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Interactions between β-lactoglobulin and dextran sulfate at near neutral pH and their effect on thermal stability

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ABSTRACT

The effect of interactions between β-lactoglobulin (β-LG) and dextran sulfate (DS) on thermal stability at near neutral pH was investigated. Samples containing 6% w/w β-LG and DS (M_w = 5–500 kDa) at different biopolymer weight ratios, pH (5.6–6.2), and NaCl concentrations (0–30 mM) were heated at 85 °C for 15 min. Turbidity results showed that the presence of DS at appropriate biopolymer weight ratio and pH significantly lowered the turbidity of heated β-LG. Solutions containing DS:β-LG weight ratios of 0.02 or less showed improved heat stability as indicated by decreased turbidity. Analysis of the unheated mixture by size exclusion chromatography coupled with multi-angle laser light scattering (SEC–MALLS) showed an interaction between β-LG and DS. The size of the aggregates increased as pH decreased. The β-LG–DS aggregates had a greater negative charge as seen from electrophoretic mobility measurement. Addition of 30 mM NaCl inhibited complex formation and the effect of DS on reducing the turbidity of heated β-LG, suggesting that the interaction was electrostatic in nature. Other than charge property, the amount and size of native aggregates appeared to be the major factor in determining how DS altered heat-induced aggregation. The presence of DS decreased denaturation temperature of β-LG, indicating that DS did not improve thermal stability of β-LG by stabilizing its native state but rather by altering its aggregation. The results provide information that will facilitate the application of whey proteins and polysaccharides as functional ingredients in foods and beverages.

1. Introduction

The functional beverages market is rapidly growing and this trend will continue for some time. Projected growth in sale of functional beverages is expected to be close to US$31 billion by 2010. Functional dairy products, along with fruit juices, hold the strongest position in terms of market segments (Lucas, Reed, & Van Goethem, 2007). Whey proteins are among the major ingredients used in sports drinks. In order to retain clarity in beverages, whey proteins must remain dispersed and soluble. The main factor that limits the use of whey proteins in beverages is the loss of protein stability after thermal processing. In this context, heat stability refers to the ability to maintain clarity and a single-phase fluid system after thermal processing. Therefore, it involves the extent of denaturation and aggregation rather than preventing the protein from denaturation. Heat denaturation and aggregation of β-lactoglobulin (β-LG), the major protein in whey, has been extensively studied (Elofsson, Dejmek, & Paulsson, 1996; Gimel, Durand, & Nicolai, 1994; Griffin, Griffin, Martin, & Price, 1993; Hoffmann, Sala, Olieman, & de Kruif, 1997; Hoffmann & vanMil, 1997; Iametti, Cairoli, Degregori, & Bonomi, 1995; McSwiney, Singh, Campanella, & Creamer, 1994; Roefs & de Kruif, 1994; Schokker, Singh, Pinder, Norris, & Creamer, 1999; Verheul, Roefs, & de Kruif, 1998). During heating, β-LG undergoes intramolecular and intermolecular changes. At near neutral pH, raising the temperature shifts the β-LG monomer–dimer equilibrium at β-LG concentrations below 10 g/L towards monomers (Verheul, Pedersen, Roefs, & De Kruif, 1999). Above 60 °C, the molecule undergoes conformational changes and partially unfolds (i.e. denatures); nonpolar groups and the thiol group are exposed and become available for intermolecular interactions (Iametti, DeGregori, Vecchio, & Bonomi, 1996). Denaturation can be followed by an irreversible aggregation. Aggregates are formed via intermolecular thio-catalysed disulfide bond interchange and non-covalent interaction. Factors affecting the aggregation of β-LG include concentration (Iametti et al., 1995, Qi, Brownlow, Holt, & Sellers, 1995), genetic variants (Holt et al., 1998; Huang, Catignani, Foegeding, & Swaisgood, 1994), pH (Hoffmann & vanMil, 1997; McSwiney, Singh, & Campanella, 1994; McSwiney, Singh, Campanella, & Creamer et al., 1994), temperature (Roefs & de Kruif, 1994), and ionic strength as well as the nature of the ions (McPhail & Holt, 1999).

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Polysaccharides are commonly used in protein beverages to increase stability and their effectiveness depends on how the polymers interact in solution. In diluted biopolymer solutions, mixing entropy dominates and mixtures of proteins and polysaccharides may be co-soluble (Schmitt, Sanchez, Desobry-Banon, & Hardy, 1998). Upon increasing the concentration of the biopolymers, two phase separation phenomena can be observed, depending on the type of the interactions between the different biopolymers and the solvent. The first one is called segregative phase separation or thermodynamic incompatibility. It appears when there is a net repulsion between the biopolymers, and the system demixes into two phases, one rich in protein and the other rich in polysaccharide (Schmitt et al., 1998). The second phase separation phenomenon is the associative phase separation or coacervation. It happens when proteins and polysaccharides show net attraction, usually through electrostatic interactions when they have oppositely charged groups, giving rise to the formation of protein–polysaccharide complexes (Bungenberg de Jong, 1949). Complexation can yield either the formation of soluble complexes or an associative phase separation having the following composition: a rich solvent phase and a rich biopolymer(s) phase forming the coacervate (Tolstoguzov, 1991).

Soluble protein–polysaccharide complexes can be produced if both biopolymers carry a net negative charge ($pH > pI_{protein}$) (Dickinson, 1998). Here, the attraction involves positively charged local patches on the protein interacting with the anionic polysaccharide (Park, Muhoberac, Dubin, & Xia, 1992). The main positively charged residues in most proteins are the $-NH_3^+$ groups. At low ionic strengths, sulfated polysaccharides of relatively high charge density can form fairly strong reversible complexes with proteins, even at pH well above pI (Dickinson, 1998). Using potentiometric titration and ultrafiltration, Girard, Turgeon, and Gauthier (2002) found that low- and high-methylated pectin formed complex with β-LG at pH 7.0. The effect of sodium chloride, urea, and temperature on complex formation suggested that the main interactions were electrostatic forces, and to a lesser extent, hydrogen bonding. Around neutral pH, bovine serum albumin (BSA) forms soluble complexes with three sulfated polysaccharides: dextran sulfate, ι-carrageenan, and κ-carrageenan as shown by size exclusion chromatography (Galazka, Smith, Ledward, & Dickinson, 1999). The complexes are dissociated by adding an electrolyte (20–100 mM NaCl), demonstrating the electrostatic character of the complexes.

