Role of Disulfide Bonds in Determining the Rheological and Microstructural Properties of Heat-Induced Protein Networks from Ovalbumin and Vicilin

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The involvement of disulfide bonds in heat-induced networks from ovalbumin and vicilin was investigated by incorporating cysteine hydrochloride (CysHCl), mercaptoethanol (ME), and N-ethylmaleimide (NEM) during network formation. Network evaluation was based on rheological properties (small amplitude oscillatory testing) and microstructure (light microscopy). Low G' (storage) and G'' (loss) moduli, elevated tan δ (G''/G') values, and a reduction in protein cross-linking at ME and NEM concentrations of 25 and 50 mM supported disulfide bond involvement in ovalbumin networks. The impact of these reagents was seen during the final stage of cooling. Vicilin networks were unaffected by these reagents, indicating no disulfide bond involvement. While disulfide bonds may contribute to the elasticity and strength of a protein network, noncovalent forces are key to initial network formation.

INTRODUCTION

Due to the high interaction energies associated with disulfide bonds, their presence should have a significant impact on protein network characteristics. The highly elastic gels resulting from heating ovalbumin in 6 M urea, a system resulting from disulfide interactions, demonstrated the potential for this type of interaction (van Kleef et al., 1978). The possibility of disulfide bond formation varies with the source of protein, and even for protein networks in which disulfide bond formation has been implicated, the literature is confusing and the exact role of these bonds has not been well established.

Investigations of disulfide bond involvement have used a number of different approaches. The solubility of networks in high levels of denaturants has been used to investigate the relative importance of noncovalent interactions and covalent disulfide bonds (Furukawa et al., 1979; Lakshmi and Nandi, 1979; Bikbov et al., 1986; van Kleef, 1986). There is little agreement, however, in the results of these studies with contradictory data for both soy and ovalbumin gels. These conflicting results may simply reflect different protein recovery procedures or differential time factors in the solubilization studies; however, they do jeopardize the credibility of this technique. In addition, the use of disulfide reducing agents alone was not sufficient to dissolve either ovalbumin (Beveridge et al., 1980; Hatta et al., 1986) or soy protein gels (Furukawa et al., 1979).

Examination of free SH groups in protein networks has also produced a range of responses for different proteins. The level of SH groups has been related to gel strength for ovalbumin (Hayakawa and Nakai, 1985) but not whey proteins (Kohnhorst and Mangino, 1985) and showed no correlation with the ability of ovalbumin to coagulate (Hayakawa and Nakai, 1985). In addition, by following the sulfhydryl/disulfide interchange reactions in β-lactoglobulin, Shimada and Cheftel (1989) found that intermolecular disulfide bonds were responsible for the elastic network at pH 7.5 but had no impact on the less elastic gel obtained at pH 2.5.

A more common technique for studying disulfide interactions is the inclusion of reagents that modify or prevent the formation of disulfide bonds prior to heating; these reagents include cysteine hydrochloride (CysHCl), 2-mercaptoethanol (ME), dithiothreitol (DTT), and N-eth-

ylmaleimide (NEM). The effectiveness of these compounds varies; twice as much ME (one SH group) as DTT (two SH groups) was required to give the same gel strength for lysozyme (Hayakawa and Nakamura, 1986), and NEM had no effect on soy protein under conditions in which ME reduced gel hardness (Utsumi and Kinsella, 1985). As was seen with the SH analysis, protein coagulation was not affected by the addition of either ME or NEM (Lakshmi and Nandi, 1979; Ma and Harwalkar, 1987). Results obtained when looking at gel hardness were also variable. In some studies, low levels of reducing agents were shown to increase gel hardness; maximum hardness for whey protein concentrate (WPC) was found in 9.7 mM cysteine (Schmidt, 1981), while maximum breaking strength for lysozyme occurred at a DTT concentration of 7 mM (Hayakawa and Nakamura, 1986). At higher concentrations, the inclusion of these reducing reagents tended to result in decreased gel hardness and torsional rigidity for both egg and soy proteins (Furukawa and Ohta, 1982; Hayakawa and Nakamura, 1986; Mori et al., 1986; Vigdorth and Ball, 1988). Interestingly, the changes in gel strength were not reflected in the microstructure observed with scanning electron microscopy for albumen gels (Vigdorth and Ball, 1988). In the study of Utsumi and Kinsella (1985), on the other hand, gel hardness for the 11S and 7S globulins decreased with increasing ME or DTT concentration as reported previously; with a soy protein isolate only an initial decrease in hardness was observed, and at concentrations greater than 0.01 M the trend was reversed so that increased values were obtained.

