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Combined Influence of NaCl and Sucrose on Heat-Induced Gelation of Bovine Serum Albumin

STEFAN K. BAIER AND D. JULIAN MCCLEMENTS*

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

The combined influence of a strongly interacting cosolvent (NaCl) and a weakly interacting cosolvent (sucrose) on the heat-induced gelation of bovine serum albumin (BSA) was studied. The dynamic shear rheology of 4 wt % BSA solutions containing 0 or 20 wt % sucrose and 0−200 mM NaCl was monitored as they were heated from 30 to 90 °C at 1.5 °C min^{-1}, held at 90 °C for 120 min, and then cooled back to 30 °C at −1.5 °C min^{-1}. The turbidity of the same solutions was monitored as they were heated from 30 to 95 °C at 1.5 °C min^{-1} or held isothermally at 90 °C for 10 min. NaCl had a similar effect on BSA solutions that contained 0 or 20 wt % sucrose, with the gelation temperature decreasing and the final gel strength increasing with increasing salt concentration and the greatest changes occurring between 25 and 100 mM NaCl. Nevertheless, the presence of sucrose did lead to an increase in the gelation temperature and final gel strength and a decrease in the final gel turbidity. The impact of NaCl on gel characteristics was attributed primarily to its ability to screen electrostatic interactions between charged protein surfaces, whereas the impact of sucrose was attributed mainly to its ability to increase protein thermal stability and strengthen the attractive forces between proteins through a preferential interaction mechanism.

KEYWORDS: Bovine serum albumin; heat denaturation; functionality; gelation; NaCl; sucrose

INTRODUCTION

Knowledge of the physicochemical basis of heat-induced protein gelation is important in the food industry because many of the sensory and textural properties of foods are a result of the structures formed during protein gelation (*1*). The rational design and production of foods with desirable quality attributes therefore depends on an improved understanding of the physicochemical factors that lead to the creation of specific gel structures. Despite being the subject of a large number of studies carried out over many years, the precise mechanism of heat-induced protein gelation is still not clearly understood (*2*).

It has been proposed that heat-induced gelation of globular proteins proceeds via a number of steps (*3*). When a protein solution is heated above a critical temperature, usually referred to as the thermal denaturation temperature (T_m), the proteins go through a conformational change that exposes amino acids normally located in the protein interior, e.g., those with nonpolar or sulfhydryl side chains (*4*). The exposure of these “reactive” amino acids increases the attractive hydrophobic forces between proteins and facilitates the formation of inter-protein disulfide bonds (*3*). Under appropriate solution conditions (pH and ionic strength), protein molecules associate to form aggregates, which can then associate with other aggregates to form a three-dimensional protein network that extends throughout the volume of the container (*5*). The structural organization of the proteins within the network, and hence the bulk physicochemical properties of the gel, are governed by the physicochemical events that occur during gelation (*6*−*12*). The appearance, rheology, and water-holding capacity of gels therefore depend on how the molecular conformation of the proteins, the nature of the attractive and repulsive interactions between the proteins, and the kinetics of the protein−protein, protein−aggregate, and aggregate−aggregate interactions change during gel formation (*13*−*17*).

The proteins in most food systems are dispersed in an aqueous medium that contains a wide variety of different water-soluble components, e.g., salts, sugars, alcohols, surfactants, and polysaccharides (*18*, *19*). It is therefore important to understand how these components influence the molecular and functional properties of globular proteins during gelation. In previous studies, we have examined the influence of various types of low molecular mass neutral cosolvents (such as sucrose, sorbitol, and glycerol) on the thermal denaturation and gelation of globular proteins (*20, 21*). These studies have shown that neutral cosolvents influence protein gelation by a number of different mechanisms, including altering the thermal stability of the protein, altering the kinetics of protein−protein encounters, and increasing the strength of protein−protein interactions. In our previous experiments, we examined the influence of cosolvent concentration at a fixed salt concentration. Salts are known to have a major impact on the gelation mechanism and on the final

* Corresponding author. Tel.: 413-545-1019. Fax: 413-545-1262. E-mail: mcclements@foodsci.umass.edu.