A number of reviews and investigations have studied the effects of protein–polysaccharide interaction on gelation and phase behavior (Doublier, Garnier, Renard, & Sanchez, 2000; Mekhloufi, Sanchez, Renard, Guillemin, & Hardy, 2005; Schmitt et al., 1998; Tuinier, Dhont, & de Kruif, 2000; Weinbreck, Nieuwenhuijse, Robijn, and de Kruif (2004)); however, much less work focuses on increasing stability after heating especially at high-protein concentration. The beneficial consequence of the complexation is the protection against loss of solubility of protein after heating (Imeson, Ledward, & Mitchell, 1977; Ledward, 1979) or high pressure treatment (Galazka, Ledward, Sumner, & Dickinson, 1997; Galazka et al., 1999). Carrageenan has been used for years to control the texture and stability of dairy products. Stabilization of calcium-sensitive caseins at the pH of milk can be achieved by carrageenan. An electrostatic complex occurs between κ-carrageenan and κ-casein without specific cations (Snoeren, Payens, & Jevnink, 1975), while a complex between κ-carrageenan and α_{s1}casein or β-casein requires the presence of calcium (Ozawa, Niki, & Arima, 1984, Skura & Nakai, 1981). Strong electrostatic interaction between dextran sulfate (DS) and BSA (1 mg/ml) facilitated the denaturation of BSA but suppressed the aggregation by preventing further oligomerization/aggregation process of denatured protein (Chung et al., 2007). Our previous work showed that at pH 6.8 the presence of low ratios of sulfate-containing polysaccharide (DS and λ-carrageenan) to β-LG (3–9% protein) decreased heat-induced aggregation as shown by lower turbidity (Zhang & Foegeding, 2003). Differential scanning calorimetry (DSC) indicated that the denaturation temperature of β-LG was 4.6 °C higher in the presence of DS, as compared with β-LG alone, whereas in the presence of λ-carrageenan the difference was about 1.2 °C (Zhang, Foegeding, & Hardin, 2004). Increasing the ratio of polysaccharide to β-LG, however, induced a significant increase in turbidity, leading to segregative phase separation. DS was also found to increase heat stability of β-LG (6% protein, pH 6.8) in the presence of 30 and 40 mM NaCl as shown by a decrease in turbidity and an increase in solubility (Vardhanabhuti & Foegeding, 2008).

Previous investigations were conducted at pH values where segregative rather than associative conditions would be favored. In this investigation, we expand to lower pH that would favor an associative mechanism. We investigated the interactions between β-LG and DS at pH 5.6–6.2 A range of molecular size of dextran sulfate and different biopolymer ratio were studied to identify regions of stabilization and destabilization. Thermal stability, defined here as the ability of protein to survive the thermal process without detrimental change, was evaluated by turbidity measurement. To gain a more extensive understanding of how dextran sulfate alters thermal stability, thermal denaturation was measured by differential scanning calorimetry and the interactions between the two biopolymers were characterized by size exclusion chromatography coupled with multi-angle laser light scattering and electrophoretic mobility measurement.

2. Materials and methods

2.1. Materials

β-LG was the gift from Davisco Foods International (Le Sueur, MN) and contained 91.64% protein based on micro-Kjeldahl nitrogen analysis ($N \times 6.38$ for β-LG). The mineral composition was determined in duplicate by inductively coupled plasma atomic emission spectroscopy (Optima 2100 DV, Perkin-Elmer, Ontario, Canada). The concentrations of Na^+, Ca^{2+}, P^+, K^+, and Mg^{++} were 8217, 1.7, 5.8, 0.4, and 0.2 ppm, respectively. DS with average M_w of 5 k, 10 k, 100 k, and 500 k were purchased from Sigma Chemical Co. (St Louis, MO). All other chemicals were of analytical grade. β-LG stock solution (15% w/w) and DS stock solution (4% w/w) were prepared by slowly dissolving the protein or polysaccharide powder in ~90% of the total deionized water (>17 MΩ). The solutions were stirred at room temperature and left overnight in the refrigerator for complete hydration. On the next day, the solutions were warmed to room temperature, and after final weight adjustment, the solutions were filtered through 0.45 μm pore size filters (Vardhanabhuti & Foegeding, 2008).

Stock solutions of β-LG and DS were mixed with NaCl solutions and deionized water at the appropriate amount such that the total weight of the mixed solutions was about 90% of the final weight. All samples contained 6% w/w β-LG and DS at DS:β-LG weight ratios ranging from 0 to 0.2. The effect of electrostatic interaction was investigated by adjusting the amount of NaCl from 0 to 30 mM. The pH of the mixed solutions was adjusted from 5.6 to 6.2, and deionized water was added to produce designated protein concentration, polymer ratios, and NaCl concentrations. The pH of the final solution was checked to assure proper pH. The mixture solutions were heated in test tubes at 85 °C for 15 min. The tubes were cooled in ice water and moved to room temperature for measurements.

2.2. Turbidity measurement

Thermal stability was evaluated by measuring the optical density at 400 nm ($OD_{400\ nm}$) on a Shimadzu UV-160 U spectrophotometer (Shimadzu Corporation, Tokyo, Japan). Water was used as the reference. All experiments were replicated at least twice.

2.3. Size exclusion chromatography and multi-angle laser light scattering (SEC–MALLS)

Separation was carried out by size exclusion chromatography using a Shodex Protein KW-804 column (Showa Denko K.K., Tokyo, Japan) attached to a Waters HPLC system (Waters Corp., Milford, MA). Solutions containing 20 mM imidazole and 0.05% NaN_3 at pH 5.6, 5.8, 6.0 or 6.2 were used as diluent buffer. For the investigation of the effect of NaCl, 10 or 30 mM NaCl were included in the imidazole buffer. Unheated samples were diluted with imidazole buffer (at corresponding pH) to a concentration of 5 mg/ml and filtered through a 0.45-μm filter (PVDF, Millipore Corporation, Bedford, MA). Sample injection volume was 100 μl, flow rate was 0.6 ml/min and separation was performed at 30 °C column temperature. Peak analysis was conducted by a photo diode array (PDA) detector (Waters Corp., Milford, MA, model 2996) operating at 280 nm. A DAWN-EOS MALLS photometer (Wyatt Technology, Santa Barbara, CA) fitted with a helium–neon laser ($\lambda = 690$ nm) and a K5-flow cell, combined with a differential refractive index (RI) detector (Waters Corp; model 2414) was used for calculation of molecular mass and size of proteins. The RI detector was used as the concentration detector. The weight average molecular mass (M_w) and the mean square radius of materials were calculated with a first order Debye fit, using the refractive index increment (dn/dc) of 0.185 (Huglin, 1972) for all samples. Light scattering data from MALLS were processed with ASTRA software (version 5.1.5.3) (Wyatt Technology, Santa Barbara, CA).

