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Effect of Sulfated Polysaccharides on Heat-Induced Structural Changes in β-Lactoglobulin

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The mechanism that leads to a decreased aggregation of β-lactoglobulin in the presence of dextran sulfate and λ-carrageenan was investigated by assessing changes in the denaturation thermodynamics and protein structure. Differential scanning calorimetry results showed that the denaturation temperature (T_p) was about 4.6 °C higher in the presence of dextran sulfate, as compared with β-lactoglobulin alone, whereas in the presence of λ-carrageenan the difference in T_p was about 1.2 °C. Changes in protein structure studies using near-UV circular dichroism (CD) provided support for the calorimetric results. The transition midpoint (T_m) for denaturation of β-lactoglobulin was about 5 °C higher in the presence of dextran sulfate than that found with β-lactoglobulin alone and about 2 °C in the presence of λ-carrageenan. Thermal modifications of the tertiary structure of β-lactoglobulin were irreversible at temperatures above 67 °C; the addition of dextran sulfate reduced the extent of such modifications. Far-UV CD studies indicated that the addition of dextran sulfate or λ-carrageenan did not affect secondary structure changes of β-lactoglobulin upon heating. These studies indicate that dextran sulfate and λ-carrageenan can enhance the stability of β-lactoglobulin and thereby inhibit heat denaturation and aggregation.

KEYWORDS: β-Lactoglobulin; thermal stability; heat denaturation; DSC; CD; dextran sulfate; λ-carrageenan

INTRODUCTION

Thermal processing is an important factor during preparation of a wide variety of milk protein-based foods, including whey protein-based nutritional drinks. Upon heating a whey protein solution, structural changes take place that lead to protein denaturation, aggregation, and possibly gelation, which can lower product quality. Thus, understanding the factors regulating the denaturation and aggregation of whey proteins would aid processors in developing whey protein-based drinks. β-Lactoglobulin (β-LG) is the primary protein in whey protein concentrates and isolates, so it contributes greatly to the thermal behavior (*1*, *2*) of the whey protein ingredients. β-LG at the pH of milk (pH 6.8) exists predominantly as a dimer, with a mass of ∼36 kDa, but dissociates into monomers with a molecular mass (M_w) of 18.3 kDa below pH 3.0 (*3*). The monomer is composed of 162 amino acid residues and contains two disulfide bonds (Cys66−Cys160 and Cys106−Cys119) and one free cysteine (C121) (*4*, *5*). Thiol/disulfide exchange reactions occur during heat-induced denaturation and aggregation of β-LG, leading to the formation of intermolecular disulfide bonds (*6*, *7*).

Extensive studies on the thermal behavior of β-LG have been reported based on various analytical techniques, such as sedimentation velocity (*8*), differential scanning calorimetry (DSC) (*9*−*13*), circular dichroism spectroscopy (CD) (*14*), electrophoresis (*15*, *16*), fluorescence (*17*), and nuclear magnetic resonance (*18*, *19*). Several methods can be used to stabilize proteins during heating. One commonly used approach is the addition of ingredients such as sugars, polyols, surfactants, salts, and polymers (*20*). In a previous study (*21*), we investigated the influence of dextran (average M_w of 10 or 580 kDa), dextran sulfate (DS) (average M_w of 5, 10, or 500 kDa), and carrageenan (car) (ι, λ, and κ) on the heat-induced aggregation (80 °C for 10 min) of β-LG at pH 6.8. The heat-induced aggregation of β-LG was decreased in solutions containing low amounts of sulfate-containing polysaccharide (DS and λ- and ι-car). Higher concentrations of DS and carrageenans and all concentrations of dextran either did not alter or increased aggregation of β-LG (*21*). Of the polysaccharides investigated, λ-car and the 5 kDa M_w DS were the most effective at decreasing aggregation. DS is an anionic derivative of dextran prepared by reacting a preselected M_w fraction of dextran with chlorosulfonic acid in pyridine; the product contains up to three ester sulfate groups per glucose unit (*22*, *23*). While not an approved food additive, DS has been used in pharmaceutical applications such as anti-HIV treatment and as a blood anticoagulant (*20*). λ-Car is a high molecular mass polysaccharide comprised of repeating

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galactose units, with little or no 3,6-anhydrogalactose, joined by alternating α-(1,3) and β-(1,4) glycosidic linkages. It contains about 35% ester sulfate (*24*). Because dextran did not decrease aggregation and λ-car was the most effective of the carrageenans (*21*), the presence of sulfate groups appears to be an important factor in inhibiting aggregation.

