Influence of Salts on the Microstructural and Rheological Properties of Heat-Induced Protein Networks from Ovalbumin and Vicilin

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The influence of salts on heat-induced networks from ovalbumin and vicilin was investigated by using dynamic rheology and light microscopy. Optimum network characteristics were obtained with 300 mM NaCl or 5 mM $CaCl_2$ for ovalbumin and 200 mM NaCl or 20–50 mM $CaCl_2$ for vicilin. At higher NaCl concentrations the storage (G') and loss (G'') moduli decreased while tan δ (G''/G') increased, indicating disruption of the network structure. Higher $CaCl_2$ concentrations resulted in increased vicilin network solubility (low G moduli and high tan δ values) and promoted ovalbumin aggregation (high G moduli and high tan δ). By use of several sodium salts in the lyotropic series, it was shown that for both ovalbumin and vicilin the promotion of intramolecular hydrophobic interactions in the protein during network formation reduced network strength. Only under highly stabilizing conditions (e.g., high concentrations of Na_2SO_4) was the influence on hydrophobic interactions sufficient to alter the structure of the network.

INTRODUCTION

The impact of salts on heat-induced protein network formation is of interest in terms of both its practical applications (inclusion of salts in food products) and fundamental investigations into the molecular interactions involved in network formation. There appears to be an optimum salt concentration for network formation. It has been suggested that at very low concentrations salts aid in protein solubilization prior to heating and provide a cross-link (particularly divalent ions) in the network (Kohnhorst and Mangino, 1985; Mulvihill and Kinsella, 1988). There is a point, however, where the masking of the net charge repulsion is the dominant factor and further salt addition simply promotes aggregation. Divalent salts tend to be more effective in promoting this aggregation than univalent ones, while the influence of polyvalent salts tends to be moderate (Nakamura et al., 1978; Varunsatian et al., 1983). Maximum gel strengths for whey proteins (e.g., β-lactoglobulin) have been reported in the range 5–20 mM for $CaCl_2$ and 75–300 mM for NaCl (Schmidt et al., 1979; Schmidt, 1981; Sone et al., 1983; Mulvihill and Kinsella, 1988), while those for egg proteins (primarily ovalbumin) are in the range 50–100 mM NaCl (Egelandsdal, 1984; Holt et al., 1984; Hayakawa and Nakamura, 1986). The maximum hardness for plant proteins can be achieved at as high as 400 mM NaCl as was reported for oats (Ma et al., 1988). With highly succinylated canola protein, an NaCl concentration of 0.7 M was not sufficient to attain maximum storage (G') and loss (G'') moduli (Paulson and Tung, 1989).

In addition to this electrostatic effect, higher concentrations of salts also exert a nonspecific influence on hydrophobic interactions. The magnitude of this influence is dependent on the properties of the salt. For example, the ranking of salts in terms of their ability to stabilize faba bean proteins (increase denaturation temperatures, T_d values, by using differential scanning calorimetry) coincides with their position in the lyotropic series (Arntfield et al., 1986; Ismond et al., 1986). As the position of a salt in this series has been related to its molal surface tension (Melander and Horvath, 1977), the dependence of T_d values on the position of the salt in the lyotropic series reflected the importance of hydrophobic interactions to the stability of these proteins. Similarly, salts in this lyotropic series have been used to probe the importance of hydrophobic interactions to network formation. The turbidity developed with egg protein in the alkaline pH region at a salt concentration of 0.5 M was dependent on the position of the anion in the lyotropic series such that $SO_4^{2-} > Cl^- > Br^- > I^- > SCN^-$ (Gossett et al., 1984). Changes in gel viscosity for the 7S and 11S soy proteins at a salt concentration of 0.75 M also depended on the lyotropic series except that $SCN^- > Br^- > Cl^- > SO_4^{2-}$ (Babajimopoulos et al., 1983). In this study, however, the enthalpy of gelation was independent of salt type, leading to the conclusion that hydrophobic interactions were of little importance in network formation. It has been shown that the inclusion of destabilizing (e.g., NaSCN or $NaClO_4$) salts from this series resulted in the formation of stronger networks than those obtained with stabilizing (e.g., Na_2SO_4 or NaCl) salts (Utsumi and Kinsella, 1985; Damodaran, 1988). This inferior network formation with the stabilizing salts was related to a greater refolding of the protein, thus reducing the number of functional groups available for interaction, rather than a nonspecific influence on hydrophobic interactions.

An additional complication in the use of salts in the lyotropic series to examine hydrophobic interactions is the binding of specific anions and the resulting impact on the charge profile. Interactions between protein and SCN^- and an increase in the net negative charge on the protein are primarily responsible for its destabilizing influence (Ismond et al., 1986). As a result, data available on the value of salts in terms of following hydrophobic interactions in protein gelation are not clear. More information is required to clarify this situation.

In the present investigation, the influence of both a univalent (NaCl) and divalent ($CaCl_2$) cation on the properties of heat-induced networks from ovalbumin and vicilin have been examined and concentrations associated with optimum network formation determined. In addition, a series of anions of sodium salts in the lyotropic series has been used to assess the importance of hydro-

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phobic interactions to the microstructural and rheological properties of ovalbumin and vicilin networks.

MATERIALS AND METHODS

Source of Material. Ovalbumin was obtained from Sigma Chemical Co. (Grade V, Lot 115F-8115) and used without further purification. Vicilin was prepared from faba bean (*Vicia faba* var. Diana) following the procedure of Ismond et al. (1985). This initial preparation of a protein isolate involved using a salt (0.5 M NaCl) extraction followed by dilution in cold water to precipitate the protein according to the method of Murray et al. (1978). Vicilin was then preferentially solubilized from this isolate by using 0.2 M sodium acetate, pH 7.5, and separated from phenolic contamination by gel filtration on Sephacryl S-300. All other chemicals were of reagent grade.

Sample Preparation. Dispersions of protein were prepared at a concentration of 10% (w/w) as this protein concentration has been shown to be sufficiently high to produce well cross-linked heat-induced networks (Arntfield et al., 1990). Salts were included in the solvent used for sample preparation. The influence of salt concentration (up to 0.5 M) was examined for both NaCl and $CaCl_2$. Anions of sodium salts in a lyotropic series, including NaSCN, $NaC_2H_3O_2$, NaCl, and Na_2SO_4, were examined at concentrations of 0.1 and 0.5 M only. For all dispersions, the pH was adjusted to 8.5 with 1.0 M NaOH. To ensure the pH was maintained, samples were equilibrated for 30 min, and the pH was rechecked prior to analysis. Only when the pH remained constant for 30 min were further analyses conducted. Samples prepared in this manner were used for calorimetric, microstructural, and rheological analyses. Network preparation from these dispersions varied depending on the type of analysis performed.