A complicating factor in examining these disulfide bond effects has been the empirical nature of the techniques used to evaluate network characteristics. In terms of fundamental rheological properties, little information is available. However, DTT has been shown to decrease G' (storage modulus) while increasing the loss tangent value for rapeseed gels; this implicated disulfide interactions as a factor in the elasticity of the network (Gill and Tung, 1978). This is in contradiction to the role of disulfide interactions based on gel solubility, where canola (genetic variant of rapeseed) gels were soluble in both 8 M urea and 6 M guanidine hydrochloride (Paulson and Tung, 1989).

To examine the role of disulfide bonds in network

Role of Disulfide Bonds in Protein Networks

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Table I. Effect of Disulfide Modifying Agents on the Thermal Properties of Ovalbumin (10%) in 0.15 M NaCl, pH 8.5[a]

concn, mM	cysteine hydrochloride		mercaptoethanol		N-ethylmaleimide	
	T_d, °C	ΔH, J/g of protein	T_d, °C	ΔH, J/g of protein	T_d, °C	ΔH, J/g of protein
0.0	85.1 ± 0.1[ab]	15.9 ± 0.1[ab]	85.1 ± 0.1[a]	15.9 ± 0.1[a]	85.1 ± 0.1[a]	15.9 ± 0.1[a]
2.5	85.0 ± 0.1[ab]	9.8 ± 1.2[a]	85.6 ± 0.1[a]	14.8 ± 0.3[a]	85.3 ± 0.2[a]	15.5 ± 1.6[a]
5.0	85.2 ± 0.4[ab]	20.6 ± 0.1[b]	85.6 ± 0.1[a]	13.1 ± 0.2[a]	84.5 ± 0.6[a]	14.0 ± 1.1[a]
10.0	84.6 ± 0.3[ab]	23.5 ± 2.6[b]	83.9 ± 0.1[b]	13.8 ± 0.7[a]	84.4 ± 0.1[a]	15.5 ± 1.6[a]
25.0	85.6 ± 0.1[a]	10.5 ± 0.1[a]	84.0 ● 0.0[b]	13.2 ± 0.2[a]	82.7 ± 0.1	12.5 ± 1.4[a]
50.0	84.3 ± 0.3[b]	13.2 ± 0.4[a]	82.1 ± 0.6[c]	12.6 ± 0.4[a]	81.1 ± 0.1	11.8 ± 2.1[a]

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

formation for ovalbumin and vicilin, a plant storage protein from faba bean (*Vicia faba*), both fundamental rheological properties and microstructure (using light microscopy) have been examined. For this purpose, three reagents that impact disulfide bond formation (CysHCl, ME, and NEM) were incorporated into the protein dispersion prior to the heat treatment. Structure development during network formation as well as characteristics of the networks formed were used to evaluate the effects of these compounds.

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 (*V. faba* var. Diana) following the procedure of Ismond et al. (1985). All other chemicals were of reagent grade.

Sample Preparation. Dispersions of protein at a concentration of 10% (w/w) were prepared in 0.15 M NaCl; this protein concentration has been shown to be sufficiently high to produce well cross-linked heat-induced networks (Arntfield et al., 1990a). Disulfide blockers (NEM) or modifying reagents (CysHCl and ME) at concentrations ranging from 2.5 to 50 mM were included in the solvent used for sample preparation. The pH of the dispersions was adjusted to 8.5 with 1.0 M NaOH, and only when this pH had been maintained for 30 min were further analyses conducted. In previous investigations (Arntfield et al., 1990b), it was shown that at alkaline pH values (e.g., pH 8.5) both vicilin and ovalbumin formed reasonably well cross-linked networks for which both rheological properties and microstructure could be readily assessed (e.g., relatively easy to section). It was necessary to use conditions producing good networks so that the effects of the various reagents on disulfide bond formation would be apparent. The 30-min time limit was somewhat arbitrary but thought to be adequate as it was noted that any drift in pH occurred during the first 10–15 min. Samples prepared in this manner were used for calorimetric, microstructural, and rheological analyses; however, further sample manipulation varied with the analysis.