In the present study, we investigated the mechanism(s) by which DS and λ-car improve the thermal stability of β-LG at neutral pH. The focus in this report will be on how λ-car and DS (5 kDa M_w) affect structural transitions in β-LG caused by heating.

MATERIALS AND METHODS

Materials. β-LG was extracted from raw skimmed milk according to the method of Mailliart and Ribadeau-Dumas (*25*). After the final isolation step, the β-LG was thoroughly dialyzed against deionized water and then freeze-dried. The lyophilized powder was stored at −20 °C prior to use.

λ-Car and DS (M_w 5 kDa) were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. All other chemicals were purchased from Fisher Scientific Company (Pittsburgh, PA). All chemicals were of analytical grade, and deionized water (>17 MΩ) was used throughout.

Preparation of Solutions. β-LG stock solutions were made by slowly dissolving the lyophilized powder in 10 mM sodium phosphate buffer (pH 6.8) containing 0.02% NaN_3. The solution was then dialyzed against the same buffer at 4 °C. The protein concentration was determined after filtration through a 0.45 μm filter. Solutions of λ-car and DS were prepared by dispersing the polysaccharide powders in 10 mM sodium phosphate buffer (pH 6.8) containing 0.02% NaN_3 followed by vigorous stirring for 30 min at room temperature. The pH of all solutions was checked and adjusted to pH 6.8 with small amounts of 0.05 M HCl or 0.05 M NaOH if needed.

Protein and Polysaccharide Concentrations. The final concentration of β-LG was determined spectrophotometrically at 278 nm using an extinction coefficient of 0.955 $cm^2\ mg^{-1}$ (*26*). The concentration of λ-car was determined using a colorimetric methylene blue method (*27*); that of DS was measured using the phenol/sulfuric acid assay (*28*).

DSC. The thermodynamic properties of β-LG were determined in the presence and absence of DS or λ-car using a Perkin-Elmer DSC-7 differential scanning calorimeter (The Perkin-Elmer Corp., Norwalk, CT). β-LG solutions were mixed with polysaccharide solutions at predetermined ratios in 10 mM sodium phosphate buffer (pH 6.8) containing 0.02% NaN_3. The final protein concentration contained 8% w/v. Sixty-five microliter sample solutions were hermetically sealed in stainless steel pans and weighed. A sealed empty pan was used as a reference. Two or three replicates of each sample suspension were scanned at a heating rate of 5 °C/min across the 20−100 °C range. Peak temperatures (T_p) and calorimetric enthalpies (ΔH_{cal}) were calculated from thermograms using the instrument data analyzer and a 18.3 kDa molecular mass of β-LG.

CD Studies. Heat-induced changes in the secondary and tertiary structures of β-LG, with and without added λ-car and DS, were determined by CD spectropolarimetry on a JASCO J600 spectropolarimeter (JASCO, Japan). The spectra were analyzed using Jasco J700 software. The sample temperatures were maintained at 25 °C or at defined intervals in the 25−87 °C range by placing the sample in either a 0.01 (far UV, 180−250 nm) or a 0.1 cm (near UV, 250−330 nm) path length-jacketed cylindrical cell connected to a temperature-controlled recirculating water bath. The final β-LG concentration in the 10 mM sodium phosphate buffer (pH 6.8) containing 0.02% NaN_3 was 0.2%, and the polysaccharide concentration was 0.06% DS or 0.003% λ-car in far-UV studies. In the case of near-UV studies, the β-LG concentration was 1% and the polysaccharide was present at 0.3% DS or 0.015% λ-car. Five scans were averaged at each spectrum. All CD data were baseline subtracted for signals due to the cell and buffer. The molar ellipticity was calculated using the molecular mass of β-LG (18.3 kDa).