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properties of globular protein gels (*7*, *13*, *22*−*25*). The objective of the current study was thus to examine the combined influence of a strongly interacting cosolvent (NaCl) and a weakly interacting cosolvent (sucrose) on the physicochemical properties of heat-induced gels formed from bovine serum albumin at neutral pH, to obtain a better understanding of the influence of solution composition on globular protein gelation. In particular, we wanted to determine if gels with specific properties could be obtained by controlling the levels of sugar and salt present during heating.

EXPERIMENTAL PROCEDURES

Materials. Analytical grade NaCl, sucrose (>99 wt % pure) and bovine serum albumin (A-7906, Lot 21K1215) 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 mass 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 1.1% and the nitrogen content of the powder was 15.8%. Distilled and deionized water was used for the preparation of all buffer solutions. The pH of the BSA solutions used in this study were measured using a pH meter to be 7.0 ± 0.1 (pH Meter 320, Corning Inc., Corning, NY).

Rheology Measurements. The influence of NaCl on the dynamic viscoelastic properties of 4 wt % BSA solutions containing 0 and 20 wt % sucrose 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 0.001, which was within the linear viscoelastic region of the material (as determined by a strain sweep).

BSA 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 heated from 30 to 90 °C at 1.5 °C min^{-1}, held for 70 min, cooled from 90 to 30 °C at −1.5 °C min^{-1}, and then held at 30 °C for 30 min. Measurements were carried out on two or three protein solutions prepared at different times from the same BSA powder (replicates). The gelation temperature (T_{gel}) of the protein solutions was defined as the temperature where the phase angle first fell below 45° during heating (*21*).

Turbidity Measurements. An UV−visible spectrometer (Ultrospec 3000pro, Amersham-Pharmacia, Uppsala, Sweden) equipped with a temperature-controlled sample holder was used to measure the change in turbidity at 600 nm of 4 wt % BSA solutions containing different NaCl and sucrose concentrations upon heating. BSA solutions were poured into 1-cm quartz cuvettes and then covered with a thin layer of mineral oil and a plastic lid to retard evaporation during the experiments. For temperature-scanning measurements, the cuvettes were placed in the sample holder of the spectrometer and allowed to equilibrate to 30 °C for 5 min. The turbidity of the solutions was then recorded as they were heated from 30 to 95 °C at 1.5 °C min^{-1}. For these measurements, the excess turbidity was defined as the turbidity measured at a particular temperature minus the turbidity measured at 30 °C: $\Delta\tau(T) = \tau(T) - \tau(30\ °C)$. For isothermal measurements, cuvettes containing protein solutions at room temperature were placed in the spectrometer sample holder set at 90 °C, and the turbidity was recorded over time. For these measurements, the excess turbidity was defined as the turbidity measured at a particular time minus the initial turbidity: $\Delta\tau(t) = \tau(t) - \tau(0\ s)$. The excess turbidity was normalized to take into account differences in the refractive indices of the aqueous solutions as described previously (*22*). Measurements were carried out on two or three protein solutions prepared at different times from the same BSA powder (replicates).

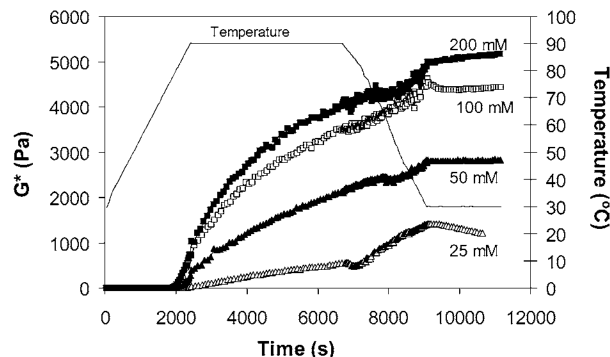

Figure 1. Influence of NaCl in the absence of sucrose on the complex shear modulus (G^*) of 4 wt % BSA solutions during a heating–cooling cycle measured using dynamic shear rheometry. Each line represents a single run. Typically, the standard deviation of repeated runs was better than 5% of G^*.