Calorimetry. The thermal properties of ovalbumin and vicilin were used as an indicator of protein conformational change resulting from the inclusion of the disulfide blocker and modifying agents. A Du Pont 9900 thermal analyzer with a 910 differential scanning calorimeter cell base was used. Thermal curves were obtained for 10–15 μL of sample at 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 by using the General Analysis Utility Program (version 2.2) available for the calorimeter. The basis for these calculations has 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 an American Optical 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 Kodak Ektachrome 160 ASA film. Details of the procedure have been published previously (Arntfield et al., 1990a).

Rheology. Heat-induced protein networks for rheological analysis were prepared in a Bohlin VOR rheometer (Bohlin Reologi, Inc., Edison, NJ 08818) 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 δ = G''/G'), a parameter reflecting the relative energy from the viscous and elastic components, was calculated. As the rheological parameters during heating were similar for all treatments, only significant changes 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 as well as its application to studying the effects of protein concentration and pH have been described previously (Arntfield et al., 1990a,b).

Dynamic properties of the networks formed in this fashion were measured as a function of oscillatory frequency (ω) at a temperature of 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 described previously (Arntfield et al., 1989, 1990a). Data comparison was based on the regression analysis of these relationships using values associated with a frequency of 1 Hz.

Statistical Analysis. All analyses were performed in duplicate and average values used in all tables and figures. Statistical differences were determined by using an analysis of variance in conjunction with a Duncan's multiple range test (Steel and Torrie, 1960).

RESULTS

Influence on Thermal Properties. The impact of including reagents to modify or block disulfide bonds on the thermal properties of ovalbumin is shown in Table I. The differences observed for both T_d and ΔH values showed no distinct trend with respect to CysHCl concentration. With ME and NEM, on the other hand, there was a decrease in the T_d value with increasing concentration. While similar trends were seen for ΔH values (particularly for ME), these differences were not significant due to the higher standard deviation association with the ΔH measurements compared to that with the T_d values. It appeared that these reagents reduced ovalbumin stability but did not induce any major conformational change to the protein.

The effect of these reagents on the thermal properties of vicilin was considerably different (Table II). With CysHCl, variations in the T_d values were minor, but ΔH values decreased with increasing concentration such that

Table II. Effect of Disulfide Modifying Agents on the Thermal Properties of Vicilin (10%) in 0.15 M NaCl, pH 8.5[a]

concn, mM	cysteine hydrochloride		mercaptoethanol		N-ethylmaleimide	
	T_d, °C	ΔH, J/g of protein	T_d, °C	ΔH, J/g of protein	T_d, °C	ΔH, J/g of protein
0.0	79.1 ± 0.9[ab]	21.5 ± 2.2	79.1 ± 0.9[a]	21.5 ± 2.2	79.1 ± 0.9[a]	21.5 ± 2.2
2.5	78.5 ● 0.1[a]	15.4 ● 1.5[a]	78.8 ± 0.0[a]	14.9 ± 3.8[a]	79.6 ± 0.5[a]	13.5 ± 0.7[a]
5.0	78.4 ± 0.1[a]	13.7 ± 1.9[a]	78.4 ± 0.1[a]	12.5 ± 0.8[a]	81.8 ● 0.2[ab]	10.5 ± 1.3[a]
10.0	79.6 ± 0.3[b]	11.2 ± 2.0[a]	78.8 ± 0.7[a]	15.6 ± 2.0[a]	81.9 ± 0.9[ab]	14.6 ● 4.6[a]
25.0	78.4 ● 0.3[a]	6.4 ± 1.2[b]	78.1 ± 0.0[a]	14.6 ± 0.6[a]	83.9 ± 1.0[b]	9.2 ± 1.2[a]
50.0	78.4 ± 0.2[a]	5.8 ± 0.3[b]	78.3 ± 0.1[a]	14.1 ± 0.1[a]	81.0 ± 0.1[a]	13.3 ● 0.6[a]

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

all were lower than the control values; values at 25 and 50 mM were significantly lower than those at 2.5 and 10 mM. The overall trend was indicative of some protein denaturation. With ME and NEM, T_d values were unaffected. Although ΔH values for all ME and NEM environments were lower than control values, there was no evidence of a concentration effect for these reagents. On the basis of these data, the impact of ME and NEM on vicilin conformation is unclear. Due to the complexity of factors contributing to the ΔH value (Murray et al., 1985) and the lack of a concentration effect, it cannot be concluded that there were changes in vicilin conformation in the presence of ME and NEM, and a relationship between their ability to react with SS or SH groups and protein conformation is questionable. As a result, the impact of cysteine hydrochloride on vicilin conformation may reflect a binding of the chloride ion and resulting charge repulsion within the molecule rather than a change in the sulfhydryl/disulfide groups within the molecule.