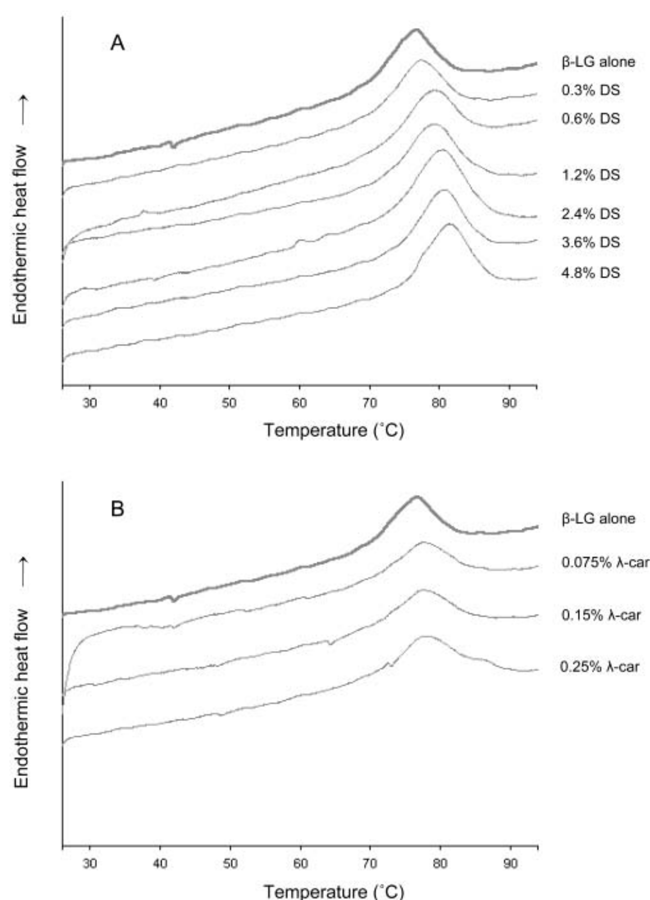

Figure 1. Effects of (**A**) DS and (**B**) λ-car on DSC thermograms of β-LG. The protein concentration was 8% in 10 mM phosphate (pH 6.8); the heating rate was 5 °C/min. Results were obtained at stated polysaccharide concentrations.

RESULTS AND DISCUSSION

DSC Properties. Figure 1 shows DSC thermograms of β-LG in the absence and presence of different concentrations of DS or λ-car. The corresponding temperatures at peak maxima (T_p) and calorimetric enthalpies (ΔH_{cal}) obtained as a function of polysaccharide concentration are reported in **Figure 2**. The calorimetric enthalpies were calculated from the area under the transition peaks and the β-LG content.

The thermal behavior of β-LG at neutral pH is complex and involves many consecutive intramolecular and intermolecular transitions. A simplified model consisting of two step reactions, denaturation ($B \leftrightarrows B^*$) and aggregation ($B^* + B^* \rightarrow B2^* \rightarrow \cdots \rightarrow B_n$) of β-LG upon heating, has been developed (*29*, *30*). The first denaturation step involves an equilibrium reaction between the native state of β-LG with a free sulfydryl group (B) and the partly unfolded state of β-LG with an exposed and reactive thiol group (B*). The second step involves several irreversible association and aggregation reactions including sulfydryl−disulfide exchange reactions (chemical aggregation) or physical aggregation of unfolded protein molecules, or a combination of chemical and physical aggregation, forming the reactive dimers (B_2^*) and the larger aggregates (B_n). The overall transition therefore becomes irreversible. The degree of irreversibility depends on experimental conditions such as scan rate, ionic strength, and protein concentration (*11*, *13*, *31*, *32*). The thermograph of β-LG obtained upon denaturation in the absence of polysaccharides (**Figure 1**) exhibited one main heat absorption peak at 70−80 °C, which had a T_p value at about 76.7 °C. This result is in agreement with published data (*33*−*35*) obtained