Calorimetry. The thermal properties of ovalbumin and vicilin were determined to assess the effects of the various salt environments on protein conformation by using a Du Pont 9900 thermal analyzer with a 910 differential scanning calorimeter cell base. Thermal curves were obtained by using 10–15 μL of sample and a heating rate of 2 °C/min with an empty pan as reference. Denaturation temperature (T_d), measured at the point of maximum heat flow, and enthalpy of denaturation (ΔH) were calculated instrumentally. The bases for these calculations have been described previously (Arntfield and Murray, 1981).

Microstructure. Heat-induced protein networks were prepared by heating samples in closed vials from 25 to 95 °C at a rate of 2 °C/min, holding at 95 °C for 5 min, and cooling to room temperature in an ice bath. These heat-induced networks were sectioned to a thickness of 7 μm by using a freezing microtome and observed in a Zeiss universal research microscope. Photomicrographs were taken by using a C35M Carl Zeiss automatic exposure 35-mm camera and a Kodak Ektachrome 160 ASA film. Details of the procedure have been published previously (Arntfield et al., 1990).

Rheology. Heat-induced protein networks for rheological analysis were prepared in a Bohlin VOR rheometer (Bohlin Reologi, Inc., Edison, NJ) equipped with a programmable water bath. Samples were heated to 95 °C at a rate of 2 °C/min, held there for 2 min, and then cooled to 25 °C at a rate of 2 °C/min. For all rheological measurements, the input amplitude strain used was 0.02, a value found to be in the linear viscoelastic region in preliminary experimentation. The sensitivity of the measurement was determined by the torque bar calibrated to 93.2 g·cm, attached to the upper plate of the rheometer. To prevent drying, samples were surrounded by paraffin oil during this procedure. The rheological characteristics, G' (storage modulus) and G'' (loss modulus), of the structure developed during this heating and cooling regime were monitored at a frequency of 0.1 Hz. These parameters represent the elastic and viscous components, respectively. In addition, the loss tangent or tan delta ($\tan \delta = G''/G'$), a parameter reflecting the relative energy from the viscous and elastic components, was calculated. Relationships between the changes in rheological parameters and protein denaturation have been reported previously (Arntfield et al., 1989). Significant changes in the rheological parameters noted during the cooling regime have been reported in this investigation. Changes in both the G' (storage) modulus and the G'' (loss) modulus as a function of temperature were best described as biphasic linear models, with negative slopes corresponding to an increase in structure development with decreasing temperature. The rationale for the use of this biphasic model and its application to studying the effects of protein concentration have been described previously (Arntfield et al., 1990).

In addition to monitoring the changes during heating and cooling, dynamic properties of the networks formed from this thermal treatment were measured as a function of oscillatory frequency (ω) at 25 °C by using the same strain amplitude and torque bar as in the thermal scans. In all cases there was a linear relationship between the log of the parameter and log ω as reported previously (Arntfield et al., 1989, 1990). The slight dependence of these dynamic parameters on ω was indicative of the viscoelastic nature of the material. Data comparison, however, was based on the regression analysis of these relationships using the value associated with a frequency of 1 Hz.

Statistical Analysis. All analyses were performed in duplicate and average values reported. Statistical differences were determined by using an analysis of variance in conjunction with a Duncan's multiple range test (Steel and Torrie, 1960) using an IBM personal computer and the Number Crunching Statistical System (NCSS) software package.

Table I. Effect of NaCl Concentration on the Thermal Denaturation of 10% Ovalbumin and 10% Vicilin, pH 8.5[a]

concn, M	ovalbumin T_d, °C	ovalbumin ΔH, J/g of protein	vicilin T_d, °C	vicilin ΔH, J/g of protein
0.1	85.4 ± 0.1[a]	22.2 ± 2.2[a]	78.1 ± 0.3	15.9 ± 2.2[ab]
0.2	85.5 ± 0.8[a]	21.3 ± 1.1[a]	82.0 ± 1.1[a]	14.8 ± 1.0[a]
0.3	86.8 ± 0.1[a]	19.6 ± 1.4[ab]	83.1 ± 0.4[ab]	16.4 ± 1.5[ab]
0.4	86.7 ± 0.1[a]	18.4 ± 1.2[ab]	84.8 ± 0.1[b]	21.4 ± 2.2[b]
0.5	86.0 ± 0.2[a]	16.4 ± 0.7[b]	84.2 ± 0.2[b]	17.1 ± 0.2[ab]

[a] Column values followed by the same letter are not significantly different ($P \leq 0.05$).

RESULTS AND DISCUSSION

Sodium Chloride. Sodium chloride is known as a stabilizing salt and has been shown to increase the thermal stability of plant proteins (Arntfield et al., 1986; Ismond et al., 1986). This increase in stability at higher salt concentrations has been related to the promotion of intramolecular hydrophobic interactions. The addition of NaCl at concentrations between 0.1 and 0.5 M, however, had no impact on the T_d values for ovalbumin and, in fact, resulted in a decrease in ΔH values, indicative of slight protein denaturation (Table I). As a result, the stabilizing influence of NaCl had little effect on the hydrophobic interactions in the native structure of ovalbumin.

Despite this, increasing the NaCl concentration resulted in a gradual increase in the tan δ values for ovalbumin networks such that in 0.1 and 0.2 M NaCl tan δ values were significantly lower than those at the higher concentrations (Figure 1). The range for these values, however, was quite narrow (0.084–0.112), and no differences were seen in the microstructure (not shown); for all NaCl concentrations, a good cross-linked network was obtained. Furthermore, there were significant decreases in the G moduli between 0.3 and 0.4 M NaCl (Figure 2). It would appear that at the higher salt concentrations, where the influence of salt has been related to the promotion of intramolecular hydrophobic interactions, the degree of interaction between proteins within the heat-induced network was greatly reduced. Although this change had little influence on the type of structure obtained (similar microstructure), the decrease in the G moduli in this range indicated that hydrophobic interactions may have an important role in determining the strength of the gel structure. In this respect, the contribution to the interactions responsible for the viscous and elastic components

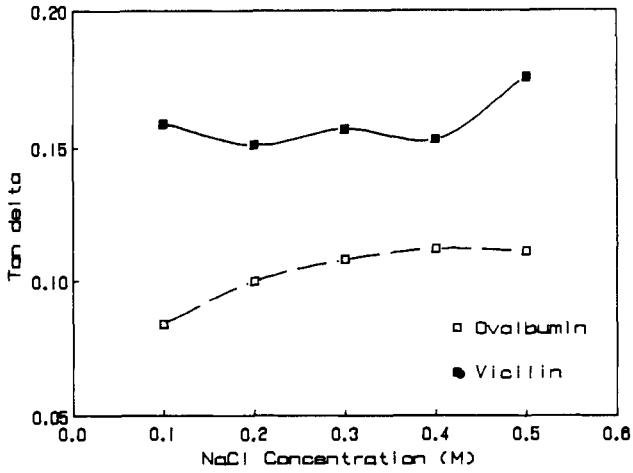

Figure 1. Effect of NaCl on tan δ (tan delta) values of heat-induced networks from 10% ovalbumin and 10% vicilin at pH 8.5.