Effect of Cysteine Hydrochloride (CysHCl) on Network Properties. The inclusion of CysHCl had little influence on the rheological properties of the heat-induced ovalbumin networks produced (Figure 1A). Tan δ values for the control and in the presence of 10 mM CysHCl were significantly higher than at other CysHCl concentrations; however, all tan δ values were less than 0.1, a value previously associated with good network formation (Arntfield et al., 1990a). Generally, the G moduli increased with the inclusion of CysHCl up to a concentration of 25 mM but then decreased. At concentrations up to 25 mM CysHCl, the microstructure was characterized by well cross-linked networks (not shown). At a concentration of 50 mM CysHCl, the cross-linking within the structure was not as uniform as at lower concentrations. Overall, changes in network characteristics due to CysHCl had little influence on the type of network formed (tan δ), though there was a gradual increase in network strength (G moduli) at concentrations below 50 mM.

Examination of the data obtained during cooling, however, indicated that the mechanism of network formation was altered by the inclusion of CysHCl (Figure 1B). For both G' and G'', there was a gradual increase in structural development during the initial cooling phase, such that the values at 25 and 50 mM CysHCl were significantly different. A gradual increase in structural development with increasing CysHCl concentration was also observed for G' and G'' during the final cooling phase except that the trend was reversed when a concentration of 50 mM CysHCl was used. In this environment, the change in the G moduli during the initial cooling phase was counteracted by this drastic decrease in structure development during the final cooling phase, resulting in slightly lower G moduli in the final product.

Changes in the tan δ values as a function of cooling temperature (Figure 2) in 50 mM CysHCl did not reflect the variations seen for the G moduli during cooling. In fact, the tan δ value resulting from the initial structure
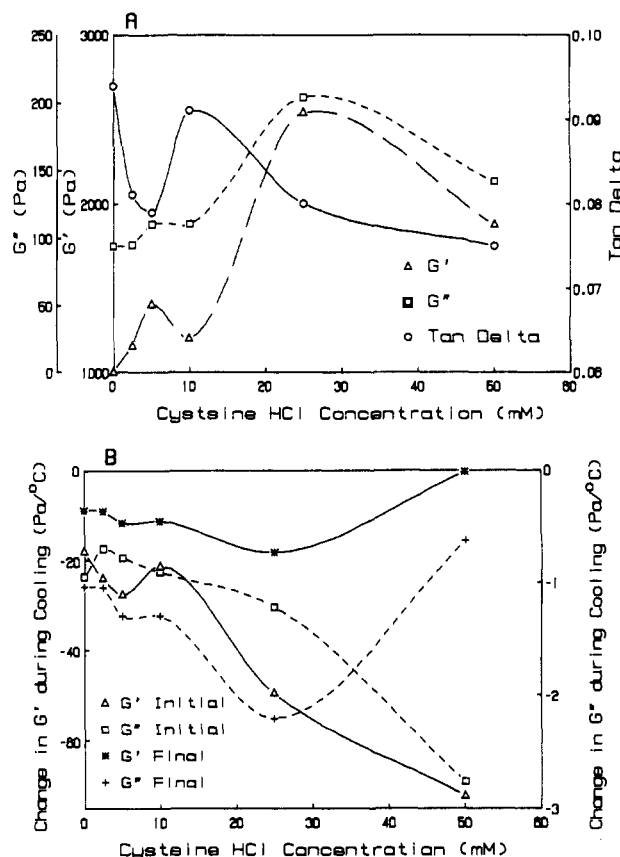

Figure 1. Effect of cysteine hydrochloride (cysteine HCl) on (A) the rheological properties and (B) structure development (change in the G moduli) during the initial and final cooling phases for 10% ovalbumin in 0.15 M NaCl, pH 8.5.

development at the onset of cooling was maintained throughout the cooling regime. The changes in the G' and G'' were parallel so that the network structure was unaffected. This was in contrast to the control, where a slight increase in tan δ values was observed during the latter stage of cooling. The net result was the lower tan δ value for the final product when 50 mM CysHCl was included.

