Impact of Preferential Interactions on Thermal Stability and Gelation of Bovine Serum Albumin in Aqueous Sucrose Solutions

Stefan Baier and David Julian McClements*

Biopolymers and Colloids Research Laboratory, Department of Food Science, University of Massachusetts, Amherst, Massachusetts 01003

The influence of sucrose (0−40 wt %) on the thermal denaturation and gelation of bovine serum albumin (BSA) in aqueous solution has been studied. The effect of sucrose on heat denaturation of 1 wt % BSA solutions (pH 6.9) was measured using ultrasensitive differential scanning calorimetry. The unfolding process was irreversible and could be characterized by a denaturation temperature (T_m), activation energy (E_A), and pre-exponential factor (A). As the sucrose concentration increased from 0 to 40 wt %, T_m increased from 72.9 to 79.2 °C, E_A decreased from 314 to 289 kJ mol^{-1}, and ln(A/s^{-1}) decreased from 104 to 94. The rise in T_m was attributed to the increased thermal stability of the globular state of BSA relative to its native state because of differences in their preferential interactions with sucrose. The change in preferential interaction coefficient ($\Delta\Gamma_{3,2}$) associated with the native-to-denatured transition was estimated. The dynamic shear rheology of 2 wt % BSA solutions (pH 6.9, 100 mM NaCl) was monitored as they were heated from 30 to 90 °C, held at 90 °C for either 15 or 120 min, and then cooled to 30 °C. Sucrose increased the gelation temperature due to thermal stabilization of the native state of the protein. The complex shear modulus (G^*) of cooled gels decreased with sucrose concentration when they were held at 90 °C for 15 min because the fraction of irreversibly denatured protein decreased. On the other hand, G^* of cooled gels increased with sucrose concentration when they were held at 90 °C for 120 min because a greater fraction of irreversibly denatured protein was formed and the strength of the protein−protein interactions increased.

Keywords: *BSA; heat denaturation; functionality; gelation; sucrose; preferential interactions*

INTRODUCTION

Globular proteins are utilized in food, pharmaceutical, and health-care products because of their unique functional attributes, for example, enzyme catalysis, surface activity, and structure formation (*1*−*5*). The expression of these functional attributes in a particular product depends on the molecular structure, chemical environment, and thermal history of the proteins (*6*−*9*). Optimization of the design and operation of processing technologies used to manufacture protein-containing materials therefore depends on a thorough understanding of the influence of processing conditions and material composition on protein properties (*10*). Nevertheless, understanding the molecular basis of protein functionality in foods is challenging because of the compositional, structural, and dynamic complexity of food materials and because of the wide range of temperatures and mechanical stresses that food materials experience during processing, storage, and consumption.

In foods, proteins are often dispersed in an aqueous phase that contains a variety of low molecular weight cosolvents, for example, sugars, minerals, and alcohols (*5*, *11*−*13*). These cosolvents can alter the conformation and interactions of proteins and, therefore, their functional properties, through a variety of different physicochemical mechanisms (*14*−*18*). Cosolvents may directly influence protein molecular and functional characteristics by binding to protein surface groups, or they may indirectly influence these characteristics by altering the structure and physicochemical properties of water (*14*, *17*−*20*). The precise nature of the interactions and their influence on protein functionality depend on the type and concentration of cosolvents present (*18*, *21*).

In this study, we focus on the influence of sucrose on the thermal stability and gelation characteristics of bovine serum albumin (BSA). BSA is a relatively large globular protein (66 kDa) with well-characterized physiochemical properties (*1*, *22*). Previous studies have shown that sugars increase the thermal denaturation temperature of globular proteins in aqueous solutions (*10*, *11*, *23*−*26*). Sugars have also been shown to reduce the extent of unfolding of globular proteins absorbed to interfaces (*27*, *28*). The mechanism proposed for the increased stability of globular proteins in the presence of sugars is that the sugar molecules are preferentially excluded from the region immediately surrounding the proteins (*15*−*18*, *23*, *29*). The origin of this exclusion is due to a combination of excluded volume and differential interaction effects (*17*, *30*). If the cosolvent concentration is less in the immediate vicinity of a protein than in the bulk aqueous solution, there must be a thermodynamic driving force that favors movement of water molecules from the cosolvent-depleted region into the cosolvent-rich region, that is, to dehydrate the protein (*18*). Protein dehydration can be achieved either by causing the protein molecules to fold more tightly or by causing them to aggregate so that they reduce their

* Corresponding author [telephone (413) 545-1019; fax (413) 545-1262; e-mail mcclements@foodsci.umass.edu].

10.1021/jf001096j CCC: $20.00
Published on Web 04/17/2001

surface area (*17*, *20*). The magnitude of the resulting thermodynamic driving force increases as the cosolvent concentration increases, and so changes in the thermal stability, conformation, or aggregation of proteins occurs when the cosolvent concentration rises (*17*−*20*). These alterations in molecular characteristics would be expected to lead to alterations in the functional attributes of proteins as food ingredients.

To act as an effective gelling agent a globular protein must be capable of aggregating with other proteins to form a three-dimensional network that extends throughout the volume of the system (*31*−*33*). The structural organization of the protein molecules within the network determines the rheology, stability, appearance, and water holding capacity of the gel (*31*, *33*, *34*). Native BSA molecules do not form gels when they are dispersed in water at pH 7 because the intermolecular repulsive interactions (mainly electrostatic, hydration, and configurational entropy) dominate the attractive interactions (mainly van der Waals and hydrophobic). The solution must be heated to a temperature at which the globular proteins unfold and expose hydrophobic groups originally located in their interior (*1*, *35*, *36*). Under appropriate solution conditions, the resultant increase in protein surface hydrophobicity is sufficient to promote aggregation and gel formation. The nature of the interactions between the proteins determines the structural organization of the protein molecules within the gel network, which determines the overall physicochemical characteristics of the gel (*31*, *32*, *35*).

Sugars can alter the protein gelation mechanism in a number of ways. First, they increase the temperature at which globular protein molecules unfold (*11*, *26*, *29*). Second, they alter the magnitude of the attractive and repulsive forces between protein molecules (*16*−*18*), which may alter the structural organization of the protein molecules within a gel as well as the strength of the bonds between the proteins. As a consequence, sugars can alter both the formation and the final physicochemical properties (appearance, texture, stability, and water holding capacity) of protein gels in a complex manner. In a previous study we examined the influence of sucrose on thermal unfolding, gelation, and emulsion stabilization of whey protein isolate (*37*). Whey protein isolate is a complex mixture of many different proteins, which makes data interpretation complicated. One of the major objectives of the present study was to obtain a better quantitative understanding of the influence of sugars on the thermal stability and gelation of food proteins by using a well-defined globular protein system (BSA) and by using kinetic and thermodynamic models to interpret the data.