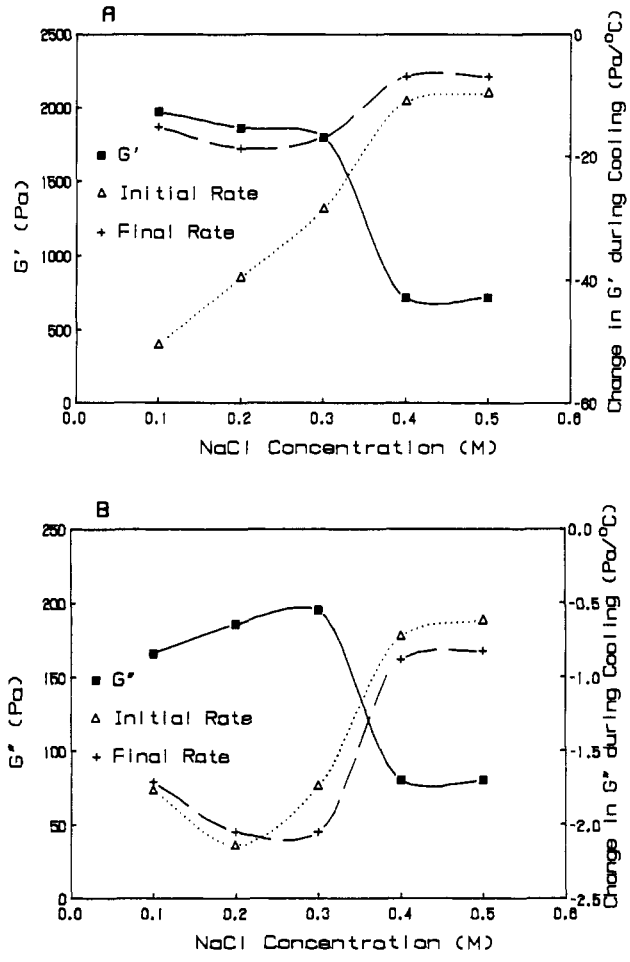

Figure 2. Effect of NaCl on the rate of structure development during the initial and final cooling phases in relation to the G moduli in the resulting networks for 10% ovalbumin, pH 8.5. (A) G' modulus; (B) G'' modulus.

in the network was the same. As a result, the promotion of hydrophobic interactions at the higher NaCl concentrations may not be sufficient to affect ovalbumin stability but does appear to influence the rheological properties of heat-induced networks produced from ovalbumin.

This influence of NaCl concentration on ovalbumin was seen during both the initial and final cooling phases (Figure 2). For both G' and G'' the rate of change at concentrations of 0.4 and 0.5 M were significantly reduced.

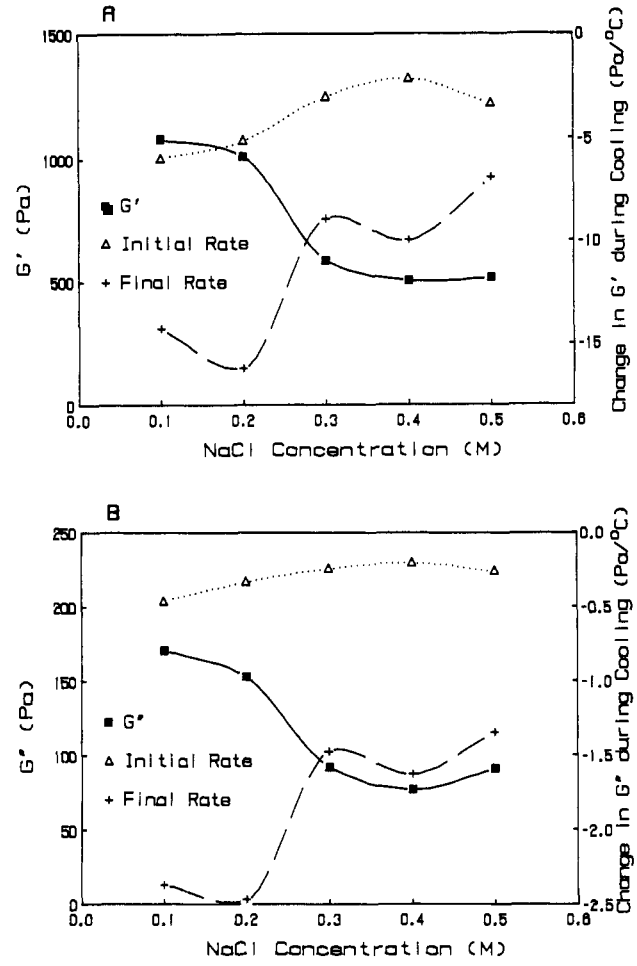

Figure 3. Effect of NaCl on the rate of structure development during the initial and final cooling phases in relation to the G moduli in the resulting networks for 10% vicilin, pH 8.5. (A) G' modulus; (B) G'' modulus.

The reductions in network strength (both G moduli) without a change in network type were related to changes during both network formation and stabilization. In addition, there was a gradual decrease in the rate of change in G' at NaCl concentrations of 0.1, 0.2, and 0.3 M. The interaction between ovalbumin and NaCl at these low salt concentrations tends to shield protein charge (von Hippel and Schleich, 1969) and may be responsible for the difference in the rate of structure development. Why a similar response was not seen for the G'' moduli is unclear.

For vicilin, the stabilizing influence of NaCl was demonstrated by the gradual increase in the T_d values with no distinct trend in terms of ΔH values (Table I). The influence of increasing NaCl concentration on the rheological characteristics of vicilin networks, however, was comparable to that for ovalbumin (Figure 1). Tan δ values were essentially unaltered with only a slight, but significant, increase in 0.5 M NaCl. Microstructure was also unaffected over this salt concentration range with good networks at all NaCl concentrations. The G moduli again showed two distinct levels, with the G' and G'' moduli at 0.1 and 0.2 M NaCl being significantly different from those at 0.3 M NaCl and above (Figure 3). For vicilin, the transition from conditions favoring the electrostatic to the lyotropic influence occurred at a lower salt concentration than for ovalbumin. Perhaps vicilin networks were more sensitive to the lyotropic influence. The fact that increased protein stability corresponded to a decrease in the G' modulus supported the concept that