2.4. Electrophoretic mobility

Protein solutions (3% w/w) were prepared in 5 mM phosphate buffer with 0.02% NaN_3 at different pH (5.8, 6.0, and 6.2) and DS:β-LG ratios (0, 0.02, 0.05, 0.1, and 0.2). Unheated samples were measured at 3% protein without further dilution. For heated samples, they were heated at 85 °C for 15 min in a temperature-controlled water bath and diluted at 1:1 (v/v) ratio with buffer at respective pH before measurements. The solutions were placed in a standard four-sided, 1 cm polystyrene cuvette and a parallel plate electrode (0.45 cm^2 square platinum plates with a 0.4 cm gap) was inserted. The cuvette was placed in a temperature-controlled holder (20 °C). The electrophoretic mobility was measured by phase analysis light scattering (ZetaPALS, Brookhaven Instruments, Holtsville, NY). Each measurement was the average of 200 (10 sets of 20) measurements and the entire experiment was conducted in triplicate.

2.5. Nano-differential scanning calorimetry

β-LG samples with or without DS were diluted to 0.5 mg/ml with 5 mM phosphate buffer having the same pH value as that of the samples. Thermodynamic properties of β-Lg were determined using an N-DSC II differential scanning calorimeter (TA Instruments previously Calorimetry Sciences Corporation, Provo, UT) at the scanning rate of 1 °C/min for a temperature range from 25 to110 °C. The protein solution was filled into the sample cell, the reference buffer in the reference cell, and a constant pressure of 3 atm was applied. For the curve analysis, buffer–buffer tracings were recorded under the same conditions and subtracted from the sample endotherms. Subsequently, curve analysis using the converter object of the CpCalc software (TA Instruments previously Calorimetry Sciences Corporation, Provo, UT) led to the excess heat capacity curve and the determination of the transition temperature at peak maximum (T_m) as well as the calorimetric enthalpy change (ΔH_m) of the unfolding process normalized for protein concentration. ΔH_m was obtained by integration of the area of the excess heat capacity endotherm.

3. Results and discussion

Based on a market survey conducted in our laboratory, of the 18 whey protein-fortified beverages, the protein content ranged between 4.2 and 7%, with an average of 6% (data not shown). β-LG concentration in this study was chosen to be at 6% (w/w) to represent high-protein beverages.

3.1. Turbidity of heated β-LG in the absence of NaCl

The optical density profiles (at 400 nm) as a function of pH of mixtures consisting of 6% w/w β-LG and DS (100 k) at different biopolymer weight ratios (from 0 to 0.2) in the absence of NaCl are shown in Fig. 1. In the absence of DS, the aggregation of heated β-LG

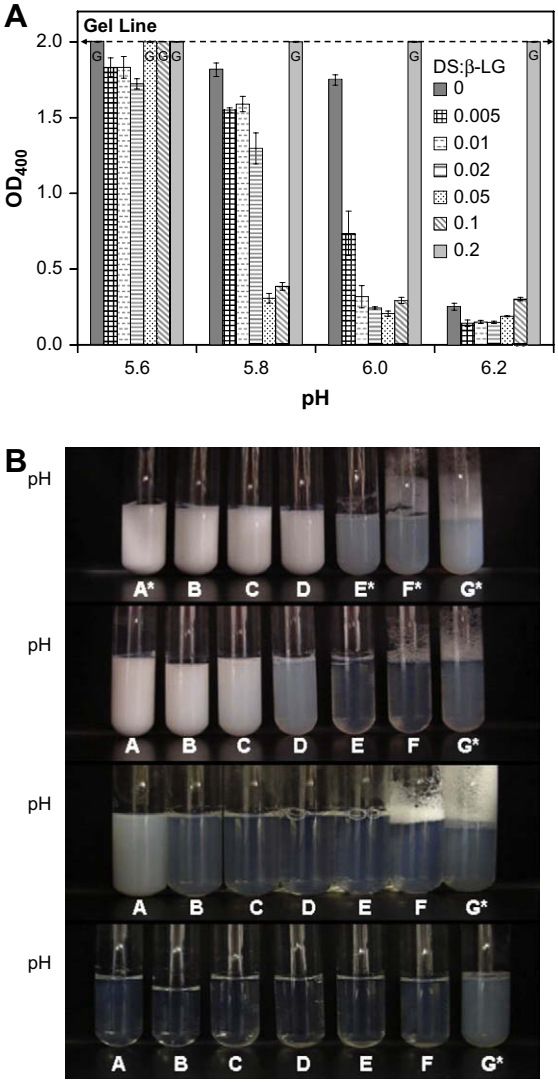

Fig. 1. Effect of pH and biopolymer weight ratio on the aggregation of β-LG. G indicates gelation of the samples.

was clearly dependent on pH. At pH 6.2, heated β-LG control appeared slightly turbid ($OD_{400} = 0.252 \pm 0.023$). Lowering the pH resulted in higher degree of aggregation as shown by an increase in turbidity, and at pH 5.6, heated β-LG control formed opaque gel. As pH approaches the isoelectric point (pI), intermolecular repulsion will be minimized, thereby enhancing molecular aggregation via short-range interactions such as hydrophobic and Van der Waals (Xiong, Dawson, & Wan, 1993).

The effect of DS on altering heat aggregation of β-LG was dependent on pH and biopolymer ratio (Fig. 1). The most noticeable effect of DS was seen at pH 5.8 and 6.0, and the highest decrease in turbidity was at pH 6.0 where only a small amount of DS (0.001 DS:β-LG mole ratio) reduced the turbidity by more than half of that of heated β-LG control. It should be noted that longer heating (2 h) slightly increased the turbidity of both β-LG control and β-LG with DS (data not shown). The turbidity of heated samples containing DS after 2 h was still much lower than heated β-LG control, indicating that the difference in turbidity of heated β-LG without and with DS was not due to differences in the kinetics of the reaction. At pH 5.6, heated samples remained very opaque even with DS addition due to less charge repulsion between molecules because of the pH being close to the pI. Only a small increase of pH to 6.2 resulted in higher charge repulsion and lower turbidity of β-LG control, so the effect of DS was minimal. The observed effect of pH on the ability of DS to alter heat aggregation of β-LG at 6% w/w was similar to that seen in BSA at 0.1% w/v (Chung et al., 2007). They found that DS lowered the turbidity of heated BSA at pH 5.1 and 6.2 but not at pH 7.5. The pH dependent behavior suggested that the interaction between proteins and polysaccharides was electrostatic driven.