MATERIALS AND METHODS

Materials. Analytical grade sucrose (S-7903, >99.5 wt % pure) and BSA (A-6793, lot 128H0458) were purchased from the Sigma Chemical Co. (St. Louis, MO). As stated by the manufacturer, the BSA was obtained using initial fractionation by heat shock (fraction V), and low molecular weight impurities were removed using charcoal and extensive dialysis. The BSA content of the lyophilized powder was determined by electrophoresis to be 98% (the remainder being mostly globulins), the decrease in mass of the protein powder upon drying was 2.4%, and the nitrogen content of the powder was 15.6%. Distilled and deionized water was used for the preparation of all solutions. The pH values of the BSA solutions used in this study were measured using a pH meter to be 6.9 ± 0.1 (pH meter 320, Corning Inc., Corning, NY).

Differential Scanning Calorimetry (DSC). The influence of sucrose on the thermal denaturation of BSA solutions was measured using an ultrasensitive differential scanning calorimeter (VP-DSC, MicroCal, Northampton, MA). Initially, a 1 wt % BSA solution was prepared by dispersing powdered protein into a sucrose solution (0−40 wt %) and stirring for at least 2 h. The protein solution was then placed in the sample cell of the DSC instrument, and a sucrose solution with the same sucrose concentration was placed in the reference cell. The heat flow required to keep the two cells thermally balanced was then recorded as their temperature was increased from 10 to 110 °C at 90 °C h^{-1}. The cells were then held at 110 °C for 15 min, cooled to 10 °C, and rescanned. The first scan was therefore of native protein, whereas the second scan was of heat-treated protein. Thermal differences in the sample and reference cells were taken into account by subtracting a water−water DSC scan from each of the sample−reference DSC scans. The molar excess heat capacity ($C_{p,xs}$) of the proteins was determined at each temperature by measuring the change in heat absorbed with temperature (dQ/dT) and normalizing with respect to the molar concentration of protein in the reference cell (M): $C_{p,xs} = 1/M(dQ/dT)$. Measurements were carried out on two or three separate samples (replicates) and reported as the average. The thermal transition temperature (T_m), defined as the temperature at which a maximum occurred in the endothermic peaks, was reproducible to within 0.4 °C.

Gelation Experiments. The influence of sucrose on the dynamic viscoelastic properties of 2 wt % BSA solutions was measured using a constant stress rheometer (Bohlin CS10, Bohlin Instruments, Cranbury, NJ). The rheometer applied an oscillating stress of specified frequency to the sample and measured the resulting strain. The magnitude of the complex shear modulus (G^*) and the phase angle (δ) were calculated from the resulting stress−strain relationship. A concentric cylinder (C25) measurement system was used, which had a rotating inner cylinder of 25-mm diameter and a static outer cylinder of 27.5-mm diameter. Measurements were made at a frequency of 0.1 Hz and at a maximum strain of 0.01, which was within the linear viscoelastic region of the material (as determined by a strain sweep).

BSA solutions were prepared by dispersing protein powder (2 wt %) into a sucrose solution (0−40 wt %) containing 100 mM NaCl and stirring for 2 h to ensure complete dissolution. These solutions were placed in the measurement cell of the rheometer and allowed to equilibrate to 30 °C for 5 min. Solutions were covered with a thin layer of mineral oil to retard evaporation during the experiments. The solutions were then heated from 30 to 90 °C at 90 °C h^{-1}, held for either 15 or 120 min, and then cooled from 90 to 30 °C at 90 °C h^{-1}. Measurements were carried out on two or three protein solutions prepared at different times from the same BSA powder (replicates).

RESULTS AND DISCUSSION

Determination of Reversibility of BSA Thermal Denaturation. The thermographs of native (first scan) and heat-treated (second scan) 1 wt % BSA solutions in water are shown in Figure 1. NaCl was not added to these solutions, as it was for the gelation studies described below, because we did not want the proteins to aggregate extensively within the DSC cells. Previous DSC studies have shown that the addition of NaCl to BSA solutions increases the protein denaturation temperature (*25*, *38*), which means that it is not possible to directly compare data from the DSC and rheology experiments. Even so, the trends in the data would be expected to be the same. The scan of the native protein exhibited an endothermic transition between 60 and 80 °C, which had a single peak at ∼73 °C, whereas the scan of the heat-treated protein exhibited no thermal transi-

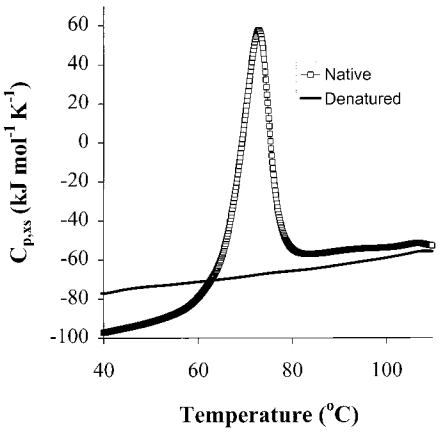

Figure 1. Heat capacity profiles of 1 wt % BSA in water solutions (pH 6.9) scanned at 90 °C h^{-1}, after baseline subtraction and normalization for concentration. The first scan represents native protein, and the second scan represents heat-treated protein.

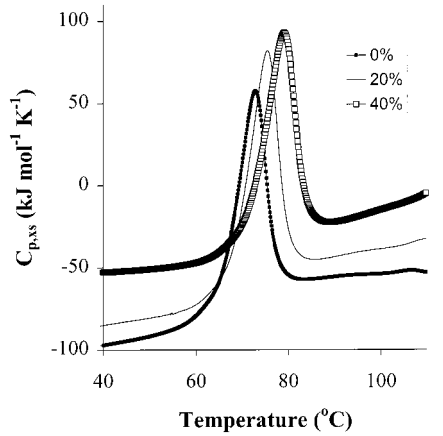

Figure 2. Heat capacity profiles of 1 wt % BSA in aqueous sucrose solutions (pH 6.9) scanned at 90 °C h^{-1}, after baseline subtraction and normalization for concentration. Aqueous phase sucrose concentrations are shown in the box.

tion (Figure 1). The molar excess heat capacity ($C_{p,xs}$) of the heat-denatured protein was appreciably greater than that of the native protein at temperatures below the thermal transition (<60 °C). An increase in $C_{p,xs}$ is indicative of an increase in the accessible surface area of nonpolar residues exposed to water after unfolding (*39*). These results indicate that BSA was irreversibly denatured during the heating process and that its surface hydrophobicity increased upon unfolding.