Table II. Effect of $CaCl_2$ Concentration on the Thermal Denaturation of 10% Ovalbumin and 10% Vicilin, pH 8.5[a]

	ovalbumin		vicilin	
concn, mM	T_d, °C	ΔH, J/g of protein	T_d, °C	ΔH, J/g of protein
0	81.8 ± 0.7[a]	12.2 ± 1.2[a]	73.9 ± 0.1[a]	14.2 ± 0.1[ab]
5	81.2 ± 0.4[a]	14.6 ± 1.3[a]	74.8 ± 0.4[a]	13.5 ± 0.7[ab]
10	81.6 ± 0.7[a]	13.6 ± 1.0[a]	75.6 ± 1.0[a]	14.7 ± 0.8[ab]
15	81.0 ± 0.2[a]	14.8 ± 0.4[a]	79.7 ± 0.5[b]	9.2 ± 1.8[a]
20	80.9 ± 0.2[a]	12.9 ± 0.8[a]	78.7 ± 1.1[b]	12.7 ± 0.1[ab]
50	80.5 ± 0.3[a]	15.7 ± 1.6[a]	79.4 ± 1.1[b]	10.6 ± 2.7[a]
100	79.7 ± 0.5[a]	13.3 ± 1.8[a]	78.5 ± 0.1[b]	14.3 ± 0.3[ab]
200	79.2 ± 0.7[a]	11.5 ± 2.3[a]	81.6 ± 0.1[c]	15.0 ± 1.9[ab]
300	79.0 ± 0.7[a]	13.3 ± 0.1[a]	81.8 ± 0.5[c]	19.0 ± 0.7[b]
400	79.2 ± 0.1[a]	16.3 ± 0.7[a]	84.7 ± 0.3[d]	17.5 ± 1.6[b]
500	79.8 ± 0.8[a]	14.5 ± 2.5[a]	84.8 ± 0.1[d]	17.1 ± 0.7[b]

[a] Column values followed by the same letter are not significantly different ($P \leq 0.05$).

the interactions which promoted protein stability were also factors in determining the strength of the network that resulted from this heat treatment. The similarities in terms of microstructure indicated that these forces did not have the same impact on network type.

The changes in the G moduli used to characterize the final network were clearly related to the changes in the rate of structure development during the final cooling phase (Figure 3); however, the rates of change in the G moduli during the initial cooling phase at concentrations of 0.1 and 0.2 M NaCl were also significantly different from those at higher salt concentrations. As was the case with ovalbumin, the importance of these interactions seemed to be associated with both network establishment and stabilization. The more obvious connection with the final cooling phase may simply reflect the greater impact of the changes during this phase on the final network characteristics.

Overall, the deterioration of ovalbumin networks began at NaCl concentrations greater than 0.3 M, while for vicilin a similar effect was seen at concentrations greater than 0.2 M. These values are in the same range as those reported previously (50–400 mM) as producing maximum strength for whey, egg, and plant proteins (Schmidt et al., 1979; Schmidt, 1981; Sone et al., 1983; Egelandsdal, 1984; Holt et al., 1984; Hayakawa and Nakamura, 1986; Ma et al., 1988; Mulvihill and Kinsella, 1988). The value of 300 mM NaCl for ovalbumin is slightly higher than the 85 mM reported by Egelandsdal (1984), but it should be noted that in the current investigation there was no difference between the G' moduli at 100 and that at 300 mM NaCl. Furthermore, the lower maximum obtained by Egelandsdal (1984) was for gels prepared at pH 4.6 compared to pH 8.5 in this study. In an earlier study (Egelandsdal, 1980), it was shown that the salt maximum was pH dependent and tended to be higher at alkaline pH values.

The deterioration of network formation at higher NaCl concentrations can be attributed to several factors. The masking of protein charge by the salt may inhibit protein–protein interactions necessary for network formation. The influence of salts on hydrophobic interactions may also be a factor. For vicilin the gradual deterioration in the properties of heat-induced networks coincided with an increase in protein stability presumably resulting from increased intramolecular hydrophobic associations. It would appear that the promotion of intramolecular hydrophobic interactions is detrimental to the formation of heat-induced networks. Although a similar scenario could apply to ovalbumin, there was no evidence of increased protein stability with the native protein at

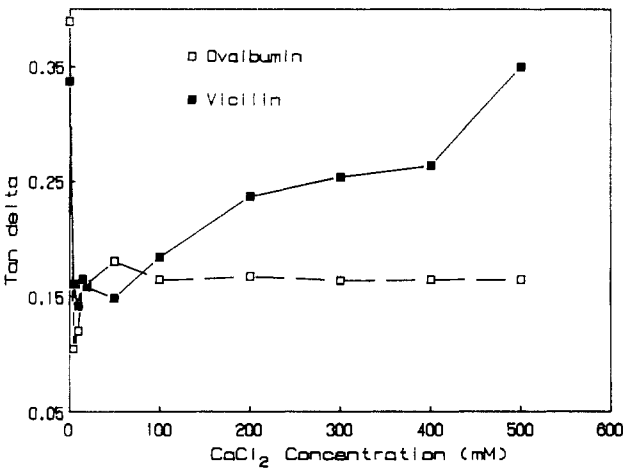

Figure 4. Effect of $CaCl_2$ on tan δ (tan delta) values of heat-induced networks from 10% ovalbumin and 10% vicilin at pH 8.5.

these salt concentrations. This does not preclude the possibility of increased hydrophobic associations in the heat-induced product.

Calcium Chloride ($CaCl_2$). The inclusion of $CaCl_2$ increases positive charge on a protein through the binding of the calcium ion. The binding of calcium has been shown to destabilize proteins, including vicilin (Arntfield et al., 1986). As such conformational changes could impact network formation, the thermal behavior was determined at the various $CaCl_2$ concentrations. There was no evidence of protein destabilization at the $CaCl_2$ concentrations used in this study (Table II). In fact, for vicilin, there was an increase in the T_d values with increasing $CaCl_2$ concentration. As a result, there was no evidence that the associating structures responsible for network formation were unfolding due to the presence of $CaCl_2$ and should therefore have no impact on network formation.

At a pH of 8.5, the initial increase in positive charge due to calcium binding should theoretically counteract the net negative charge so that a situation would result in which the effective charge would be neutralized and aggregation such as that seen around the isoelectric point would be expected. Further addition of $CaCl_2$ would increase the net positive charge, leading to conditions favoring network formation and eventually solubilization. The effect of $CaCl_2$ on the tan δ values for ovalbumin and vicilin did not support this theory (Figure 4). There was no evidence of poor network structure at low $CaCl_2$ concentrations. In fact, for ovalbumin the lowest tan δ value, indicative of a network in which the elastic component was relatively high compared to the viscous component, was obtained in 5 mM $CaCl_2$. Higher $CaCl_2$ concentrations resulted in an increase in tan δ such that it returned to a value of approximately 0.16 at concentrations of 15 mM and above. The microstructure associated with these conditions contained aggregated masses of protein (i.e., no protein network formed). The tan δ values for vicilin also decreased significantly with the inclusion of 5 mM $CaCl_2$ but remained essentially the same up to concentrations of 50 mM. At higher concentrations, tan δ values continued to increase. Interestingly, the microstructure associated with these high tan δ values was not an aggregated product, as was the case with ovalbumin, but tended to increase in solubility with increasing $CaCl_2$ concentration.