Addition of DS at low concentrations decreased the turbidity of heated β-LG at all pH values (Fig. 1). Higher DS concentration led to an increase in turbidity and gelation of the samples even at pH 6.2. Several possible phenomena could occur at high DS concentration leading to loss of thermal stability. First, the biopolymers could phase separate at higher polymer concentration due to thermodynamic incompatibility from the competition for solvent between macromolecules (Tolstoguzov, 1986). Second, soluble complexes between the biopolymers could crosslink during heating, resulting in turbidity increase and gelation. In this study, phase separation was not observed (i.e. no increase in turbidity or two layers separation) in unheated samples even at highest DS concentration (0.2 DS:β-LG). Therefore, destabilization was caused by reactions occurring during heating. Loss of heat stability in samples containing higher DS concentration was different from the heated β-LG control. The heated β-LG control samples were opaque and formed gels only at pH 5.6, while samples with the highest amount of DS were translucent gels at all pH values (Fig. 1B). Dextran sulfate alone at these concentrations and pH values did not form a gel. It is more likely that heating forms a combination of β-LG and β-LG/DS aggregates at high DS concentrations that interact and form a gel network. The general ability of DS to alter heat stability of protein has been observed; however, previous investigations were either conducted at low protein concentration (0.1% w/v, Chung et al., 2007) or the polysaccharide only caused the small decrease in turbidity (Chung et al., 2007; Vardhanabhuti & Foegeding, 2008; Zhang & Foegeding, 2003). This study revealed a large reduction of turbidity of heated β-LG even at high-protein concentration.

Molecular size of polysaccharides is one of the major factors influencing their role in altering protein aggregation and phase stability (Schmitt et al., 1998). At pH 6.0, the most effective weight ratio of different M_w of DS was similar; however, 100 k DS appeared to be the most effective in lowering the turbidity of heated 6% β-LG as shown by the lowest turbidity values (Fig. 2). Though the optimum weight ratio of 5 k and 100 k DS was also at 0.05 DS:β-LG, they were less effective at some biopolymer ratios compared to

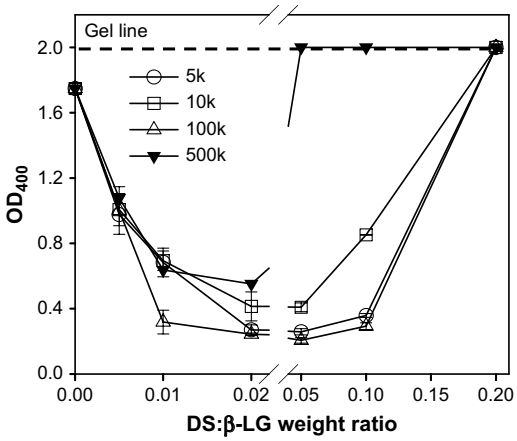

Fig. 2. Effect of DS molecular weight on β-LG aggregation. Dash line indicates gelation of the samples.

100 k DS. The highest M_w (500 k) DS had the most narrow range of effective weight ratios in decreasing β-LG aggregation. The narrow range of effective weight ratio of high M_w of polysaccharide on inhibiting β-LG aggregation was also observed at pH 6.8 by Zhang et al. (2004). In theory, an increase in the molecular weight of the biopolymers is expected to lower the biopolymer compatibility in solution by lowering the combinatorial entropy of mixing (Schmitt et al., 1998). This could explain why 500 k DS exhibited narrow range of effective weight ratio. Using light scattering, Semenova (1996) suggested that increasing dextran M_w favored coacervation with soy globulin because larger size polysaccharide was more accessible for the protein. This may explain the better effect of 100 k DS over the lower M_w DS at low DS:β-LG ratio but not at high biopolymer ratio. It is likely that charge density and charge distribution along the DS chain other than molecular size also play an important role in inhibiting or altering protein aggregation.

3.2. SEC–MALLS

The chromatograms of native β-LG control alone and dextran sulfate alone are presented in Fig. 3. Native β-LG peak eluted around 18 min from the photo diode array (PDA) and refractive index (RI) detectors (Peak 1). Dextran sulfate does not absorb at 280 nm; therefore, it only appeared at the baseline in Fig. 3A. As expected with polysaccharides, chromatograms of DS from RI and MALLS detectors (not shown) showed that DS sample did not contain molecules of single size but rather a distribution of sizes with the weight average molecular mass of 100 k, based on the MALLS calculation.

When DS was mixed with native β-LG (Fig. 4), there was a reduction in the β-LG peak and an increase in the peak eluted between 10 and 16 min on all detectors (peak 2). This indicated that the addition of DS led to a decrease in β-LG and a formation of large aggregates. It is likely that these large aggregates were complexes between β-LG and DS. Though both biopolymers carried the net negative charge at pH 6.0, DS could interact with positive patches on β-LG molecules and form complexes (Park et al., 1992). The higher the amount of DS, the higher the amount of complex formation. The weight average molecular mass (M_w) distribution of β-LG with and without DS at pH 6.0 is shown in Fig. 5. It can be seen that 90% of β-LG control samples had an M_w of 3.5×10^4 or less, indicating that it was mainly dimeric β-LG. This is in agreement with results previously reported (Hoffmann, Sala et al., 1997; Hoffmann, van Miltenburg et al., 1997; Schokker et al., 1999; Vardhanabhuti & Foegeding, 2008), which was expected of native β-LG dimer. Raising DS concentration shifted the distribution to higher

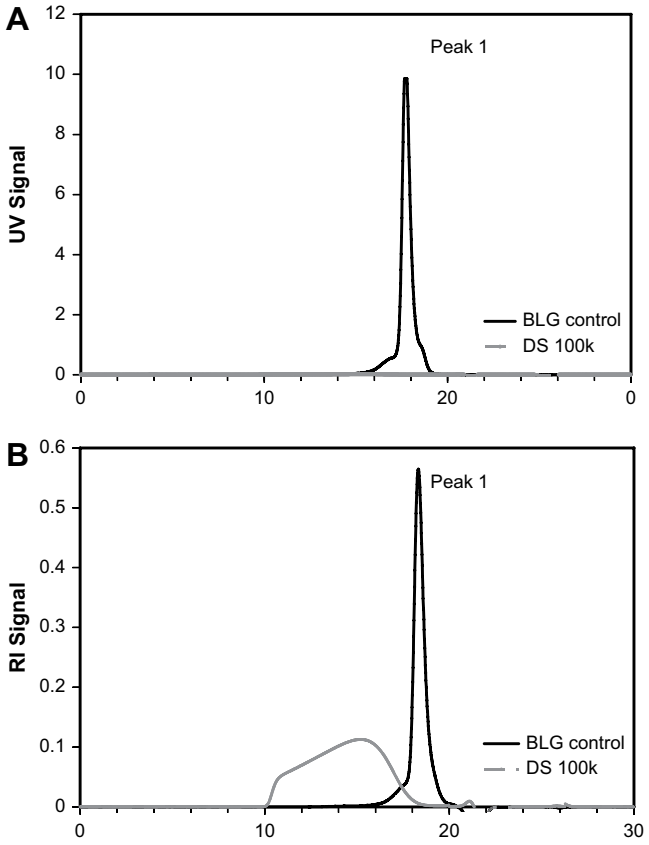

Fig. 3. SEC–MALLS chromatograms of native β-LG control and DS 100 k from PDA (A) and RI (B) detectors.