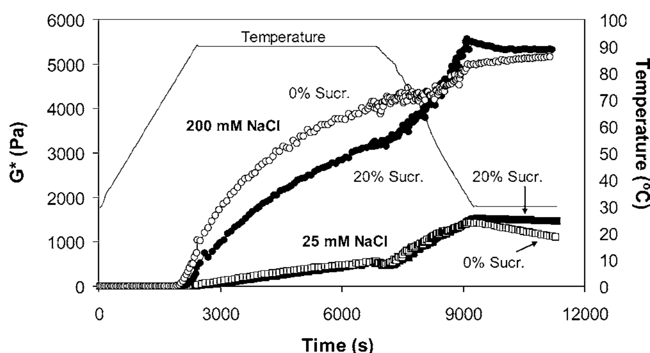

Figure 2. Combined effect of NaCl and sucrose on the complex shear modulus (G^*) of 4 wt % BSA solutions during a heating–cooling cycle measured using dynamic shear rheometry. Each line represents a single run. Typically, the standard deviation of repeated runs was better than 5% of G^*.

RESULTS AND DISCUSSION

Rheological Properties of Gels. The shear modulus (G^*) and phase angle (δ) of 4 wt % BSA solutions containing either 0 or 20 wt % sucrose and different NaCl concentrations (25−200 mM) was measured. The time-dependence of the temperature and G^* for selected protein solutions heated from 30 to 90 °C, held at 90 °C for 70 min, cooled back to 30 °C, and then held at 30 °C for 30 min are shown in **Figures 1** and **2**. No gelation was observed during heating for NaCl concentrations below 25 mM NaCl, suggesting that the electrostatic repulsive forces between the proteins were sufficiently strong to prevent the formation of a gel network (*26*).

The influence of NaCl on the evolution of G^* of the BSA solutions during the heating−cooling cycle in the absence of sucrose is shown in **Figure 1**. The shear modulus remained close to zero when the solutions were heated from 30 to around 70 °C, which indicated that the solutions remained fluid below this temperature. Upon further heating, the shear modulus increased steeply, indicating that the solutions formed gels. During the isothermal holding period at 90 °C the shear modulus continued to increase, indicating that gelation was occurring and that gel formation was not completed, even after holding the protein solutions for 2 h at this elevated temperature. G^* increased during the cooling period, which may have been caused by

further incorporation of proteins into the gel network or by strengthening of the bonds between the proteins already trapped within the gel network. The shear modulus of most of the gels remained relatively constant throughout the final isothermal holding period at 30 °C, which suggested that no further gel formation took place. However, the shear modulus of the gels containing 25 mM NaCl decreased significantly with time during this isothermal holding period, suggesting that they became weaker. As reported in previous studies (*21*, *22*), the phase angle of the BSA solutions was high (>45°) and erratic during heating from 30 to around 70 °C, rapidly decreased to a relatively low value (<2°) when the gelation temperature was exceeded and remained low during further heating and isothermal storage at 90 °C (data not shown). When the gels were cooled, the phase angle increased slightly with decreasing temperature, reaching a value of around 5° at 30 °C, which indicated that the gels became slightly less elastic at lower temperatures. The phase angle of the gels then remained relatively constant throughout the final isothermal holding period at 30 °C, indicating that no further gelation occurred.

The presence of salt in the protein solutions also had a major impact on the gelation kinetics during the 90 °C isothermal holding period (**Figure 1**). The initial rate of the increase of G^* with time increased as the salt concentration increased from 25 to 200 mM, which can again be attributed to the ability of the salt to screen the electrostatic repulsion between the protein molecules, thereby increasing the fraction of collisions that leads to aggregation. The influence of the NaCl was fairly similar in the presence and absence of sucrose, with G^* at the end of the 90 °C holding period falling from 3.9 to 3.5 to 0.5 kPa at 0 wt % sucrose and from 3.2 to 2.7 to 0.5 kPa at 20 wt % sucrose as the NaCl concentration decreased from 200 to 100 to 25mM NaCl.