Influence of Sucrose on Thermal Denaturation. For all of the sucrose concentrations used (0−40 wt %) the heat-treated protein showed no thermal transition, which indicated that BSA was irreversibly denatured by the heating process in the presence and absence of sucrose. The influence of sucrose concentration on the temperature dependence of the molar excess heat capacity of 1 wt % BSA solutions is shown in Figure 2. The maximum in the DSC scans (T_m) shifted to higher temperatures as the sucrose concentration increased from 0 to 40 wt % (Table 1). There was also a significant change in the magnitude and slope of the heat capacities of the native ($T < T_m$) and heat-denatured ($T > T_m$) states, which indicated that sucrose caused an alteration in the interaction between the protein surface and the surrounding aqueous phase (*39*, *40*). The increase in the molar excess heat capacity with increasing sucrose concentration suggests that the contact between the protein surface and the aqueous phase becomes more unfavorable in the presence of sucrose (*39*).

The fact that the thermal transition was not reversible meant that equilibrium models could not be used to analyze the DSC data (*40*). Nevertheless, it was still possible to determine the denaturation temperature (T_m) and to obtain valuable information about the kinetics of the process by analyzing the DSC scans. The rate constant characterizing an irreversible transition is described by the Arrhenius equation (*40*)

$$k = A \exp(-E_A/RT) \tag{1}$$

where E_A is the activation energy (J mol^{-1}), A is a pre-exponential factor (s^{-1}), R is the gas constant (8.31 J mol^{-1} K^{-1}), and T is the absolute temperature (K). The irreversible thermal denaturation of BSA can be considered to involve a two-step process

$$\mathrm{N} \overset{K}{\rightleftharpoons} \mathrm{I} \overset{k}{\rightarrow} \mathrm{D}$$

where N, I, and D are the native, intermediate, and denatured states of the protein and K and k are rate constants (*38*). The first step involves the reversible unfolding of the native state of the protein into an intermediate state. The second step involves the irreversible conversion of the intermediate state into a denatured state. If it is assumed that the irreversible reaction is much slower than the reversible reaction ($k \ll K$) and that k is a first-order rate constant that changes with temperature according to the Arrhenius equation, then k can be determined from the DSC data using the equation (*41*)

$$k = \sigma[C_{p,xs}/(\Delta H_{cal} - Q(T))] \tag{2}$$

where σ is the scan rate (dT/dt), T is the temperature, t is the time, $Q(T)$ is the amount of heat produced by the transition when the temperature is scanned to temperature T, and ΔH_{cal} is the total enthalpy change produced by the transition. The value of $C_{p,xs}$ is obtained directly from the DSC scan, whereas $Q(T)$ and ΔH_{cal} are obtained by integration of the DSC scan over the appropriate temperature ranges. Once the rate constant has been calculated from the above expression, it is possible to determine the activation energy (E_A) and pre-exponential factor (A) using eq 1 by plotting ln k versus $1/T$. For all of the systems studied there was an excellent correlation ($r^2 > 0.99$) between the experimental data and the Arrhenius equation (Figure 3). This

Table 1. Thermal Properties of 1 wt % BSA Samples Determined by Analysis of Rheology or DSC Thermograms As Described in the Text

sucrose wt %	T_{gel} (°C)	T_m (°C)	$T_{0.5}$ (°C)	E_A (kJ mol^{-1})	ΔH_{cal} (kJ mol^{-1})	ln(A/s^{-1})	$\Delta\Gamma_{32}$ (mol mol^{-1})
0	78.1 ± 0.4	72.9 ± 0.1	6.9 ± 0.1	314 ± 5	102 ± 3	104 ± 2	0
10	80.5 ± 0.2	73.8 ± 0.1	7.1 ± 0.1	315 ± 5	103 ± 4	104 ± 2	−0.8
20	83.3 ± 0.8	75.3 ± 0.1	7.3 ± 0.1	306 ± 5	112 ± 5	101± 2	−2.1
30	86.8 ± 0.4	76.9 ± 0.2	7.5 ± 0.1	285 ± 5	117 ± 5	93 ± 2	−3.3
40	89.4 ± 0.7	79.2 ± 0.2	7.5 ± 0.1	289 ± 5	119 ± 5	94 ± 2	−5.0

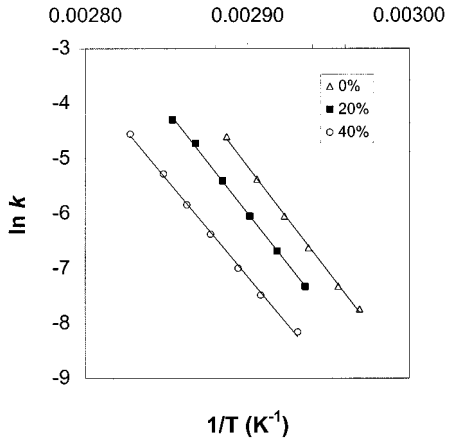

Figure 3. Linear dependence of ln k on $1/T$ used to calculate the activation energy and pre-exponential factor for the irreversible thermal transitions of 1 wt % BSA in aqueous sucrose solutions scanned at 90 °C h^{-1}.

approach of determining rate constants of protein denaturation is considerably easier and less time-consuming than approaches that involve heating the protein isothermally at different temperatures and times and determining the fraction of denatured protein (*42*).