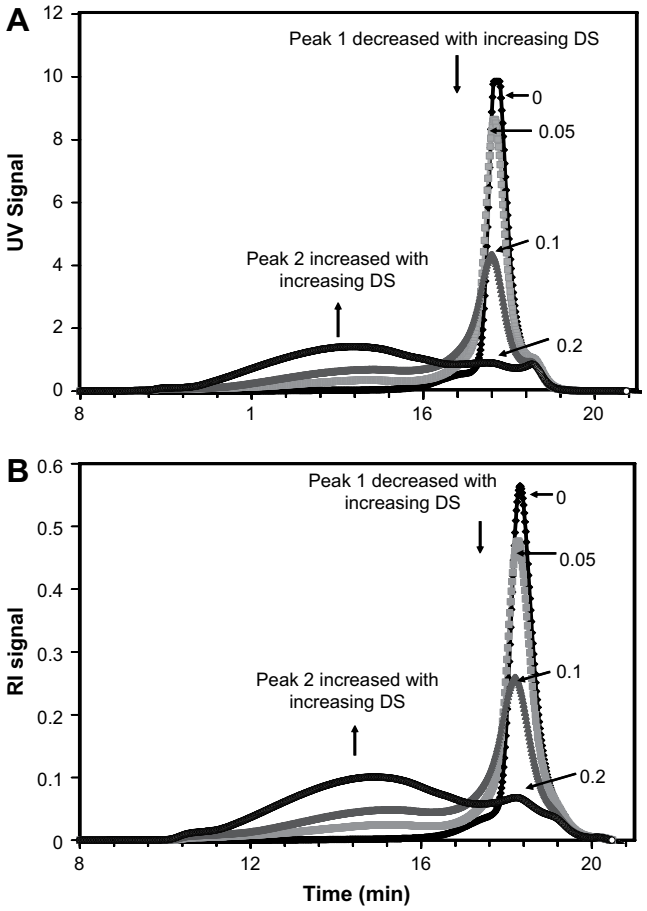

Fig. 4. SEC–MALLS chromatograms of β-LG without and with DS at different weight ratios (0, 0.05, 0.1, and 0.2 DS:β-LG). Signals are from PDA (A) and RI (B) detectors.

M_w. At 0.05 DS:β-LG ratio, where the highest thermal stability was observed, about 70% of the sample was still in the native β-LG dimer form, while only 30% of β-LG interacted with DS. When the mixture contained higher DS more native β-LG was involved in complex formation and at 0.2 DS:β-LG only 25% of β-LG remained in the native dimer form. This suggested that β-LG was in excess in all the mixtures. Using turbidity and light scattering measurements, Weinbreck et al. (2004) found that around pH 5.5 unheated whey proteins and gum arabic formed soluble complexes with an excess of β-LG over a wide range of biopolymer ratio. It was suggested that complexes occurred between a single polyelectrolyte chain and a given amount of proteins. Similar scenario may be applied to mixtures of β-LG and DS in this study in that a certain amount of β-LG forms complex along DS chain and the mixtures are in excess of β-LG.

The effect of pH on complex formation is shown in Fig. 6. β-LG without DS at different pH values gave similar cumulative weight fraction distribution (Fig. 6A) with 90% of the samples having an M_w of 3.5×10^4 or less. On the other hand, pH had a big influence on complex formation of samples with DS. Decreasing pH led to a higher amount of large M_w complex in samples containing 0.05 and 0.2 DS:β-LG ratio (Fig. 6B and C, respectively). For example, 80% weight fraction of sample at 0.05 DS:β-LG ratio had a M_w below 3.5×10^4 at pH 6.2, whereas about 20% of samples at pH 5.6 had a M_w below 3.5×10^4. The pH effect was even more pronounced in samples having the highest DS concentration (0.2 DS:β-LG ratio). SEC–MALLS results of unheated samples were in agreement with the turbidity experiment of heated samples that β-LG and DS interactions and their effect on β-LG heat aggregation were pH dependent. The effect of pH on complex formation has been reported in many protein–polysaccharide mixtures such as in whey proteins–gum arabic (Weinbreck, de Vries, Schrooyen, & de Kruif, 2003), β-LG–chitosan (Guzey & McClements, 2006), and BSA–dextran sulfate (Chung et al., 2007).

If we assume that formation of β-LG and DS complex and their heat stability at high concentration (6% protein as in turbidity experiment) follow those at low concentration (as in SEC–MALLS), these results suggest that there is an optimum size for β-LG–DS complex or optimum degree of complex formation to improve heat stability. At 0.05 DS:β-LG (lowest turbidity at pH 6.0), only 30% of native β-LG formed complex with DS. More β-LG formed complex with additional DS at higher DS concentration, suggesting that the

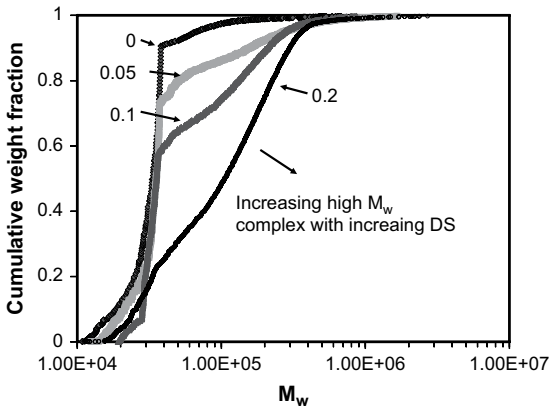

Fig. 5. Cumulative weight fraction distribution of β-LG without and with DS at different DS:β-LG ratio (0, 0.05, 0.1, 0.2) at pH 6.0.