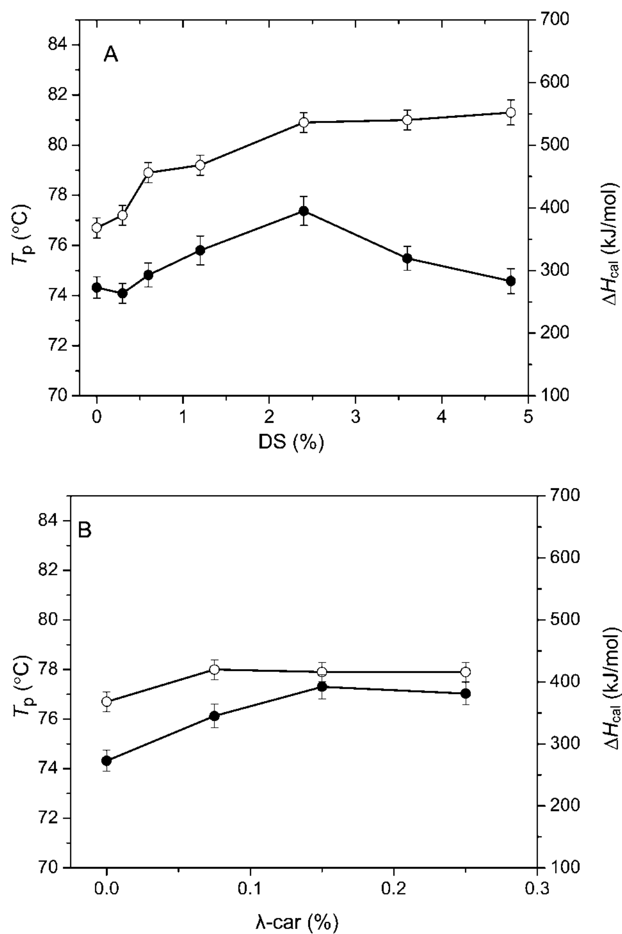

Figure 2. Peak temperatures (T_p) for β-LG denaturation (opened symbols) and the corresponding calorimetric enthalpies (ΔH_{cal}) (solid symbols) determined by DSC as a function of polysaccharide concentration. (**A**) Effect of DS; (**B**) effect of λ-car. Results are the means of three or four replications.

under similar conditions (relatively high protein concentration, low ionic strength, and neutral pH); slight discrepancies are probably due to different heating rates or minor differences with respect to protein samples (e.g., source, genetic variants) (*11*, *35*). The calorimetric enthalpy change obtained with β-LG (273 ± 17 kJ/mol, shown in **Figure 2**) at 0% polysaccharide concentration was similar to the values obtained by Dannenberg and Kessler (*36*), Gotham et al. (*37*), and Puyol et al. (*38*). To estimate whether the denaturation of β-lg is reversible under these conditions, the sample temperature was quenched to 20 °C after the first heating step from 20 to 100 °C, followed by a second scan at the same heating rate. No transition peaks were detected during the second attempt, confirming that the overall thermal denaturation of β-LG is irreversible under these conditions.

In the presence of DS or λ-car, the heat absorption peak shapes were similar to those obtained with β-LG alone (**Figure 1**). Moreover, adding DS or λ-car did not alter the irreversibility of β-LG thermal denaturation (not shown). Upon increasing the DS concentration from 0.3 to 4.8% and the λ-car concentration from 0.075 to 0.25%, the heat absorption peaks shifted to higher temperatures. In the case of β-LG/DS mixtures, when the DS concentration was 2.4%, the T_p value reached a maximum and then remained essentially constant at approximately 81.3 °C within the experimental concentration range as shown in **Figure 2**. When the λ-car concentration was 0.075% in β-LG/λ-car mixtures, a maximum T_p value of about 77.9 °C was obtained; above this concentration, the data tended toward plateaulike behavior. The maximum temperature (T_p) was, therefore, about 4.6 °C higher in the presence of DS as compared to that of β-LG alone, whereas in the presence of λ-car the difference in T_p was about 1.2 °C. This indicates a significantly increased stability of β-LG in the presence of DS relative to added λ-car. This conclusion supports previous results determined by following changes in turbidity of β-LG/DS and β-LG/λ-car complexes (*21*). However, in other studies by Galazka et al., the addition of DS ($M_w \approx 500\,000$) to the native β-LG (1:1, w/w) at neutral pH led to no significant change in T_p (*39*). This discrepancy could be the result of different experimental conditions, e.g., different ratios of DS to β-LG and a different average M_w of DS, both of which have been reported to affect the thermal stability of β-LG significantly (*21*).

Increasing the DS or λ-car concentration affected the enthalpy change (ΔH_{cal}) of β-LG as shown in **Figure 2**. The larger ΔH_{cal} value suggests that β-LG with DS or λ-car required more energy to denature than β-LG alone. As mentioned above, β-LG in solution at neutral pH unfolds when heated, followed by aggregation, polymer formation, and, under some circumstances, gelation. The thermodynamics of denaturation are influenced by variations in pH, ionic strength, protein concentration, and scanning rate during calorimetric data acquisition (*11*, *32*). In our study, we used typically high protein concentrations (8%) and a relatively fast scanning rate (5 °C/min) at neutral pH. This means that aggregation might play a significant role as scanning occurs (*31*). Denaturation is an endothermic process, while heat-induced aggregation is expected to be slightly exothermic (*32*). Thus, the overall enthalpy change at high protein concentrations is probably the result of the summation of endothermic (denaturations) and exothermic processes (aggregations) (*13*).