The activation energy (E_A) and pre-exponential factor (A) of the thermal transition decreased as the sucrose concentration in the aqueous phase increased, whereas the transition temperature (T_m) and peak width of the transition at half-height ($T_{0.5}$) increased (Table 1). The peak width at half-height provides an indication of the cooperativity of a thermal transition (*43*). The increase in $T_{0.5}$ with increasing sucrose concentration suggests that the denaturation process became less cooperative at higher sugar contents. The increase in the transition temperature indicated that the thermal stability of the proteins was increased by the presence of sucrose. It has been proposed that the origin of this effect is the difference in preferential interactions of the native and heat-denatured states of proteins with the sugar and water molecules in their immediate vicinity (*15*−*20*). Sucrose molecules are preferentially excluded relative to water molecules through a combination of excluded volume and differential interaction effects (*30*). The excluded volume effect occurs because sucrose molecules are considerably larger than water molecules. As a result, the volume available to protein molecules is reduced in an aqueous solution when sucrose molecules are present, which reduces the configurational entropy of the fluid mixture. This effect occurs for both the native and heat-denatured states of the protein, but the magnitude of the effect is greater for the heat-denatured state because it has a larger surface area. Consequently, the excluded volume effect shifts the equilibrium between the native and heat-denatured states toward the native state, which increases the thermal stability of the proteins. The differential interaction effect is due to differences in the relative magnitudes of protein−sucrose and protein−water interactions, for example, electrostatic, van der Waals, hydrogen bonding, and hydrophobic interactions (*16*, *17*). This effect may either favor or oppose protein unfolding depending on the change in protein−solution interactions upon denaturation in water and in sucrose solutions. Recent experimental and theoretical studies indicate that the major cause of the stabilizing effect of sucrose molecules on thermal unfolding of globular proteins is the excluded volume effect (*30*). Nevertheless, the differential interaction effect may either attenuate or accentuate the excluded volume stabilization effect depending on the temperature (*44*−*46*).

Estimation of Changes in Preferential Interactions Resulting from Protein Unfolding. The impact of cosolvents on an equilibrium involving a biopolymer molecule can be quantified in terms of a preferential interaction coefficient, $\Gamma_{3,2}$ (*17*, *18*):

$$\Gamma_{3,2} = \lim_{m_2 \to 0} (\delta m_3/\delta m_2)_{T,P,\mu_3} \qquad (3)$$

Here, m_3 and m_2 are the molal concentrations of cosolvent and protein, respectively, T is the absolute temperature, P is the pressure, and μ_3 is the cosolvent chemical potential. The preferential interaction coefficient provides quantitative information about preferential exclusion or accumulation of cosolvent molecules at a protein surface (*18*). If a cosolvent is preferentially excluded, then $\Gamma_{3,2}$ is negative, but if it is preferentially accumulated, then $\Gamma_{3,2}$ is positive. At room temperature, measured preferential interaction coefficients of globular proteins become increasingly negative as sugar concentration increases, indicating that sugar molecules are progressively more excluded from the vicinity of the protein surface (*23*, *29*).

The influence of nonionic cosolvents on the denaturation temperature of proteins can be related to $\Gamma_{3,2}$ by thermodynamic analysis of a reversible thermal transition (*18*):

$$\Delta T_m \cong \frac{-R(T_m^0)^2}{\Delta H_{cal}^0} \Delta\Gamma_{3,2} \left(1 + \frac{1}{m_3} \int_0^{m_3} \epsilon_3 \, dm_3\right) \qquad (4)$$

Here, T_m^0 and ΔH_{cal}^0 are the denaturation temperature and enthalpy of the transition in the absence of cosolvent, ΔT_m is the increase in denaturation temperature in the presence of cosolvent ($T_m - T_m^0$), and $\Delta\Gamma_{3,2}$ ($=\Gamma_{3,2}^D - \Gamma_{3,2}^N$) is the difference in preferential interaction coefficients of the native and denatured states. The ϵ_3 parameter can be calculated from the change in activity coefficient of the cosolvent with cosolvent concentration in the absence of protein: $\epsilon_3 = \delta \ln \gamma_3/\delta \ln m_3$ (*18*). The values of T_m^0, ΔH_{cal}^0, and ΔT_m can be determined directly from the DSC scans of the thermal transition. Values of ϵ_3 can be determined from the dependence of the activity coefficient of sucrose on molality for aqueous sucrose solutions at the appropriate temperature. We used a modified UNIQUAC model (*47*) to calculate the water activity of aqueous sucrose solutions at the transition temperature (T_m^0). We then used the Gibbs−Duhem equation to calculate the dependence of the activity coefficient of sucrose on molality (*48*). Finally, we determined the integral in eq 4 by modeling the ϵ_3 versus m_3 dependence using a polynomial: $\epsilon_3 = a_0 + a_1 m_3 + a_2 m_3^2 + a_3 m_3^3 + a_4 m_3^4$.

The values of $\Delta\Gamma_{3,2}$ for the native to heat-denatured transition of the BSA were calculated from the experimental data using eq 4 (Table 1). The $\Delta\Gamma_{3,2}$ values became increasingly negative as the sucrose concentration increased (Figure 4), which indicated that the presence of sucrose favored the native state over the denatured state. Nevertheless, it is not possible to

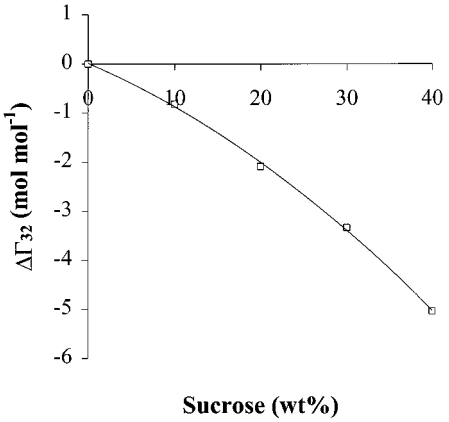

Figure 4. Change in preferential interaction coefficient associated with the native to denaturated thermal transition of BSA becomes more negative with increasing sucrose concentration.

ascertain the precise origin of this stabilization mechanism from the DSC measurements alone. For example, it may be that there is more preferential exclusion of sucrose from the denatured state than from the native state or that there is more preferential accumulation of sucrose to the native state than to the denatured state (*17*). It has recently been shown that cosolvents that are preferentially excluded from the vicinity of a globular protein at relatively low temperatures may become preferentially accumulated at higher temperatures (*44*−*46*). To identify whether the observed change in $\Delta\Gamma_{3,2}$ was due to changes in preferential exclusion or accumulation, it would be necessary to measure $\Gamma_{3,2}$ for the native and denatured states separately (*44*−*46*, *49*, *50*).