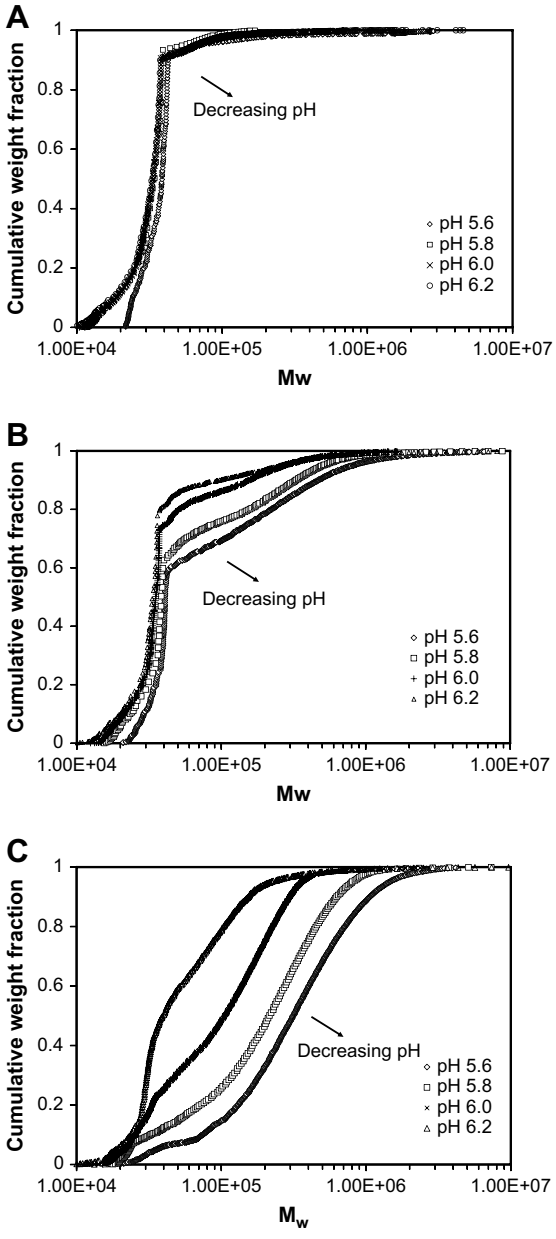

Fig. 6. Effect of pH on the distribution of molecular weight of β-LG control (A) and β-LG with DS (B: 0.05 DS:β-LG weight ratio and C: 0.2 DS:β-LG weight ratio).

mixtures were in excess of β-LG. It is likely that several β-LG molecules interact with DS along the polysaccharide chain. Higher DS likely led to a different type of aggregation and gelation of the heated samples.

3.3. Effect of NaCl

Salts such as NaCl are a major factor affecting heat aggregation of proteins (Varunsatian, Watanabe, Hayakawa, & Nakamura, 1983). The presence of salts leads to charge shielding and facilitate protein aggregation. As previously discussed, at pH 6.0, heated β-LG in the absence of salt appeared opaque, and interaction with DS decreased the turbidity of heated samples. In the presence of 10 mM NaCl, the effect of DS on lowering the turbidity of heated β-LG decreased as shown by an increase in turbidity in all samples. The effect of DS disappeared in the presence of 30 mM NaCl, as all samples turned into gel after heating (Fig. 7).

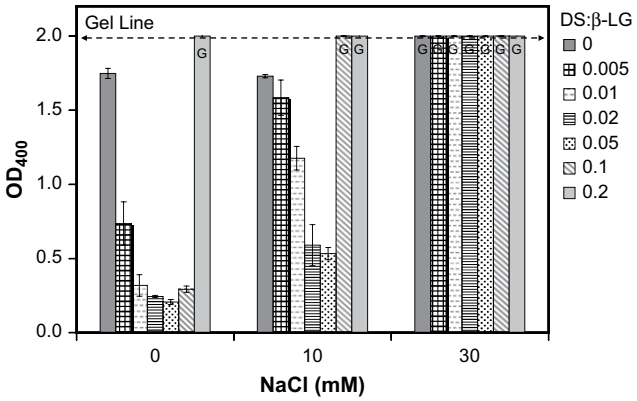

Fig. 7. Effect of NaCl on turbidity of heated β-LG without and with different concentration of 100 k DS. G indicates gelation of the samples.

The suppression of the interactions between β-LG and DS was also shown by SEC–MALLS. In Fig. 8A, unheated β-LG without DS showed similar M_w distribution in the presence of 0–30 mM NaCl. As previously described, β-LG and DS mixtures at highest DS concentration (0.2 β-LG:DS ratio) showed the presence of large molecular weight complexes (Fig. 8B, 0 mM NaCl). With increasing NaCl, the cumulative weight fraction distribution of M_w shifted towards lower M_w values. In the presence of 30 mM NaCl, the cumulative weight fraction distribution of the mixtures approached that of β-LG dimer, with 60% of the samples having an

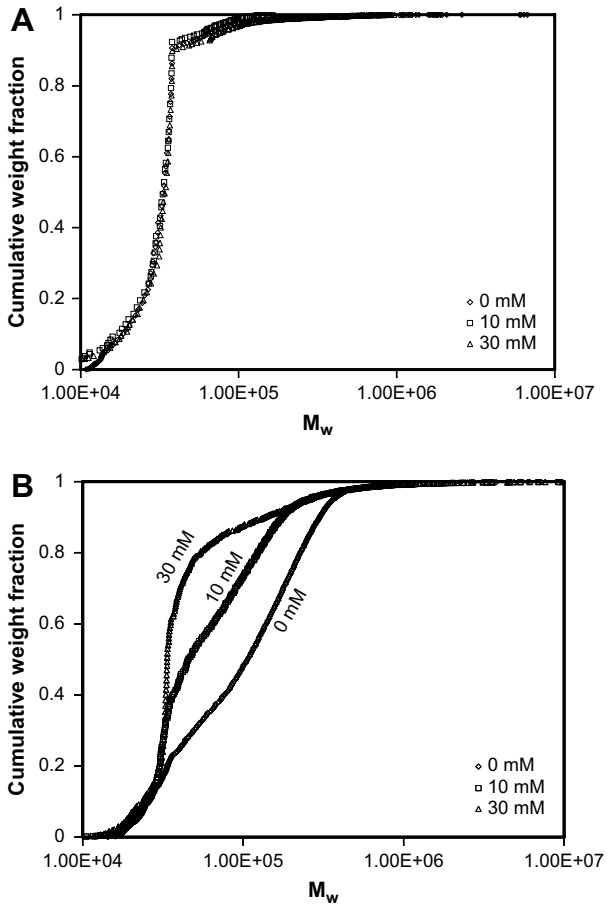

Fig. 8. Effect of NaCl on M_w distribution of native β-LG control (A) and β-LG with DS at 0.2 DS:β-LG ratio (B).

M_w of 3.5×10^4 or less. Similarly, addition of salt dissociates the BSA–sulfated polysaccharide complex(es), and the protective effect of polysaccharide is then lost (Galazka et al., 1999). Complex formation between whey proteins and exopolysaccharides EPS B40 was suppressed with the addition of NaCl (Weinbreck, Nieuwenhuijse, Robijn, & de Kruif, 2003). The effect of NaCl on complex formation and heat stability of β-LG and DS solutions indicated the electrostatic interactions as the major force in β-LG and DS interactions.