Previous studies demonstrated that low ratios of sulfate-containing polysaccharides to β-LG stabilized protein against heat-induced aggregation (*21*). Likewise, it was noted that at maximum T_p in the presence of DS or λ-car (**Figure 2**), the corresponding ΔH_{cal} of β-LG also reached a maximal value. A further increase in DS concentration resulted in a drastic decrease in the ΔH_{cal} of β-LG, indicating that the enthalpy change was dependent on the concentration of DS. In contrast, the ΔH_{cal} of β-LG did not vary significantly with a further increase in λ-car concentration. The ratio of DS to β-LG influences the rate of heat-induced aggregation of β-LG (*21*). The aggregation was inhibited at low ratios of DS to β-LG, and increasing the ratio increases the aggregation rate, eventually producing a bulk phase transition (*21*). Thus, the decrease in ΔH_{cal} at a DS concentration above 2.4% is most likely the result of an increase in the aggregation rate of β-LG.

Spectroscopic Properties. To better understand what types of structural transformations occur when β-LG is heated in the presence of DS or λ-car, CD spectroscopic analyses were carried out at a low protein concentration. Ratios of polysaccharide to β-LG, 0.3 for the DS/β-lg mixture and 0.015 for the λ-car/β-LG mixture, correspond to those at which the calorimetric T_p reaches its maximal value.

Figure 3 shows the near-UV CD spectra of β-LG without and with DS or λ-car at neutral pH in the 25−82 °C range. The sample solution was equilibrated for 10 min at the required temperature, and then, the spectra were recorded in 7 min, for a total heat treatment of 17 min.

The main chromophores in the near-UV (240−320 nm) in proteins are tryptophan, tyrosine, and phenylalanine (*40*), so