The changes in the G' modulus with $CaCl_2$ concentration also supported the formation of protein networks at

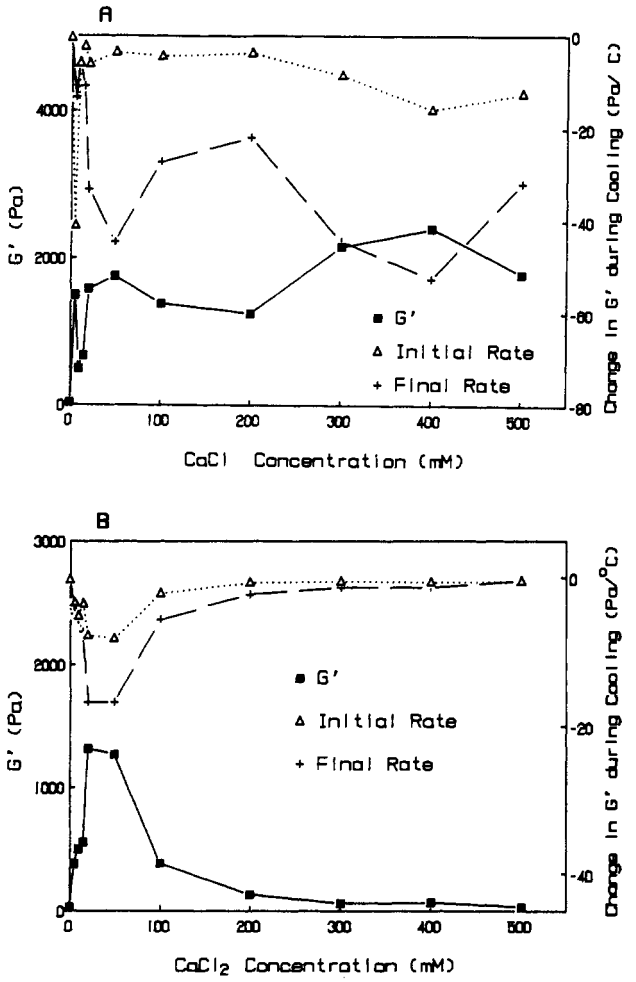

Figure 5. Effect of $CaCl_2$ on the rate of structure development during the initial and final cooling phases in relation to the G' modulus in the resulting networks. (A) 10% ovalbumin, pH 8.5; (B) 10% vicilin, pH 8.5.

low $CaCl_2$ concentrations (Figure 5). For ovalbumin there was a distinct increase in G' with the inclusion of 5 mM $CaCl_2$ (Figure 5A). At concentrations of 10–15 mM $CaCl_2$, the G' modulus was again low, but increased again as the $CaCl_2$ concentration was elevated to conditions associated with higher tan δ values and aggregation. The increase in the G' modulus in this situation appeared to be associated with an increase in the strength of the aggregated mass. The changes in the initial and final cooling phases demonstrated the impact of the changes in G' during cooling on the type of structure obtained. Only with 5 mM $CaCl_2$, where low tan δ values were obtained, was the rate of change in G' during the initial cooling phase influenced by $CaCl_2$ concentration. At higher concentrations, the increase in the G' modulus was reflected in increased rates of structure development during the final cooling phase. This supported the observation made during an investigation into the effect of charge on these two proteins (Arntfield et al., 1989) that changes during the initial cooling phase were associated with the formation of a well cross-linked three-dimensional network.

For vicilin, the response to $CaCl_2$ was somewhat different (Figure 5B). The maximum G' modulus was obtained at $CaCl_2$ concentrations of 20–50 mM. This was in the range where relatively low tan δ values were obtained. Higher $CaCl_2$ concentrations resulted in a significant decrease in the G' modulus, which continues to decrease with increasing concentration. This concurs with the tan δ values and microstructure observed and would be expected if the product was becoming soluble. Interestingly, the maximum G' modulus was associated with changes during both the initial and final cooling phases, though changes during the final cooling phase were greater. This type of response has been shown previously for vicilin when good networks are formed (Arntfield et al., 1989); significant changes during the final phase of cooling appear to be characteristic of vicilin network formation.

The effect of $CaCl_2$ was different for the two proteins in this study. For ovalbumin, optimum network formation occurred at a very low $CaCl_2$ concentration (5 mM). Presumably, even this low level of calcium binding was sufficient to attain the balance of attractive and repulsive forces necessary for network formation. Further calcium binding was associated with increased protein aggregation and a high G' modulus. In this situation, the calcium may have served as a bridge and thus promoted the attractive forces associated with aggregation.

For vicilin, optimum network formation was obtained at slightly higher $CaCl_2$ concentrations (20–50 mM). The optimum $CaCl_2$ concentrations for both ovalbumin and vicilin network formation were comparable to those reported previously for whey proteins (Schmidt and Morris, 1984; Mulvihill and Kinsella, 1988). Increasing the $CaCl_2$ concentration did not result in increased aggregation but rather produced a soluble product as would result from an increase in the net positive charge. It is possible that the conformation of the associated vicilin molecule is such that the binding calcium ions cannot serve the same bridging function that was observed with ovalbumin. This may account for the different responses to the inclusion of $CaCl_2$ for ovalbumin and vicilin.

Anions of Sodium Salts. The use of various anions of a given salt is one of the most valuable tools for investigating the importance of hydrophobic interactions to a given system. Even with this technique, it is not always possible to separate the influence of hydrophobic interactions from other changes that may result from the inclusion of these anions. This is particularly true for anions that destabilize the protein by binding. Not only can this lead to conformational changes, but the net charge on the protein may also be altered, thus making it impossible to isolate a response that can be attributed to hydrophobic interactions. In an attempt to account for possible charge effects, the anions have been examined at concentrations of 0.1 and 0.5 M. Differences seen at 0.5 M but not at 0.1 M should provide a better evaluation of the importance of hydrophobic interactions, as the lyotropic influence should not be a significant factor at the lower salt concentration.