It should be stressed that the above method of determining $\Delta\Gamma_{3,2}$ is only approximate for an irreversible transition because the derivation of eq 4 assumes that the transition is reversible. Nevertheless, we believe that this method gives a reasonable approximation of $\Delta\Gamma_{3,2}$ because calculations of the heat capacity versus temperature carried out using the values of E_A and A determined experimentally (Table 1) showed that ΔT_m changed by <10% for scan rates between 0.5 and 5 °C min^{-1}, even though T_m changed appreciably. In addition, studies by other workers have found that the thermal transition of BSA is reversible up to T_m but becomes irreversible at higher temperatures (*38*). Thus, the measured ΔT_m values reported in this study should be similar to those for a reversible transition. Even so, the values of $\Delta\Gamma_{3,2}$ calculated from the measured ΔT_m values probably correspond to the N → I transition rather than the N → D transition. It should also be noted that the measured $\Delta\Gamma_{3,2}$ value is the net result of many different changes in the interaction of the protein surface with the molecules in the surrounding solution (*17*). Upon a conformational change, there may be changes in the number of ionic, polar, and nonpolar groups exposed to the solution, and some of these changes may be unfavorable to unfolding, whereas others may be favorable. The experimentally observed change in $\Delta\Gamma_{3,2}$ for the native−denatured transition indicates that the overall effect of the sucrose was to make the unfolding process less favorable, but it does not provide information about the molecular origin of this effect.

Influence of Sucrose on Gelation. The dependence of the dynamic shear modulus (G^*) and phase angle (δ)

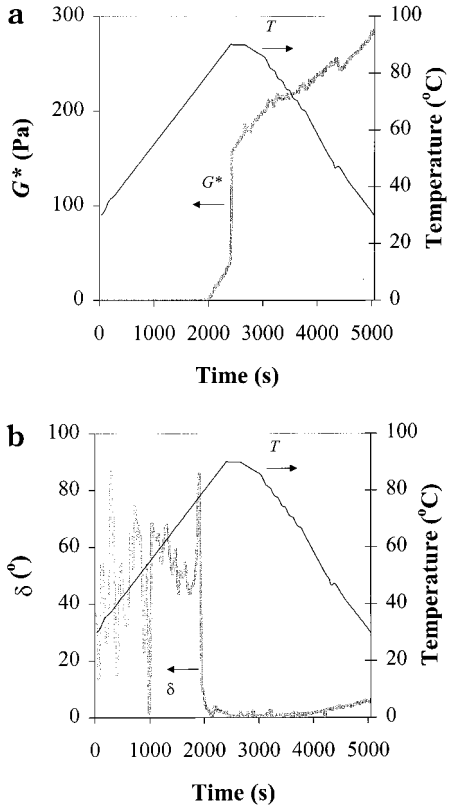

Figure 5. Time dependence of temperature and dynamic shear rheology of 2 wt % BSA dispersed in water (pH 6.9) scanned at 90 °C h^{-1}: (a) complex shear modulus; (b) phase angle.

of 2 wt % BSA solutions containing different sucrose concentrations (0−40 wt %) on temperature and holding time was measured. The time dependence of the temperature, G^*, and δ for protein solutions containing no sucrose that were heated from 30 to 90 °C, held at 90 °C for 15 min, and then cooled to 30 °C are shown in Figure 5. The shear modulus of the solutions was close to zero, and the phase angle was high and erratic up to temperatures around 70 °C, which indicated that the solutions remained fluid below this temperature. Above ~73 °C, the shear modulus rose steeply and the phase angle fell steeply, indicating that the solutions gelled around this temperature. The apparent gelation temperature was defined as the temperature at which the phase angle fell below 45°. The rigidity of the gels continued to increase when they were held at 90 °C and when they were cooled to room temperature (Figure 5a). After gelation had occurred, the phase angle of the gels remained low and relatively constant during holding at 90 °C but increased slightly during cooling to 30 °C (Figure 5b). The increase in gel rigidity upon cooling suggests that decreasing the temperature either increases the attractive forces between protein molecules (e.g., van der Waals, hydrogen bonds, and hydrophobic), decreases the repulsive forces (e.g., electrostatic and hydration), or decreases the entropy loss ($T\Delta S$) associated with trapping protein molecules within the gel network. The hydrophobic attraction decreases and the hydration repulsion increases with decreasing temperature, and therefore changes in these interactions cannot account for the increased gel rigidity upon cooling.

There were appreciable differences between the temperature dependencies of the rheological characteristics

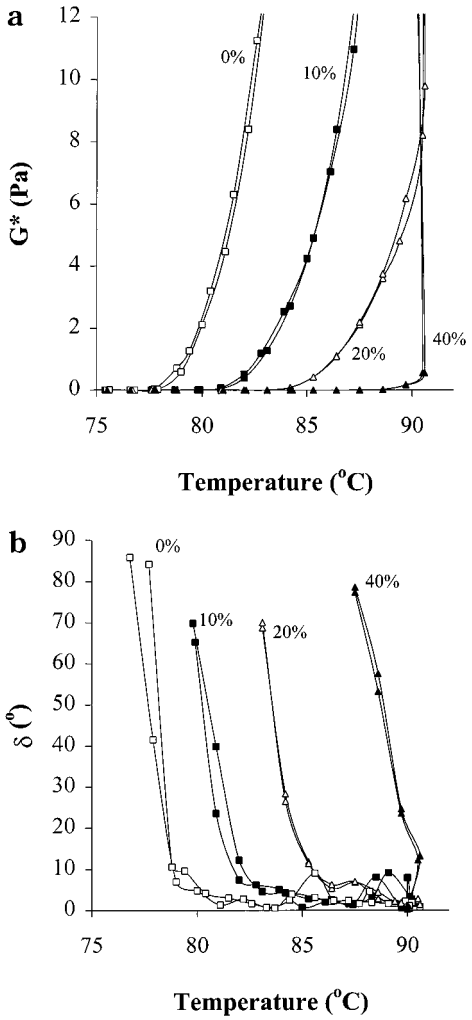

Figure 6. Dynamic shear rheology of 2 wt % BSA in aqueous sucrose solutions scanned at 90 °C h^{-1} around the thermal transition: (a) complex shear modulus; (b) phase angle. Solutions were held for 15 min at 90 °C before cooling.

