Blotting. Western blotting analysis confirmed distinct staining of β-lg monomers, dimers, and trimers with a faint detection of

Figure 3. TEM micrographs of heated β-lg dispersions at pH 7 (**A**) and pH 3.35 (**B**) and TEM micrograph of pepsin hydrolyzed β-lg (24 h, 37 °C) (**C**). All samples were heated at 8% (w/w) solids, 85 °C for 3 h. Scale bar is 200 nm in **A**, **C**, and 100 nm in **B**.

1495

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Figure 5. SDS-PAGE of pepsin hydrolysis of β-lg, pH 3.35 (**A**), and Western blotting of pepsin hydrolysis of β-lg (**B**). Lanes: 1, marker; 2, untreated β-lg; 3, preheated at 85 °C, 15 min; 4, control, 24 h, 37 °C; 5, (+) pepsin 24 h, 37 °C; 6, control 48 h, 37 °C; 7, (+) pepsin 48 h, 37 °C; 8, control 24 h, heated at 85 °C, 3 h; 9, (+) pepsin (24 h) heated at 85 °C, 3 h.

oligomers (Figure 5B). Thus, the identity of β-lg antigen, associated with each sized protein species, was confirmed, although the antiserum did not bind to the smaller end products generated either by acid hydrolysis or by cleavage with pepsin. Likely, the small size of the β-lg fragment was too small to elicit antibody binding activity.

Proposed Mechanism for Cold-Thickening Mechanism of β-Lg at Acidic pH. At low pH, such as 2 and 3.35, β-lg is positively charged and undergoes electrostatic repulsion. During heating, conformational changes take place, and buried hydrophobic residues become solvent accessible. Because aggregation and thickening at low pH are observed only during heating, hydrophobic interactions appear to play an important role. At a pH value near the isoelectric point, particulated networks with random aggregates are formed, whereas at pH away from the isoelectric point, fine-stranded networks are formed because of controlled and limited aggregation. Therefore, at low pH, aggregation appears to result from a subtle balance among electrostatic and hydrophobic interactions.

Independently, hydrolytic reactions result in the formation of smaller fragments, which alter the overall protein conformation; therefore, hydrophobic interactions and charge distributions affect aggregation phenomena. Acid hydrolysis is favored at low pH,

whereas disulfide interactions are favored at higher pH. Therefore, the aggregation mechanism of β-lg under various pH conditions becomes a combination of all these factors, thus affecting network characteristics, particle size, interparticle interactions, and, finally, the rheology of heated protein dispersions.

■ AUTHOR INFORMATION

Corresponding Author
*E-mail cdaubert@ncsu.edu; phone (919) 513-2092; fax (919) 515-7124.

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1497

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