Variations in tan δ values for ovalbumin and vicilin networks with the inclusion of various sodium salts are given in Figure 6. The salts have been presented in terms of their position in the lyotropic series, with the more stabilizing salts [on the basis of the molal surface tension increments as suggested by Melander and Horvath (1977)] on the right-hand side of the graph. At a concentration of 0.1 M, tan δ values for ovalbumin were all approximately 0.1 or less, values which in the previous experiments represented well cross-linked networks. Within this range, however, the tan δ value of the network formed in NaSCN was significantly lower than in other salts, while in Na_2SO_4 the value was significantly higher. According to DSC data (Table III), the NaSCN environment resulted in significant ovalbumin destabilization which can be attributed to preferential binding of the thiocyanate to the protein (Arakawa and Timasheff, 1982). The increase in the net negative charge on the protein resulting from the interaction between NaSCN

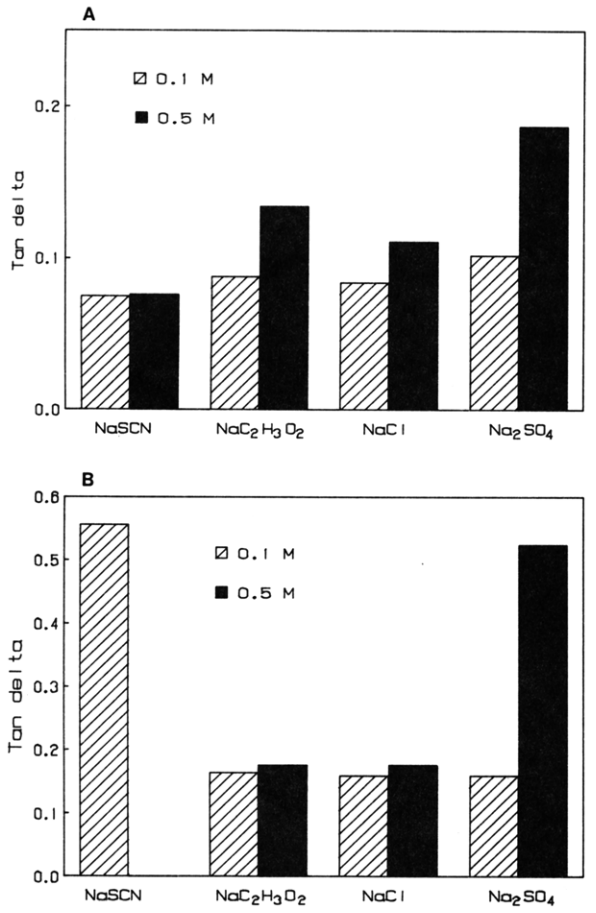

Figure 6. Effect of anions of sodium salts (0.1 and 0.5 M) on the tan δ (tan delta) values for heat-induced protein networks. (A) 10% ovalbumin, pH 8.5; (B) 10% vicilin, pH 8.5.

Table III. Effect of Various Anions of Sodium Salts (0.1 M) on the Thermal Denaturation of 10% Ovalbumin and 10% Vicilin, pH 8.5[a]

	ovalbumin		vicilin	
salt	T_d, °C	ΔH, J/g of protein	T_d, °C	ΔH, J/g of protein
NaSCN	83.9 ± 0.0[a]	16.6 ± 0.6[a]	75.2 ± 0.5	12.5 ± 1.8[a]
$NaC_2H_3O_2$	85.7 ± 0.2[b]	15.1 ± 0.8[a]	78.3 ± 0.2[a]	13.3 ± 0.7[a]
NaCl	85.4 ± 0.1[b]	14.1 ± 0.9[a]	78.1 ± 0.3[a]	15.9 ± 0.9[a]
Na_2SO_4	86.1 ± 0.3[b]	10.5 ± 0.7	80.6 ± 0.6	15.6 ± 1.3[a]

[a] Column values followed by the same letter are not significantly different ($P \leq 0.05$).

and the protein may account for the low tan δ values observed in this environment. The higher tan δ value in the presence of 0.1 M Na_2SO_4 may result from the higher ionic strength (μ = 0.3) associated with this salt. The differences in the tan δ values were also reflected in the microstructure (Figure 7). As expected, the NaSCN environment produced an intense well cross-linked network. With $NaC_2H_3O_2$ and NaCl, the networks were again characterized as well cross-linked strands, but the degree of cross-linking appeared to be slightly lower as evidenced by the number of strands that stopped abruptly and were not connected to an adjacent strand. The poor network in the sulfate environment—many strands but little evidence of cross-linking—was also worse than expected considering the tan δ value was only slightly higher than those for the other salts.

The trend in terms of tan δ values at a salt concentration of 0.5 M was similar in that the tan δ value was significantly lower in the thiocyanate environment and significantly higher in the sulfate environment. The tan δ value in the acetate environment was also higher than in chloride but lower than in sulfate, though the reason for this was not clear. In all cases, the values were higher than at the lower salt concentration, supporting the observation made with NaCl that increased salt concentrations result in networks that were not as well structured. The similarity to the 0.1 M results was also evident in the microstructure (not shown). Structures for each salt tended to be a little more open than at 0.1 M, with marked deterioration in strand formation for the sulfate environment. Due to the similarities in the results with 0.1 and 0.5 M salts, it appeared that hydrophobic interactions have little influence on the type of structure that is formed in heat-induced ovalbumin networks.

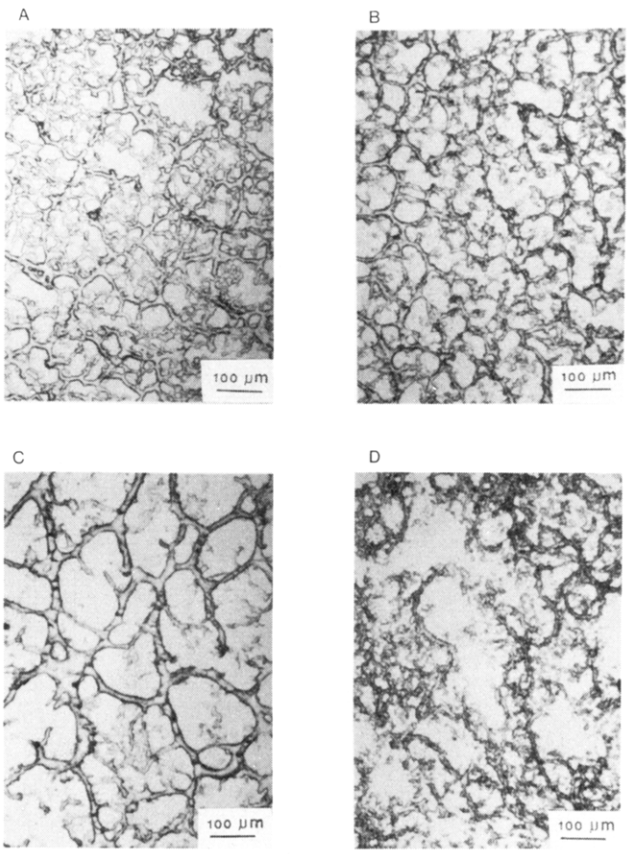

Figure 7. Photomicrographs showing the effect of anions of sodium salts (0.1 M) on heat-induced networks from 10% ovalbumin, pH 8.5. (A) NaSCN; (B) $NaC_2H_3O_2$; (C) NaCl; (D) Na_2SO_4.

With the exception of NaSCN, the inclusion of various anions of sodium salts at the 0.1 M level had no impact on the tan δ values for heat-induced vicilin networks (Figure 6B). As with ovalbumin, the thiocyanate environment resulted in protein destabilization (significantly lower T_d value; Table III) due to binding of the thiocyanate anion, as has been reported previously (Ismond et al., 1986). Unlike ovalbumin, however, the binding did not result in improved gel structure; the high tan δ value due to this increase in charge represented a situation in which the charge repulsion was so high that the protein tended to be soluble. This was also evident from the microstructure, where highly hydrated structures were observed.