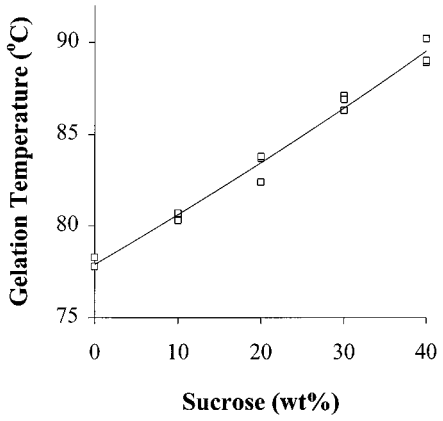

Figure 7. Dependence of gelation temperature on sucrose concentration for 2 wt % BSA in aqueous sucrose solutions.

of gels containing different amounts of sucrose. The addition of sucrose to the BSA solutions caused an increase in the temperature at which gelation occurred (Figures 6 and 7). Sucrose also caused a significant change in the rigidity of gels once they had been cooled to 40 °C (Figure 8). When protein solutions were held for 15 min at 90 °C before cooling, the presence of sucrose decreased the gel strength (Figure 8a), but when

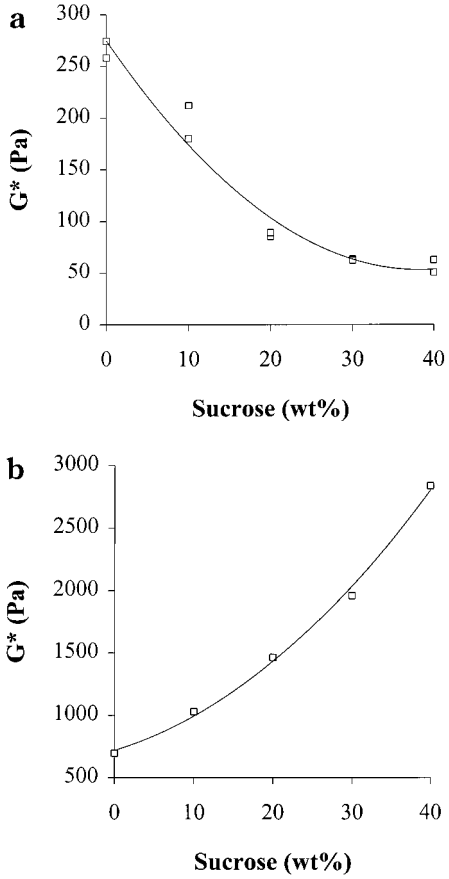

Figure 8. Dependence of shear modulus (at 40 °C during cooling) on sucrose concentration for 2 wt % BSA in aqueous sucrose solutions: (a) held for 15 min at 90 °C; (b) held for 120 min at 90 °C.

they were held for 15 min at 90 °C before cooling, the sucrose increased the gel strength (Figure 8b). The increase in the gelation temperature can be attributed to the fact that a higher temperature had to be reached before the globular protein molecules unfolded when sucrose was present (Table 1). Protein unfolding is an integral part of the gelation process; it leads to the exposure of nonpolar amino acids that were originally located in the interior of the globular molecule (*31*, *35*). The exposed nonpolar groups cause a strong hydrophobic attraction between whey protein molecules, which leads to aggregation under suitable solution conditions (*51*). If the protein molecules do not unfold, then these groups are not exposed and the molecules are not able to form a gel. The fact that the gelation temperature of the BSA solutions was appreciably higher than the denaturation temperature of the BSA molecules could have been due to a number of reasons. First, the presence of NaCl in the gelation experiments increased the thermal denaturation temperature of the proteins. Second, the proteins may have to be converted from the native state to the denatured state (via the intermediate state) before protein aggregation can occur. Third, a certain amount of network formation may have to take place before a gel is formed.

The rigidity of a gel depends on the concentration of particles incorporated into the gel network and the strength of the interactions between the particles (*52*). The presence of sucrose in the aqueous phase of the BSA solutions influences the fraction of protein molecules incorporated into the gel structure and the strength of

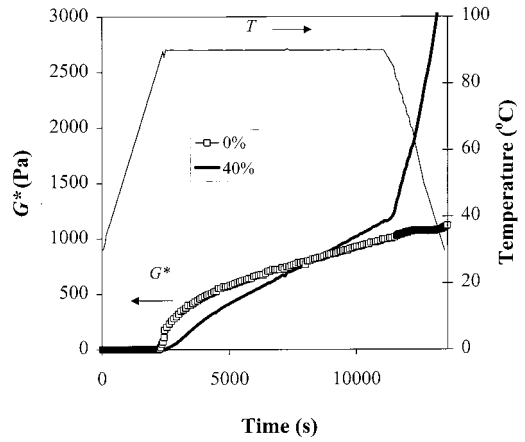

Figure 9. Time dependence of temperature and shear modulus for 2 wt % BSA in aqueous solutions containing 0 or 40 wt % sucrose. Solutions were held for 120 min at 90 °C before cooling.

the attraction between the protein molecules, and would therefore be expected to have a major impact on gel rigidity. The combined influence of sucrose concentration and holding time on gel rigidity is demonstrated by the time dependence of G^* during the heating−holding−cooling cycle (Figure 9). The BSA solution containing 0% sucrose gelled at a lower temperature than the one containing 40 wt % sucrose, and therefore it had a higher gel rigidity at shorter holding times (up to ∼100 min at 90 °C). At longer holding times the rigidity of the BSA solution containing 40 wt % sucrose increased above that of the solution containing 0 wt % sucrose. This accounts for the observation that the rigidity of BSA solutions heated to 90 °C for 15 min decreased with sucrose concentration (Figure 8a), whereas those that were heated to 90 °C for 120 min increased with sucrose concentration (Figure 8b). It should also be noted that there was a steep increase in the rigidity of gels containing 40 wt % sucrose upon cooling, whereas the rigidity of the gels containing no sucrose increased only slightly upon cooling. This suggests that the enhancement of protein−protein interactions due to the presence of sucrose increased with decreasing temperature.

The complex dependence of the rigidity of cooled gels on sucrose concentration and holding time can be attributed to the influence of sucrose on protein unfolding and the kinetics and thermodynamics of protein−protein interactions. We postulate that the ability of proteins to aggregate and be incorporated into a gel network depends on the proteins being in the denatured state, rather than the intermediate state. The transformation of the protein from the intermediate state to the denatured state is relatively slow compared to protein unfolding. Thus, at the shorter holding time (15 min) it is likely that only a fraction of the native BSA molecules had time to be converted to the denatured state and therefore participate in gel formation. In addition, the presence of sucrose increases the viscosity of the continuous phase and therefore decreases the frequency of protein−protein encounters, leading to a slower gelation rate (*53*). On the other hand, the presence of sucrose increases the attraction between protein molecules because preferential interaction effects favor those molecular arrangements that reduce the contact area between proteins and the surrounding solution (*17*). Thus, if globular protein molecules are given sufficient time to be converted to the denatured state and aggregate, the overall rigidity of the gel formed should be stronger in the presence of sucrose because the protein−protein attractive forces are greater. These factors would explain the observation that the BSA solution with the lower sucrose concentration had a higher rigidity at short holding times but a lower rigidity at longer holding times (Figure 8).