The salt also had a major impact on the gelation kinetics during the cooling period from 90 to 30 °C (**Figure 1**). In the absence of sucrose, the increase in G^* during the cooling of gels containing 25 mM NaCl was appreciably higher than in the isothermal holding period at 90 °C. On the other hand, the increase in G^* during the cooling of gels containing 200 mM NaCl was fairly similar to that in the isothermal holding period at 90 °C. In the presence of 20 wt % sucrose, there was a much steeper increase in G^* with time upon cooling than in the isothermal holding period at 90 °C at all salt concentrations (compare **Figures 1** and **2**). This strong temperature dependence of the shear modulus of protein gels containing high cosolvent concentrations was also observed in our studies with sucrose, sorbitol, and glycerol (*21*, *22*). This observation suggests that these cosolvents are more effective at increasing the attraction between protein molecules at low temperatures than at high temperatures. This hypothesis is supported by experimental measurements of the temperature-dependence of the preferential interaction coefficients of various cosolvents with globular proteins, which demonstrate that there is an appreciable change in the sign and magnitude of the preferential interaction coefficients with temperature (*30*−*32*). The preferential interaction coefficient provides quantitative information about the preferential accumulation or exclusion of cosolvent molecules at the protein surface (*19*). A negative preferential interaction coefficient indicates preferential exclusion of the cosolvent from the protein surface. A positive preferential interaction coefficient indicates preferential accumulation of the cosolvent at the protein surface. Preferentially excluded cosolvents, such as sucrose and NaCl, will favor the aggregated state, due to a reduced surface area from which the cosolvent has to be excluded, which is thermodynamically more favorable.

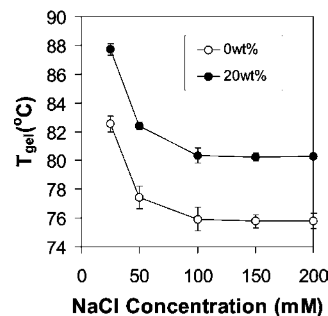

Figure 3. Influence of NaCl on gelation temperature (T_{gel}) of 4 wt % BSA solutions (pH 7.0) in the absence (0%) and presence (20%) sucrose.

The concentration of NaCl in the protein solutions had a major impact on the rheological characteristics of the BSA gels. In the absence of sucrose, the gelation temperature decreased by about 7 °C when the NaCl concentration was increased from 25 to 200 mM, with the most substantial decrease occurring between 25 and 50 mM NaCl (**Figure 3**). The salt could potentially influence the gelation temperature of BSA through a number of mechanisms, e.g., by altering the protein denaturation temperature, protein−protein interactions, or protein aggregation kinetics (*20*−*22*). Differential scanning calorimetry studies have shown that the thermal denaturation temperature (T_m) of globular proteins increases with increasing NaCl concentration (*23*, *27*), which has been attributed to screening of the intramolecular electrostatic repulsive forces in the BSA molecule (*28*). An increase in T_m would be expected to increase the gelation temperature because the protein needs to unfold before it can gel. Studies with β-lactoglobulin have shown that this increase is about 2−3 °C when the NaCl concentration is increased from 0 to 300 mM (*18*). Salt also screens the electrostatic repulsion between proteins (*29*); hence, the fraction of collisions leading to protein aggregation and network formation should increase with increasing NaCl concentration, which would be expected to increase the gelation rate. Electrostatic repulsive interactions are primarily responsible for preventing the association of protein molecules, and the effect of electrostatic screening on protein functionality can be characterized in terms of the Debye−Hückel screening parameter (*35*). Electrostatic screening interactions have been shown to have a significant impact on the conformation and association of proteins (*36*, *37*). The fact that we observed a decrease in gelation temperature with increasing NaCl concentration suggests that the major impact of the salt was on the strength of the protein−protein interactions, by reducing the charge repulsion between the negatively charged groups of the protein molecules at pH 7 (*37*). The presence of NaCl in the aqueous phase of the solutions would only have increased the solution viscosity slightly, i.e., <2%, and thus should not have a major impact on the gelation rate (*20*).

The dependence of the gelation temperature on NaCl concentration followed a similar trend in the protein solution containing 20% sucrose, with the decrease in gelation temperature being about 7 °C when the NaCl concentration was increased from 25 to 200 mM (**Figure 3**). Nevertheless, the gelation temperature was about 5 °C higher in the protein solutions containing 20 wt % sucrose than in those containing 0 wt % sucrose. This phenomenon can be attributed to the ability of sucrose to increase the thermal denaturation temperature of BSA through a preferential interaction effect (*19*−*21*).

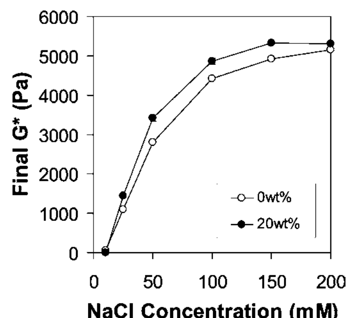

Figure 4. Influence of NaCl on final complex shear modulus (G^*) of 4 wt % BSA solutions (pH 7.0) in the absence (0%) and presence (20%) of sucrose. G^* was measured at the end of the 30 °C isothermal holding period.