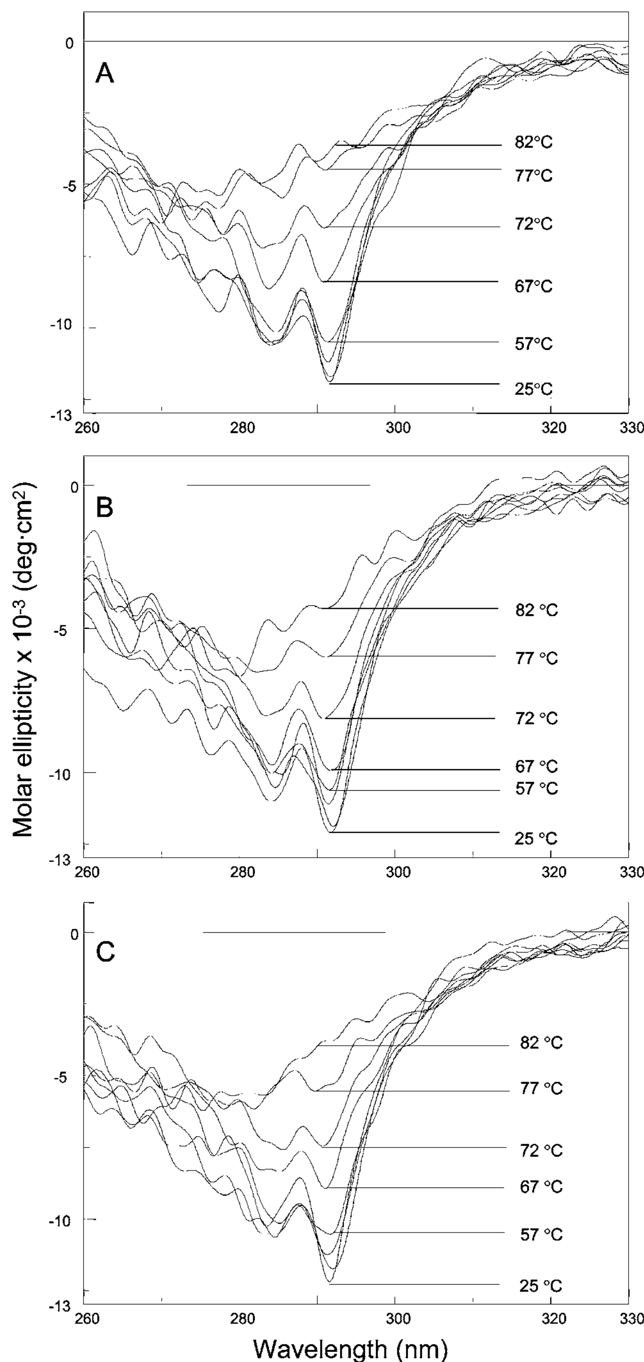

Figure 3. Near-UV CD spectra of β-LG in the absence and presence of DS or λ-car as a function of incubation temperature. Sample solutions were equilibrated at the indicated temperature for 10 min, and then, spectra were recorded. (**A**) Sample containing 1% β-LG in 10 mM sodium phosphate (pH 6.8) alone, (**B**) in the presence of 0.3% DS, or (**C**) in the presence of 0.015% λ-car. Spectra were acquired at 25, 37, 47, 57, 67, 72, 77, and 82 °C (from bottom to top at 292 nm). Each spectrum is the smoothed average of five repeat scans.

the ellipticity in this region is sensitive to the existence of tertiary structure. Signals at 262 and 269 nm are attributed to transitions of phenylalanine; those at 286 and 292 nm are interpreted as the transitions of tryptophan (*40*). In bovine β-LG, Trp 61 is located on the protein surface and not considered to be a significant source of the CD tryptophan band (*14*, *41*), while the indole ring of Trp19, which is buried within the hydrophobic binding cavity or calyx (*5*), is suggested to be a main contributor at 286 and 292 nm (*14*, *42*).

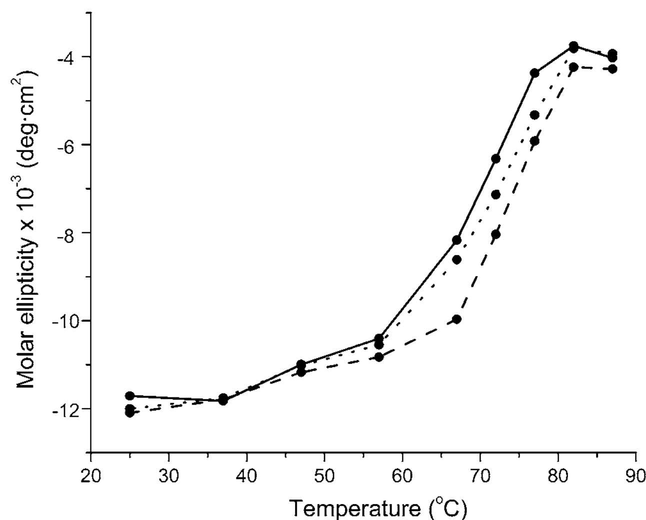

Figure 4. Molar ellipticities corresponding to β-LG in the absence (solid line) and presence of DS (dashed line) or λ-car (dotted line) at 292 nm plotted as a function of temperature. Results are the means of two or three replications.

With increasing temperature, the intensities of the CD bands of β-LG (**Figure 3A**) decreased. A dramatic decrease in intensity of the CD bands at 286 and 292 nm occurred at temperatures above 57 °C. This result agrees with a previous study in which modifications in the near-UV CD spectra were only evident at temperatures above 50 °C (*43*). Likewise, the large CD bands essentially disappeared when the temperature was above 77 °C. In the presence of DS (**Figure 3B**) and λ-car (**Figure 3C**), similar spectral trends were observed. In the presence of DS, the major change in the intense CD bands began at temperatures above 67 °C, and the signal disappeared at about 82 °C. Adding λ-car also inhibited the reduction in intensity at elevated temperatures, although the effect was not as evident as observed with DS. These results indicate that heat-induced exposure of hydrophobic aromatic residues from the protein interior upon unfolding was inhibited by the addition of DS and λ-car.

The most intense CD band at 292 nm, which originates from the Trp19 residue, was used for comparison among treatments. The changes in ellipticity are plotted as a function of temperature for β-LG in the absence and presence of DS or λ-car (**Figure 4**). The midpoint temperature of the thermal transition curve is abbreviated as T_m and usually referred to as the "melting temperature". The T_m temperatures, at which the molar ellipticicity was about -7.7×10^{-3} deg cm^2, were approximately 69 °C for β-LG alone, 71 °C for β-LG with λ-car, and 74 °C for β-LG with DS.