3.4. Electrophoretic mobility

The interactions between β-LG and DS were further characterized by examining the effect of pH and DS:β-LG ratio on electrophoretic mobility of β-LG and mixed β-LG and DS. To avoid gelation of the heated samples, the concentration used in this measurement was at 3% (w/w) protein. Unheated samples were measured at 3% protein, while heated samples were diluted 1:1 with buffer at respective pH before measurement, due to the high turbidity after heating. The electrophoretic mobility of unheated β-LG control became more negative as the pH was increased from 5.8 to 6.2 (Fig. 9A) corresponding to the increased negative charge on the molecule (e.g., deprotonation of amine and carboxylic acid residues). The values were in agreement with those reported by Guzey and McClements (2006). The mobility of β-LG solutions also became more negative as the DS concentration was increased at all pH values, suggesting a formation of a β-LG and DS complex that was more negatively charged. However, considerably less change in the mobility of β-LG solutions in the presence of DS was seen at pH 6.2, implying less interaction at this pH. This is in agreement with results from SEC–MALLS showing less complex formation at higher pH, which was due to higher electrostatic repulsion between β-LG and DS. Higher negative mobility was also observed in complexes between β-LG and acacia gum at pH 5.6–4.0 (Mekhloufi et al., 2005).

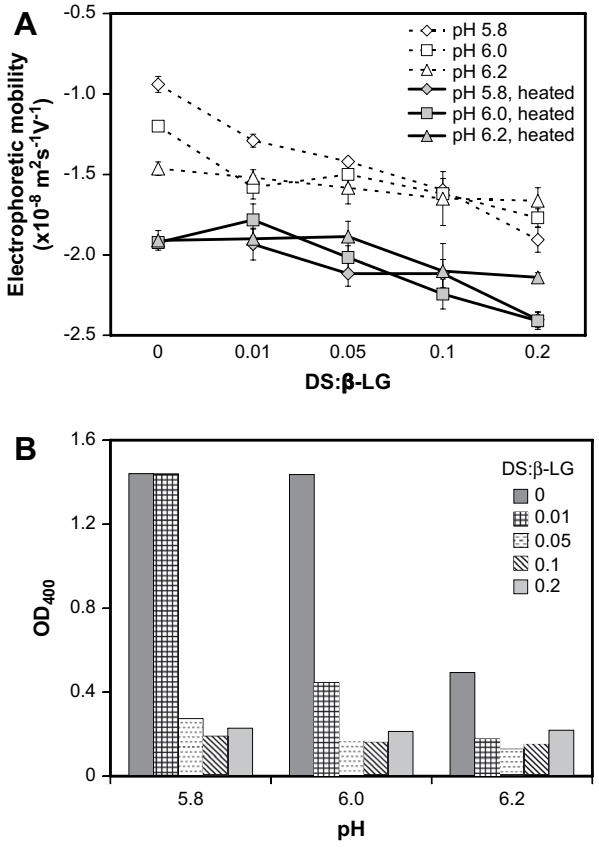

Fig. 9. Electrophoretic mobility of unheated and heated DS and BLG at different biopolymer ratio and pH (A). Turbidity of heated 3% (wt.) β-LG with or with out DS at different pH (B).

Heated β-LG control at pH 5.8 was very turbid even after dilution such that its mobility could not be measured. The mobility of all other samples was more negative after heating, indicating that heat-induced aggregates were more negatively charged. Similarly, Vardhanabhuti, Foegeding, McGuffey, Daubert, and Swaisgood (2001) reported that heated whey protein isolates at neutral pH were more negatively charged than unheated proteins. Increasing DS concentration in β-LG solutions led to more negative mobility of heated samples at all pH values. However, the effect of pH at different DS:β-LG ratio was not apparent and no particular pH produced samples with lower mobilities than others. It is interesting to note that though samples with the highest DS concentration were most negative both before and after heating (Fig. 9A), they were not the least turbid after heating (Fig. 9B). For example, the electrophoretic mobility of β-LG at pH 6.0 does not change when 0.01% DS is added but the turbidity decreases dramatically. Therefore, electrophoretic mobility alone cannot predict how it will alter heat-induced aggregation. Moreover, it supports the concept that the size of the complex and/or the degree of complexation also plays an important role.

3.5. Nano-differential scanning calorimetry (nDSC)

Fig. 10 shows the nDSC thermograms of β-LG in the absence and presence of different DS concentrations at different pH values including pH 6.8. The calorimetric scans of β-LG obtained upon denaturation in the absence of polysaccharide exhibited one main heat adsorption peak, which had a T_m value at about 78.8 °C at pH 6.8. This result was in agreement with Matheus, Friess, and Mahler (2006) who, using nDSC, reported T_m at 77.6 °C for β-LG at pH 7.2. Published T_m values of β-LG at neutral pH from standard DSC equipment ranged from 70 to 84.7 °C (Boye & Alli, 2000; Hoffmann & vanMil, 1997; Hoffmann, van Miltenburg, & vanMil, 1997; Imafidon, Ngkwaihang, Harwalkar, & Ma, 1991; Qi et al., 1995). Discrepancies in T_m values are probably due to differences in protein concentration, pH (Ma & Harwalkar, 1996), buffer, scanning rate, genetic variant (Imafidon et al., 1991), and other solute present in the samples (Ma & Harwalkar, 1996). As pH was lowered from 6.8 to 5.8, the T_m values increased approximately 7 °C from 78.8 to 86.2 °C. The effect of pH on thermal behavior of β-LG as measured calorimetrically has been studied. β-LG has been shown to be thermally more stable at low pH (Boye & Alli, 2000; Boye, Ismail, & Alli, 1996, Ma & Harwalkar, 1996, Qi et al., 1995). Qi et al. (1995) reported a 6–7 °C increase in T_m as pH was lowered from 8.05 to 6.75. This increase in thermal stability has been attributed to extra hydrogen bonding (Kella & Kinsella, 1988).

At pH 5.8–6.2, the addition of DS produced a general trend of reduction in T_m, narrower peak, and higher ΔH_m (Fig. 9 and Table 1). The decrease in T_m suggested that β-LG–DS mixtures started to unfold at a lower temperature, indicating a destabilzation. In contrast, at pH 6.8, the T_m did not change but there were variations in ΔH_m. A similar change was observed for bovine serum albumin–DS mixtures where the apparent T_m decreased by 8 °C and no change in apparent T_m was observed at a pH where protein–polysaccharide complexes were not formed (Chung et al., 2007). In contrast, Zhang et al. (2004) reported a 4.6 °C increase in T_m of β-LG in the presence of DS at pH 6.8, indicating that DS helped in the stabilization of β-LG. Their results were different from what we have shown here that DS had no effect on T_m at pH 6.8. The reason for the different calorimetry results is not clear; however, it may be related to the use of higher protein concentration in the study of Zhang et al. (2004). Addition of carrageenan, guar gum and pectin