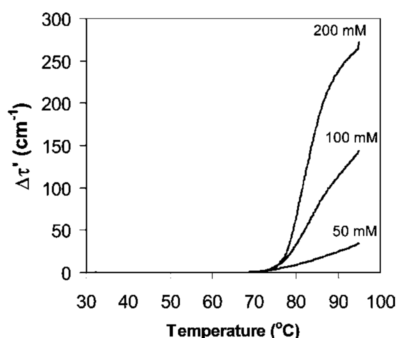

Figure 5. Influence of NaCl concentration on the normalized turbidity as a function of temperature (at 600 nm) for 4 wt % BSA solutions (pH 7.0) in the absence of sucrose.

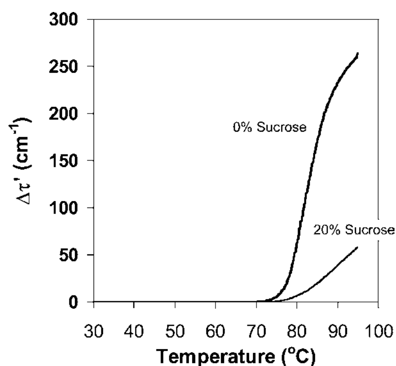

Figure 6. Influence of sucrose on the normalized turbidity as a function of temperature (at 600 nm) of 4 wt % BSA solutions (pH 7.0, 200 mM NaCl).

The presence of NaCl also had an appreciable impact on the final rigidity of the gels at the end of the final isothermal holding period at 30 °C (**Figure 4**). The final gel rigidity increased steeply from 25 to 100 mM NaCl, after which it remained fairly constant, which supports previous studies (*10*, *17*). Sucrose caused a slight increase in the final gel strength, which has been attributed to its ability to increase protein−protein interactions through a preferential interaction mechanism (*21*, *33*).

Optical Properties of Gels. The temperature-dependence of the normalized turbidity (at 600 nm) of 4 wt % BSA solutions containing different NaCl concentrations (50−200 mM) and sucrose concentrations (0 and 20 wt %) was measured (**Figures 5** and **6**). There was little change in the turbidity of the solutions when the temperature was increased from 30 to 65 °C, which indicated that no large ($r > 10-20$ nm) protein aggregates were formed. At higher temperatures, there was an appreciable increase in turbidity with increasing temperature, which was

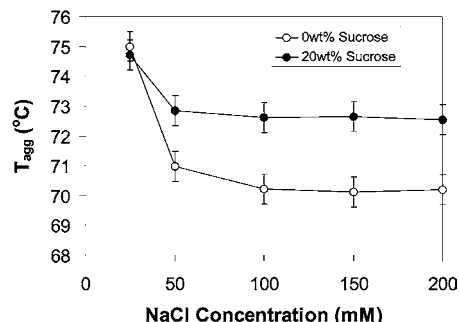

Figure 7. Influence of NaCl and sucrose on the aggregation temperature (determined from turbidity measurements) of 4 wt % BSA solutions (pH 7.0).

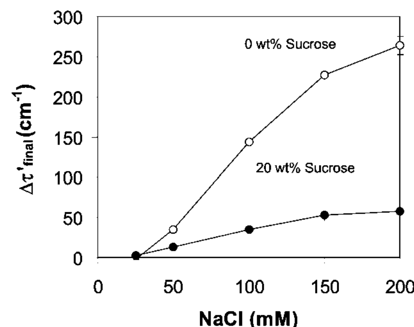

Figure 8. Influence of NaCl and sucrose on the final normalized turbidity (measured at 95 °C) of 4 wt % BSA solutions (pH 7.0).

attributed to protein aggregation. In the absence of sucrose, the turbidity increase with temperature was more extensive and occurred more rapidly as the salt concentration was increased (**Figure 5**), which can be attributed to the ability of the salt to screen electrostatic interactions between the protein molecules (*28*, *11*). At a constant salt concentration (200 mM NaCl), turbidity increase with temperature was greater and occurred more rapidly for 0% sucrose than for 20% sucrose (**Figure 6**). This effect may be attributed to the ability of sucrose to increase the thermal stability of the protein molecules through a preferential interaction mechanism and to slow protein−protein encounters by increasing solution viscosity (*21*).