Results with 0.5 M salts were somewhat complex. With NaSCN, vicilin remained soluble during the heat treatment so that no rheological or microstructural data could be obtained. The T_d values for vicilin in $NaC_2H_3O_2$ and NaCl were not significantly different, but significantly lower than that for Na_2SO_4 (Table IV). This is in agreement with data reported previously (Ismond et al., 1986).

Table IV. Effect of Various Anions of Sodium Salts (0.5 M) on the Thermal Denaturation of 10% Ovalbumin and 10% Vicilin, pH 8.5[a]

	ovalbumin		vicilin	
salt	T_d, °C	ΔH, J/g of protein	T_d, °C	ΔH, J/g of protein
NaSCN	79.2 ± 0.1	13.7 ± 1.5[a]	73.7 ± 0.1	13.1 ± 0.9[a]
$NaC_2H_3O_2$	86.5 ± 0.1[a]	18.4 ± 0.7[a]	85.1 ± 0.3[a]	18.1 ± 4.0[a]
NaCl	86.0 ± 0.2[a]	16.4 ± 0.7[a]	84.2 ± 0.1[a]	14.6 ± 2.7[a]
Na_2SO_4	89.6 ± 0.6	17.1 ± 3.6[a]	92.2 ± 0.1	17.9 ± 3.8[a]

[a] Column values followed by the same letter are not significantly different ($P \leq 0.05$).

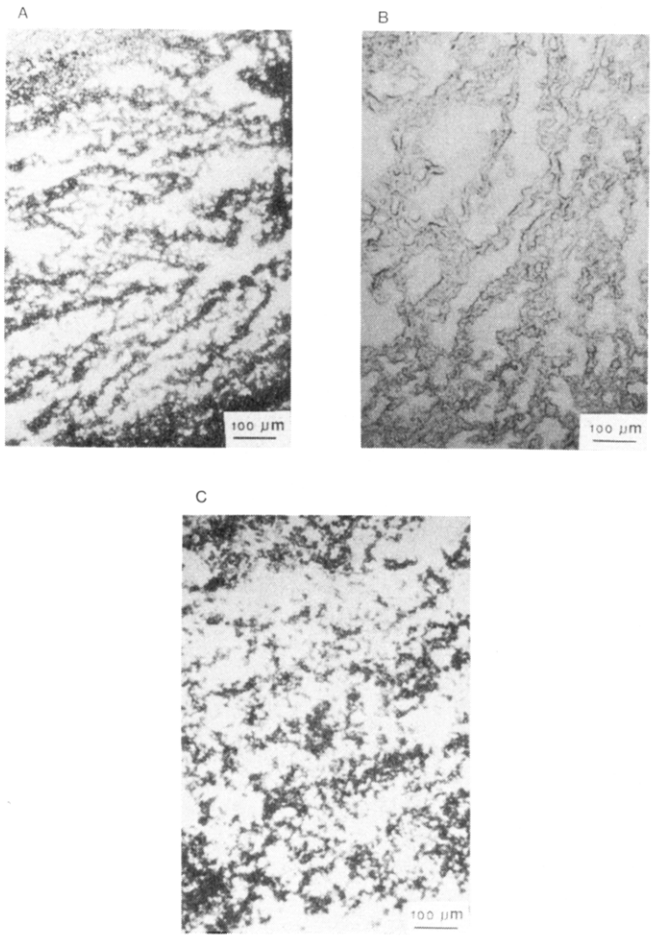

Figure 8. Photomicrographs showing the effect of anions of sodium salts (0.5 M) on heat-induced networks from 10% vicilin, pH 8.5. (A) $NaC_2H_3O_2$; (B) NaCl; (C) Na_2SO_4.

Tan δ values in $NaC_2H_3O_2$ and NaCl were the same, while values in the Na_2SO_4 environment were significantly higher (Figure 6B). In terms of microstructure, none of these environments produced particularly good networks (Figure 8). With NaCl, there were clear protein strands, while the degree of cross-linking was minimal. Despite a similar tan δ value, the alignment of protein in the acetate environment did not give the distinct strands seen with chloride and the extent of cross-linking was again poor. With Na_2SO_4, only protein aggregates were present as expected from the tan δ values. Despite the poor microstructure associated with higher salt levels, there was little evidence to demonstrate an influence of hydrophobic interactions on the type of heat-induced network formed with vicilin.

The strength of the networks can be assessed by examining the G moduli. At a concentration of 0.1 M the G moduli for ovalbumin were unaffected by anion type (Figure 9A). Higher salt concentrations, however, had a profound effect. Those salts at the destabilizing end of the series, especially NaSCN, produced high G' and G'' moduli which decreased with the inclusion of the more stabilizing salts such that with Na_2SO_4 the G moduli were extremely low. This relationship between G moduli and the position of a salt in the lyotropic series at a concentration of 0.5 M, but not 0.1 M, provided strong evidence for the involvement of hydrophobic interactions as factors in determining the strength of heat-induced ovalbumin networks. Interestingly, this influence of hydrophobic interactions on network strength did not appear to impact the type of network formed.

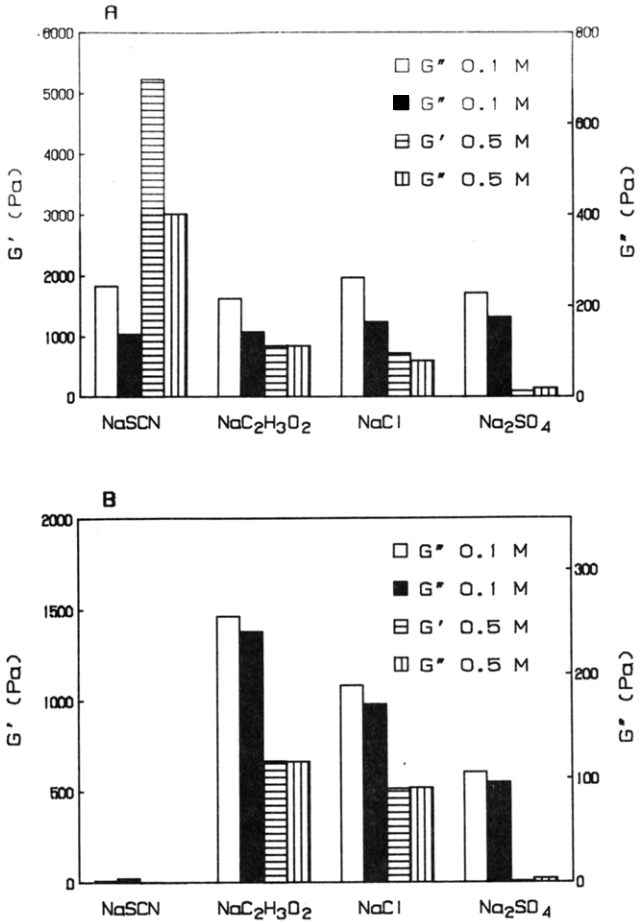

Figure 9. Effect of anions of sodium salts (0.1 and 0.5 M) on the G moduli for heat-induced protein networks. (A) 10% ovalbumin, pH 8.5; (B) 10% vicilin, pH 8.5.