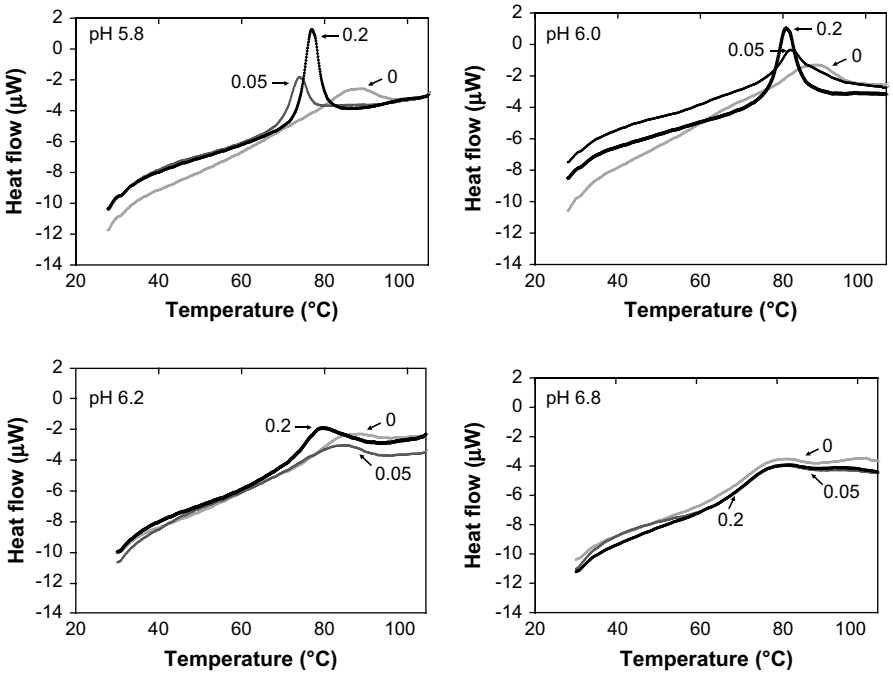

Fig. 10. Heat flow of β-LG (0.05 mg/ml) without and with DS (0, 0.05 or 0.2 DS:β-LG) at different pH.

increased T_m and ΔH_m of whey protein isolate (Ibanoglu, 2005). Another investigation showed minimal change in T_m when κ-carrageenan, λ-carrageenan, guar gum, or xanthan gum were added to β-LG at pH 6 (Baeza & Pilosof, 2002). Studying the interaction between β-LG and DS induced by high pressure, Galazka, Dickinson, and Ledward (1996) found that the addition of DS ($M_w = 500$ k) to the native β-LG at neutral pH led to no significant change in T_m. In other studies, destabilization of proteins by polysaccharides was observed by Galazka et al. (1997), Galazka et al. (1999), and Hianik, Ponikova, Bagel'ova, and Antalik (2006). Formation of complexes between human serum albumin and polyanions heparin and/or dextran sulfate at pH 7.6 led to a reduction in T_m (Hianik et al., 2006). Galazka et al. (1997) reported that both the endothermic peak temperature and the value of ΔH_m for BSA and DS mixtures were reduced compared to those of BSA alone. They suggested that interaction between BSA and DS led to a lowering of the activation (free) energy for the heat-induced conversion of the native protein into the denatured form via the partially unfolded transition state.

The overall enthalpy change is the result of the summation of endothermic (denaturation) and exothermic (aggregation) processes. Nano-differential scanning calorimetry is designed to operate under dilute solutions conditions (0.5 mg/ml) to minimize the contribution of aggregation. Electrostatic interaction and hydrogen bonding are endothermic, while hydrophobic is exothermic (Myers, 1990). It is possible that β-LG–DS complexes had an increase in electrostatic interactions as well as hydrogen bonds and a reduction in hydrophobic interactions, resulting in the higher increase in ΔH_m. The narrower peak in the presence of DS indicated that the denaturation was more cooperative. The lower T_m of β-LG in the presence of DS at lower pH values suggested that DS did not stabilize β-LG by preventing its denaturation.

Table 1
T_m and ΔH_m of β-LG in the presence of DS at different biopolymer ratio and pH.

pH	DS:β-LG	T_m (°C)	ΔH_m (kcal/g)
5.8	0	86.2 ± 0.2	1.23 ± 0.11
	0.05	73.7 ± 0.1	1.15 ± 0.02
	0.2	77.7 ± 1.0	2.61 ± 0.14
6	0	86.9 ± 0.2	2.02 ± 0.16
	0.05	81.3 ± 0.6	2.40 ± 0.26
	0.2	80.9 ± 0.3	3.02 ± 0.04
6.2	0	85.7 ± 0.8	1.41 ± 0.26
	0.05	83.4 ± 0.0	1.82 ± 0.19
	0.2	79.5 ± 0.1	2.48 ± 0.11
6.8	0	78.8 ± 0.0	1.70 ± 0.02
	0.05	78.6 ± 0.1	1.20 ± 0.01
	0.2	78.4 ± 0.1	1.43 ± 0.03

The nDSC and electrophoretic mobility data showed that binding of DS to β-LG alters charge and stability. However, these properties continued to change relatively linearly while turbidity showed a decrease and abrupt increase. Using near-UV CD analysis and DSC, Chung et al. (2007) also reported that electrostatic binding of DS to BSA at pH above pI of proteins did not stabilize the native state of BSA but rather facilitated denaturation of BSA. It was suggested that DS could suppress heat aggregation of BSA by preventing the oligomerization. While that may explain the decrease in turbidity, it does not explain the abrupt increase in turbidity at higher DS concentrations. Our results reveal that the effectiveness of DS in inhibiting heat-induced aggregation depends on solutions conditions (pH and salts) and amount of DS. While turbidity decrease can be explained by the consequences of protein–polysaccharide interaction, the reason for the turbidity increase appears to be related to polysaccharide molecular weight and other factors.

4. Conclusions

At near neutral pH, though β-LG has net negative charge, interactions between β-LG and DS exist between positively charged patches on proteins and negatively charged polysaccharides. These interactions lead to the alteration of heat aggregation of β-LG. The effect of DS on protein aggregation is influenced by pH, biopolymer ratio, DS molecular weight, and NaCl. A drastic decrease in turbidity after heating is observed even at high-protein concentration when the mixtures are at appropriate pH, biopolymer ratio, and low salt concentration. Formation of soluble complexes having large M_w

and higher negatively charge was confirmed by SEC–MALLS and electrophoretic mobility measurement. Complex formation and the effect of DS on heat stability are influenced by pH and the presence of NaCl, indicating that the interactions between the biopolymers are electrostatic driven. The nDSC results reveal that the presence of DS lowered denaturation temperature; therefore, DS does not improve heat stability of β-LG by stabilizing its native state but rather by altering its aggregation. At low DS concentrations, a large decrease in turbidity was observed, coinciding with an increase in the negative charge of the complexes. At higher DS concentrations, more complexes with larger M_w and more negatively charged are formed; however, the turbidity of the samples increases or gelation occurs. This study illustrates that, by varying the degree of interactions between β-LG and DS, and the amount of DS, the aggregation process can be directed to form solutions with lower turbidity or gels. The results could be applied in food formulations.

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