These results were somewhat different from the peak temperatures T_p determined by DSC (**Figures 1** and **2**). This is reasonable since these two methods measure either released or absorbed heat or structural transformations, respectively, and can be significantly different (*20*). DSC measurements were carried out at higher β-LG concentrations, where the thermal transition signal was thought to be the sum of endothermic protein unfolding and exothermic aggregation. In contrast, the near-UV CD measurements were carried out at relatively low β-LG concentrations, so that T_m at 292 nm for the Trp residue signal was mainly related with the increasing exposure of buried hydrophobic groups.

The results in **Figure 4** show that the T_m of β-LG was affected by the addition of λ-car or DS. When λ-car was added, the T_m was about 2 °C higher than that of β-LG alone. The addition of DS resulted in an increase of about 5 °C. This trend matched

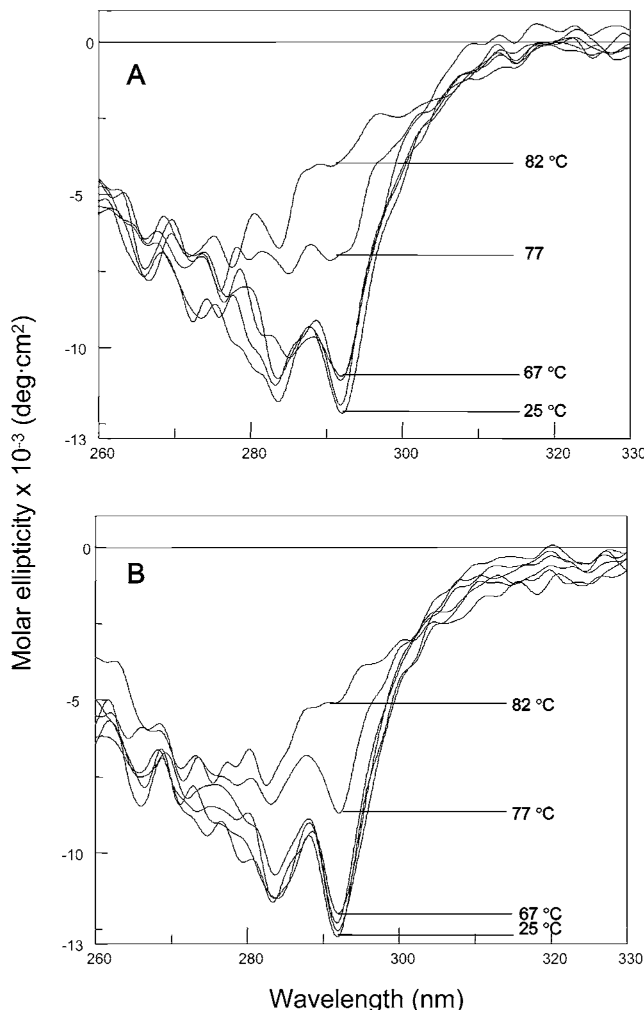

Figure 5. Near-UV CD spectra of β-LG in the absence and presence of DS at different incubation temperatures. Sample solutions were heated and then cooled, and spectra were recorded at 25 °C. (**A**) Sample containing 1% β-LG in 10 mM sodium phosphate (pH 6.8) alone or (**B**) in the presence of 0.3% DS. Incubation temperatures were 25, 47, 57, 67, 77, and 82 °C (from bottom to top at 292 nm). Each spectrum is the smoothed average of five repeat scans.