Examination of the cooling curve data for 0.5 M salts showed that changes in G' during both the initial and final cooling phases followed the same trend as the G' modulus for the final product (Figure 10A). The more stabilizing salts were associated with a lower rate of structure development, thus accounting for the lower G' modulus in the final product. A similar trend was noted for the G'' modulus, though not shown. From these results, it was apparent that the influence of salts in the lyotropic series on the strength of heat-induced ovalbumin networks was in effect during both the establishment and strengthening of the network.

With vicilin, the very low G moduli in the 0.1 M NaSCN environment were consistent with the trend toward protein solubilization (Figure 9B). For the other anions, there was a gradual decrease in the G moduli with the inclusion of the more stabilizing salts. Although vicilin stability was not affected at this low salt concentration, the interactions in the heat-induced networks were. It is pos-

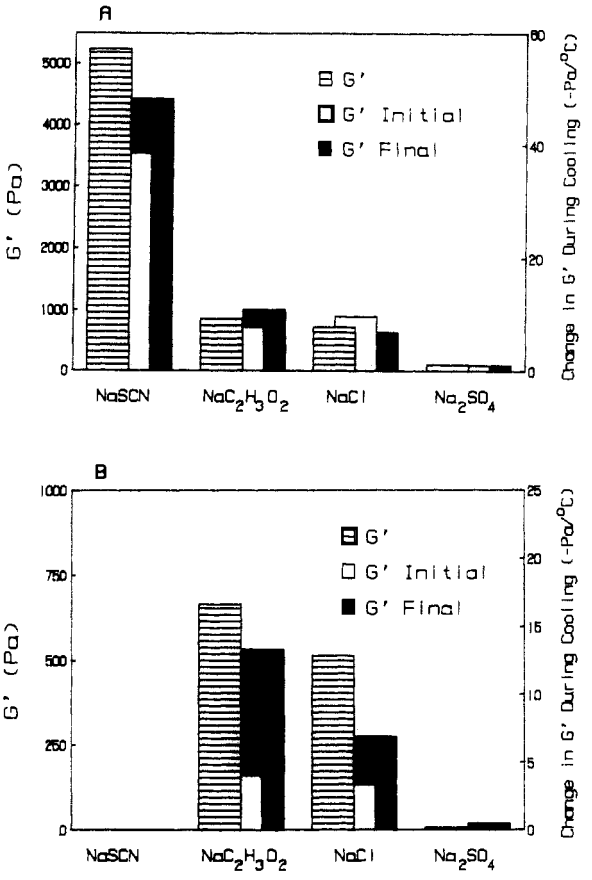

Figure 10. Influence of anions of sodium salts (0.5 M) on the rate of structure development during cooling in relation to the G' modulus in the resulting heat-induced protein networks. (A) 10% ovalbumin, pH 8.5; (B) 10% vicilin, pH 8.5.

sible that under certain conditions even this low salt concentration may impact hydrophobic interactions. The G moduli in 0.5 M salts also decreased in the presence of more stabilizing anions as was observed with ovalbumin. This influence was evident during both the initial and final cooling phases (Figure 10B). In this respect, the response was similar to that for ovalbumin and implicated the importance of hydrophobic interactions during both the establishment and stabilization of heat-induced vicilin networks.

Although it is difficult to isolate the contribution of hydrophobic interactions to any protein system, the relationship between the G moduli in the 0.5 M salts and the position of the salts in the lyotropic series for both ovalbumin and vicilin provided strong evidence in support of a significant role for hydrophobic interactions in heat-induced network formation. The impact of these interactions on network strength was evident during both phases of the cooling regime, indicating involvement during the establishment as well as the strengthening of the protein network. The possibility of hydrophobic interactions influencing the type of network formed was not well supported by these data. Extreme stabilizing conditions (Na_2SO_4) were required to change the ovalbumin network from a gelled to an aggregated product. It is possible that the higher ionic strength of this salt and not just the influence on hydrophobic interactions was responsible for this response. With vicilin, evidence linking network type to hydrophobic interactions was stronger; however, even this was questionable due to the poor networks at the high salt concentration required to produce a lyotropic effect.

To determine how hydrophobic interactions are involved in network formation, the principles behind the lyotropic influence must first be examined. The hydrophobic interaction results from the unfavorable association between water and nonpolar groups. With native protein structure, this manifests itself in a burial of hydrophobic residues within the molecule. The extent of hydrophobic interaction formation has been attributed to effects on the structure of water. The lyotropic series have been correlated to the effect of salts on the surface tension of water (Melander and Horvath, 1977). As surface tension is increased, a compact protein structure is energetically favored, thus accounting for the increase in stability.

The same rationale does not apply to intermolecular protein interactions. If the higher surface tension in the stabilizing salts favored intermolecular interactions, then an increase in gel strength would have been expected. This was not the case; the more stabilizing salts reduced the G moduli. In consideration of the theory that network formation with globular protein is through the interaction of corpuscular structures (Clark and Lee-Tuffnell, 1986), it is plausible to conclude that in the stabilizing environments hydrophobic interactions were promoted within the corpuscular structure. In this way, the number of nonpolar residues available for intermolecular interactions was reduced, thus accounting for the low G moduli.

As a result, it would appear that for both ovalbumin and vicilin hydrophobic interactions represent an attractive component in the balance of attractive and repulsive forces required for network formation. Under most conditions, this force adds to the strength of the network even during its initial formation. It is the electrostatic profile, however, that seems to control the type of network formed. Only with highly stabilizing conditions (e.g., sulfate environment) did hydrophobic interactions appear to have an impact on network type. It may be hypothesized that the need to bury nonpolar residues in this environment could not be accommodated simply by a tightening of the corpuscular structure and further burial was via intermolecular hydrophobic associations. In this situation, an aggregated product was obtained as these attractive intermolecular forces may have disrupted the balance with repulsive electrostatic influence. In this respect, the data agreed with those obtained by Paulson and Tung (1989), where they found the minimum tan δ values were associated with an intermediate surface hydrophobicity value for the heated dispersion (S_e). When S_e was low, there were insufficient interactions to form a three-dimensional network, while at high S_e values, aggregation was promoted. In the current investigation, the high level of intermolecular hydrophobic interactions in the sulfate environment promoted aggregation, while suppression of surface hydrophobicity within the corpuscular structure with other salt environments merely reduced network strength rather than prevented network formation.

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Registry No. NaCl, 7647-14-5; CaCl, 10043-52-4; NaSCN, 540-72-7; $NaC_2H_3O_2$, 127-09-3; Na_2SO_4, 7757-82-6.