Conclusions. This study has shown that ultrasensitive DSC is a powerful tool for characterizing the influence of cosolvents on the thermal stability of globular food proteins, especially when used in combination with thermodynamic and kinetic analysis. Sucrose increases the thermal denaturation temperature of BSA because its preferential interactions with the denatured state of the protein are thermodynamically less favorable than its interactions with the native state. The influence of the cosolvent on the thermal stability of the protein can be quantified in terms of the change in the preferential interaction coefficient ($\Delta\Gamma_{3,2}$) of the native and denatured states (or more likely the intermediate state). The ability of sucrose to increase the thermal stability of BSA caused an increase in gelation temperature. The presence of sucrose had a more complicated influence on gel rigidity, because it could influence the fraction of proteins that could participate in gel formation, the kinetics of protein−protein encounters, and the strength of protein−protein interactions. This study indicates the potentially complex nature of the impact of sugars on the gelation of globular proteins in foods and highlights the need for a more fundamental understanding of the molecular basis of protein functionality. In future studies, it would be useful to relate small strain deformation measurements of protein gels to their failure analysis, because most applications of food gels depend on their failure characteristics, for example, cutting, slicing, spooning, and mastication.

ACKNOWLEDGMENT

We thank Prof. Helmut Strey for useful advice and discussions about osmotic stress effects on biopolymer functionality.

LITERATURE CITED

(1) Kinsella, J. E.; Whitehead, D. M. Proteins in whey: Chemical, physical and functional properties. *Adv. Food Nutr. Res.* **1989**, *33*, 343−438.
(2) Phillips, L. G.; Whitehead, D. M.; Kinsella, J. E. *Structure−Function Properties of Food Proteins*; Academic Press: San Diego, CA, 1994.
(3) Dalgleish, D. G. Food emulsions. In *Emulsions and Emulsion Stability*; Sjoblom, J., Ed.; Dekker: New York, 1996.
(4) Huffman, L. M. Processing whey protein for use as a food ingredient. *Food Technol.* **1996**, *50*, 49−52.
(5) Nakai, S.; Modler, H. W. *Food Proteins: Properties and Characterization*; VCH Publishers: New York, 1996.
(6) Kinsella, J. E. Protein structure and functional properties. In *Food Protein Deterioration*; Cherry, J. P., Ed.; American Chemical Society: Washington, DC, 1982.
(7) Damodaran, S.; Kinsella, J. E. Effects of ions on protein conformation and functionality. In *Food Protein Deterioration*; Cherry, J. P., Ed.; American Chemical Society: Washington, DC, 1982; pp 327−357.
(8) Damodaran, S. Amino acids, peptides and proteins. In *Food Chemistry*, 3rd ed.; Fennema, O. R., Ed.; Dekker: New York, 1996; p 321.