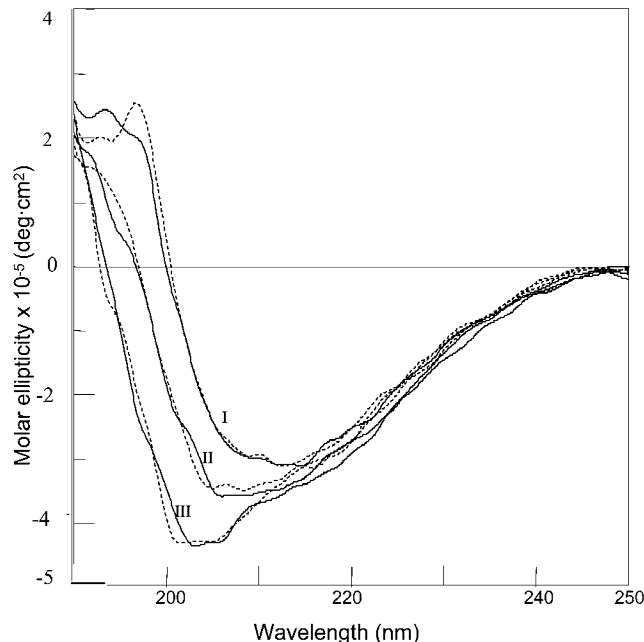

Figure 6. Far-UV CD spectra of β-LG (0.2%) in the absence (solid line) and presence of DS (0.06%) (dashed line) at 25 (I), 67 (II), and 77 °C (III) in 10 mM sodium phosphate (pH 6.8). Sample solutions were equilibrated at the required temperature for 10 min, and then, spectra were recorded. Each spectrum is the smoothed average of five repeat scans.

the pattern obtained in the DSC experiments. They indicate that DS can prevent the loss of the β-LG tertiary structure in the vicinity of tryptophan residues somewhat better than λ-car and therefore enhance the stability of β-LG with respect to heating. The difference between DS and λ-car could be due to the difference in charge density and molecular mass (*21*).

To clarify whether the thermal modifications of β-LG tertiary structure were reversible, near-UV CD studies were also carried out at neutral pH at which protein samples were first heated at the predetermined temperatures, ranging from 25 to 82 °C, for 15 min and then cooled to 25 °C and spectra were acquired at 25 °C. The CD spectra of β-LG in the absence and presence of DS are shown in **Figure 5**. No significant changes occurred in the CD bands of β-LG across the 25−67 °C temperature range (**Figure 5A**), whereas when the temperature was increased above 67 °C (for example at 77 °C), there was a significant decrease in the intensity of the CD bands. In comparison with the spectra in **Figure 3A**, the CD results indicated that β-LG molecules reverted to a nativelike tertiary structure when heated to temperatures in the 25−67 °C range and then cooled back to 25 °C, whereas at temperatures >67 °C the thermal modification of β-LG was irreversible. These results agreed with previous CD data and fluorescence measurements, which indicated that irreversible changes occurred at the emission wavelength of tryptophans at temperatures above 70 °C (*13*, *17*).

The addition of DS did not change the minimum temperature required for irreversible denaturation of β-LG (**Figure 5B**). However, at temperatures >67 °C, the decrease in CD band intensity of β-LG was less in the presence of DS. These results suggest that the mechanism for DS-induced stabilization only occurs when β-LG is heated to temperatures causing irreversible structural modifications.

CD spectra were also acquired in the far-UV range (250 to 190 nm). **Figure 6** shows the spectra of β-LG obtained at 25, 67, and 77 °C in the absence and presence of DS. At 25 °C, the native β-LG showed a broad peak around 213 nm; there was no effect on secondary structure with the addition of DS to native β-LG. Increasing the temperature to 67 and 77 °C increased the negative ellipticity of β-LG and the position of the spectrum peak shifted to 209 and 203 nm, respectively, indicating changes in secondary structures (α-helix and β-sheet structures). The added DS did not produce any significant change in the CD spectra. Similar results were also obtained in the case of adding λ-car (data not shown). These results indicate that the addition of DS or λ-car does not influence the changes in the secondary structure of β-LG upon heating.

The model previously discussed consisted of denaturation, producing a partly unfolded reactive monomer (B*) with an exposed reactive thiol group, followed by aggregation (*17*, *29*, *30,*). The structural change in producing the reactive monomer is also known to increase hydrophobic surfaces (*17*, *43*). DS and λ-car appeared to have an affect on the reactive monomer. Added DS and λ-car inhibited changes in the tertiary structure and decreased the aggregation rate as indicated by CD and turbidity studies (*21*). The type of interactions between DS and β-LG are less clear. The isoelectric point of β-LG is 5.13 (*3*), so at neutral pH it has a net negative charge. DS is also

negatively charged as a result of its ester sulfate groups, so formal charge–charge interactions between β-LG and DS would depend on the ability of DS to interact with a positively charged surface of β-LG. It has been reported that sulfates do not generally interact directly with the charged groups on the surface of proteins and are preferentially excluded from the protein surface, suggesting that sulfates perturb the water structure surrounding the proteins (*44*, *45*). Clearly, more work is needed before the types of interactions can be established.

In conclusion, DS and λ-car enhance the thermal stability of β-LG by causing an increase in the denaturation temperature associated with changes in the tertiary structure. Changes in the secondary structure appear to be unaffected.

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