An "aggregation temperature" (T_{agg}) was defined as being the temperature where proteins first formed aggregates large enough to scatter light during heating and was determined by finding the temperature where the turbidity first exceeded 0.05. For both 0 and 20 wt % sucrose, the aggregation temperature decreased when the salt concentration was increased appreciably from 25 to 50 mM NaCl, but maintained a plateau level between 50 and 200 mM NaCl (**Figure 7**). However, T_{agg} was about 2 °C higher in the presence of sucrose than in its absence. These trends were therefore fairly similar to those observed for T_{gel} determined from the rheology measurements (**Figure 3**), except that the values of T_{agg} were less than those of T_{gel}, because a certain amount of protein aggregation needs to occur before a gel network is formed.

An indication of the influence of NaCl and sucrose on the appearance of the gels was obtained by plotting the turbidity of the gels at the end of the heating process, i.e., at 95 °C (**Figure 8**). The final turbidity of the gels increases with NaCl concentration in both the presence and absence of salt. This can be attributed to the change from a filamentous gel structure at low salt concentrations (i.e., thin protein aggregates that do not scatter light efficiently) to a particulate structure at high salt concentrations (i.e., relatively large protein aggregates that

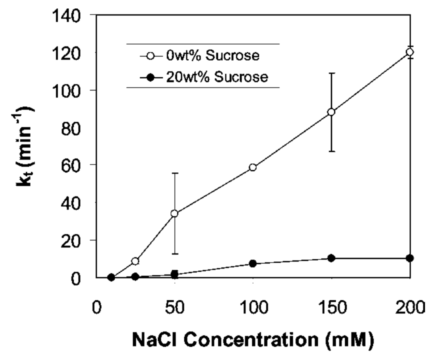

Figure 9. Influence of NaCl and sucrose on the isothermal gelation rate at 90 °C of 4 wt % BSA solutions (pH 7.0).

scatter light efficiently) due to electrostatic screening effects (*34*). The presence of sucrose causes a large decrease in the normalized turbidity of the gels, which suggests that it suppressed the formation of large protein aggregates. This effect is probably due to its ability to stabilize the globular structure of BSA and reduce the protein–protein collision frequency (*21*).

We also carried out isothermal measurements of the turbidity of 4 wt % BSA solutions placed in a 90 °C measurement chamber. A measure of the rate of aggregate formation was obtained by determining the slope of the normalized turbidity versus temperature ($k_\tau = \delta\Delta\tau'/\delta T$) profiles in the region from 1 to 4 min as described previously (*22*). In both the absence and presence of sucrose, k_τ increased with increasing NaCl concentration (**Figure 9**), which can be attributed to the salts ability to screen electrostatic interactions. At the same NaCl concentration, the value of k_τ was appreciably lower in systems containing 20 wt % sucrose than 0 wt % sucrose (**Figure 9**), which is probably due to the ability of sucrose to stabilize the globular structure of the proteins and reduce the protein–protein collision frequency (*21*).

CONCLUSIONS

The utilization of binary cosolvent systems may be useful for obtaining protein gels with specific physicochemical or sensory properties. Many attributes such as gel rigidity, water-holding capacity, flavor retention and release, and appearance are directly associated with the microstructure of the protein–protein aggregates. The presence of NaCl and sucrose in the aqueous solution surrounding globular proteins affects protein aggregation in numerous ways: (1) alteration of the thermal stability of globular proteins (NaCl and sucrose both increase T_m), (2) alteration of the magnitude of protein–protein interactions (NaCl reduces electrostatic repulsion, while sucrose increases attraction through a preferential interaction mechanism), and (3) alteration of the frequency of protein–protein collisions by increasing the viscosity of the aqueous phase (NaCl has little effect, while sucrose increases viscosity appreciably). The properties of protein gels can therefore be controlled by altering the concentrations of these two different cosolvents in the aqueous phase prior to heating. At low NaCl concentrations (<50 mM) a filamentous type gel structure predominates (transparent gels), whereas at high salt concentrations a particulate type gel structure predominates (opaque gels). The addition of sucrose to the gels altered the final gel strength and appearance of the gels. An improved knowledge of protein–cosolvent interactions may enable food scientists to design protein gels with a range of different desirable physicochemical characteristics by manipulating the aqueous phase composition.

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