(9) Kilara, A.; Harwalkar, V. R. Denaturation. In *Food Proteins: Properties and Characterization*; VCH: New York, 1996.
(10) Arntfield, S. D.; Ismond, M. A. H.; Murray, E. D. Thermal analysis of food proteins. In *Thermal Analysis of Foods*; Harwalkar, V. R., Ma, C. Y., Eds.; Elsevier: London, U.K., 1990; pp 51−91.
(11) Harwalkar, V. R.; Ma, C. Y. Effects of medium composition, preheating and chemical modification upon thermal behavior of oat globulin and β-lactoglobulin. In *Food Proteins*; Kinsella, J. E., Soucie, W. G., Eds.; American Oil Chemists' Society: Champaign, IL, 1989; p 210.
(12) Harwalkar, V. R.; Ma, C. Y. Thermal analysis: Principles and practice. In *Food Proteins: Properties and Characterization*; VCH: New York, 1996.
(13) Gaonkar, A. G. *Ingredient Interactions: Effects on Food Quality*; Dekker: New York, 1995.
(14) Wyman, J.; Gill, S. J. *Binding and Linkage: Functional Chemistry of Biological Molecules*; University Science Books: Mill Valley, CA, 1990.
(15) Timasheff, S. N.; Arakawa, T. Stability of protein structure by solvents. In *Protein Structure: A Practical Approach*; Creighton, T. E., Ed.; Oxford University Press: Oxford, U.K., 1989.
(16) Timasheff, S. N. The control of protein stability and association by weak interactions with water: How do solvents affect these processes. *Annu. Rev. Biophys. Biomol. Struct.* **1993**, *22*, 67−97.
(17) Timasheff, S. N. Control of protein stability and reactions by weakly interacting cosolvents: The simplicity of the complicated. *Adv. Protein Chem.* **1998**, *51*, 356−432.
(18) Record, M. T.; Zhang, W.; Anderson, C. Analysis of effects of salts and uncharged solutes on protein and nucleic acid equilibria and processes: A practical guide to recognizing and interpreting polyelectrolyte effects, Hofmeister effects and osmotic effects of salts. *Adv. Protein Chem.* **1998**, *51*, 282−355.
(19) Creighton, T. E. *Proteins*, 2nd ed.; Freeman: New York, 1993.
(20) Parsegian, V. A.; Rand, R. P.; Rau, D. C. Macromolecules and water: Probing with osmotic stress. *Methods Enzymol.* **1995**, *259*, 43−94.
(21) Kumosinski, T. F. Thermodynamic Linkage and Non-Linear Regression Analysis: A Molecular Basis for Modeling Biomolecular Processes. *Adv. Food Nutr. Res.* **1990**, *34*, 203−298.
(22) Peters, T. J. *All About Albumin Biochemistry, Genetics and Medical Applications*; Academic Press: San Diego, CA, 1996.
(23) Lee, J. C.; Timasheff, S. N. The stabilization of proteins by sucrose. *J. Biol. Chem.* **1981**, *256*, 7193−7201.
(24) Dumay, E. M.; Kalichevsky, M. T.; Cheftel, J. C. High-pressure unfolding and aggregation of β-lactoglobulin and the baroprotective effects of sucrose. *J. Agric. Food Chem.* **1994**, *42*, 1861−1868.
(25) Boye, J. I.; Alli, I.; Ismail, A. A. Interactions involved in the gelation of bovine serum albumin. *J. Agric. Food Chem.* **1996**, *44*, 996−1004.
(26) Jou, K. D.; Harper, W. J. Effect of disaccharides on the thermal properties of whey proteins determined by differential scanning calorimetry (DSC). *Milchwissenschaft* **1996**, *51*, 509−512.
(27) Nino, M. R. R.; Wilde, P. J.; Clark, D. C.; Husband, F. A.; Patino, J. M. R. Rheokinetic analysis of protein films at the air-aqueous interface. 2. Bovine serum albumin adsorption from sucrose aqueous solutions. *J. Agric. Food Chem.* **1997**, *45*, 3016−3021.
(28) Nino, M. R. R.; Wilde, P. J.; Clark, D. C.; Patino, J. M. R. Surface dilational properties of protein and lipid films at the air−water interface. *Langmuir* **1998**, *14*, 2160−2166.
(29) Arakawa, T.; Timasheff, S. N. Stabilization of protein structure by sugars. *Biochemistry* **1982**, *21*, 6536−6544.
(30) Saunders, A. J.; Davis-Searles, P. R.; Allen, D. L.; Pielak, G. J.; Erie, D. A. Osmolyte-induced changes in protein conformational equilibria. *Biopolymers* **2000**, *53*, 293−307.
(31) Mulvihill, D. M.; Kinsella, J. E. Gelation characteristics of whey proteins and β-lactoglobulin. *Food Technol.* **1987**, *41*, 102−111.
(32) Zeigler, G. R.; Foegeding, E. A. The gelation of proteins. *Adv. Food Nutr. Res.* **1990**, *34*, 203−298.
(33) Clark, A. H. Gels and gelling. In *Physical Chemistry of Foods*; Schartzberg, H. G., Hartel, R. W., Eds.; Dekker: New York, 1992; pp 263−306.
(34) Hutchings, J. B. *Food Color and Appearance*, 2nd ed.; Aspen Publishers: Gaithersburg, MD, 1999.
(35) Mulvihill, D. M.; Donovan, M. Whey proteins and their thermal denaturation−A review. *Irish J. Food Sci. Technol.* **1987**, *11*, 43−75.
(36) Maruta, M.; Tani, F.; Higasa, T.; Kitabatake, N.; Doi, E. Heat-induced transparent gel formation of bovine serum albumin. *Biosci., Biotechnol., Biochem.* **1993**, *57*, 43−46.
(37) Kulmyrzaev, A.; Bryant, C. M.; McClements, D. J. Influence of sucrose on thermal denaturation, gelation and emulsion stabilization of whey proteins. *J. Agric. Food Chem.* **2000**, *48*, 1593−1597.
(38) Giancola, C.; De Sena, C.; Fessas, D.; Graziano, G.; Barone, G. DSC studies on bovine serum albumin denaturation: Effects of ionic strength and SDS concentration. **1997**, *20*, 193−204.
(39) Freire, E. Thermodynamics of partly folded intermediates in proteins. *Annu. Rev. Biophys. Biomol. Struct.* **1995**, *24*, 141−165.
(40) Leharne, S. A.; Chowdhry, B. Z. In *Biocalorimetry: Applications of Calorimetry in the Biological Sciences*; Ladbury, J. E., Chowdhry, B. Z., Eds.; Wiley: New York, 1998; pp 157−182.
(41) Sanchez-Ruiz, J. M.; Lopez-Lacomba, J. L.; Cortijo, M.; Mateo, P. L. Differential scanning calorimetry of the irreversible thermal denaturation of thermolysin. *Biochemistry* **1988**, *27*, 1648−1652.
(42) De Cordt, S.; Hendrickx, M.; Tobback, P. DSC and protein-based time−temperature integrators: Case study of α-amylase stabilized by polyols and/or sugar. *Fluid Phase Equilib.* **1994**, *96*, 33−50.
(43) Myers, C. D. Study of thermodynamics and kinetics of protein instability by thermal analysis. In *Thermal Analysis of Foods*; Harwalkar, V. R., Ma, C. Y., Eds.; Elsevier: London, U.K., 1990; pp 16−50.
(44) Xie, G. F.; Timasheff, S. N. The thermodynamic mechanism of protein stabilization by trehalose. *Biophys. Chem.* **1997**, *64*, 25−43.
(45) Xie, G. F.; Timasheff, S. N. Mechanism of the stabilization of ribonuclease A by sorbitol: Preferential hydration is greater for the denatured than for the native protein. *Protein Sci.* **1997**, *6*, 211−221.
(46) Xie, G. F.; Timasheff, S. N. Temperature dependence of the preferential interactions of ribonuclease A in aqueous co-solvent systems: Thermodynamic analysis. *Protein Sci.* **1997**, *6*, 222−232.
(47) Catte, M.; Dussap, C. G.; Achard, C.; Gros, J. B. Excess properties and solid−liquid equilibria for aqueous solutions of sugars using a UNIQUAC model. *Fluid Phase Equilib.* **1994**, *96*, 33−50.
(48) McQuarrie, D. A.; Simon, J. D. *Physical Chemistry: A Molecular Approach*; University Science Books: Sausalito, CA, 1997.
(49) Lee, J. C.; Gekko, K.; Timasheff, S. N. Determination of preferential interaction coefficients using density measurements. *Methods Enzymol.* **1979**, *61*, 26−49.
(50) Zhang, W.; Capp, M. W.; Bond, J. P.; Anderson, C. F.; Record, M. T. Thermodynamic characterization of interactions of native bovine serum albumin with highly excluded (glycine betaine) and moderately accumulated (urea) solutes by a novel application of vapor pressure osmometry. *Biochemistry* **1996**, *35*, 10506−10516.

(51) Bryant, C. M.; McClements, D. J. Molecular basis of protein functionality with special consideration of cold-set gels derived from heat-denatured whey. *Trends Food Sci. Technol*. **1998**, *9*, 143−151.
(52) Narine, S. S.; Maragoni, A. G. Mechanical and structural model of fractal networks of fat crystal at low deformations. *Phys. Rev. E* **1999**, *60*, 6991−7000.
(53) Kulmyrazaev, A.; Cancalliere, C.; McClements, D. J. Influence of sucrose on cold-gelation of heat-denatured whey protein isolate. *J. Sci. Food Agric.* **2000**, *80*, 1314−1318.

Received for review September 6, 2000. Revised manuscript received February 8, 2001. Accepted February 27, 2001. This material is based upon work supported by the United States Department of Agriculture (Hatch Award 745).

JF001096J