At the levels of CysHCl used in this investigation, there were no significant changes in the G moduli of heat-induced vicilin networks (Table III). However, tan δ values at concentrations of 25 and 50 mM CysHCl were slightly lower than with 5 mM CysHCl. This slight increase in the relative elasticity at the higher CysHCl concentration may be related to the partial denaturation of vicilin under these conditions. It has been shown previously that conditions which promote protein unfolding (i.e., low pH) can promote the formation of a more elastic network (Arntfield et al., 1990b). Presumably, this is due to a change in the conformation of the associating protein structure from a globular to a more linear form. These changes, however, were minor as was reflected in the microstructure, where the only difference was a slight increase in cross-linking
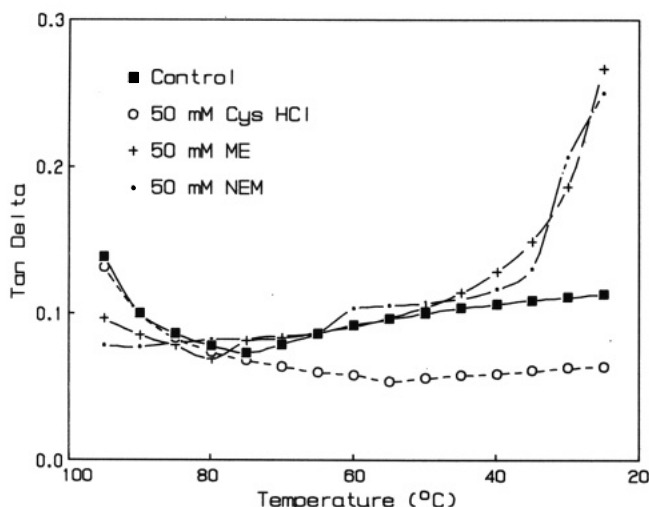
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Figure 2. Influence of disulfide modifying reagents on tan delta (tan δ) values during cooling for heat-induced networks from ovalbumin. Curves were calculated from the biphasic linear models used to describe the changes in the G' and G'' moduli. Control was a sample of 10% ovalbumin in 0.15 M NaCl, pH 8.5; other samples used the same solvent but also included the disulfide modifying reagents indicated.

Table III. Effect of Cystine Hydrochloride on the Rheological Properties of Heat-Set Vicilin (10% in 0.15 M NaCl, pH 8.5)[a]

concn, mM	G', Pa	G'', Pa	tan δ
0.0	1221 ± 91[a]	161 ± 5[a]	0.132 ± 0.006[b]
2.5	910 ± 196[a]	141 ± 41[a]	0.157 ± 0.006[ab]
5.0	719 ± 57[a]	116 ± 16[a]	0.161 ± 0.010[a]
10.0	1024 ± 24[a]	161 ± 10[a]	0.157 ± 0.006[ab]
25.0	881 ± 10[a]	123 ± 3[a]	0.140 ± 0.002[b]
50.0	700 ± 24[a]	98 ± 4[a]	0.140 ± 0.000[b]

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

Figure 3. Photomicrographs showing the effect of cysteine hydrochloride on heat-induced networks for 10% vicilin in 0.15 M NaCl, pH 8.5. Cysteine hydrochloride concentrations were (A) 5 mM, where a space between protein strands is indicated by the arrow, (B) 10 mM, (C) 25 mM, and (D) 50 mM, where an example of cross-linking is indicated by the arrow.

when 50 mM CysHCl was included (Figure 3). Unlike the situation with ovalbumin, there were no differences in the G moduli during the initial and final phases of the cooling regime (not shown).

Effect of Mercaptoethanol (ME) on Network Properties. The G moduli for ovalbumin networks in 2.5 mM ME were higher than the control values. The inclusion of increasing concentrations of ME, however, resulted in a gradual decrease in the G moduli up to a concentration of 10 mM, after which the G moduli were not significantly different (Figure 4A). The type of structure, as indicated by the tan δ value, was not affected by the initial changes in the G moduli, but differences in the viscous and elastic components at higher ME concentrations resulted in a significant increase in the tan δ values for network at 25 and 50 mM ME. The microstructure at low ME concentrations (2.5 and 5 mM) exhibited well cross-linked networks (Figure 5A,B). The appearance of amorphous areas within the network at 5 mM ME may account for the lower G moduli. The structure at 10 mM ME (not shown) was similar to that at 5 mM. At higher ME concentrations the amorphous regions had disappeared and strands that remained were not as well cross-linked (e.g., 50 mM, Figure 5D). This decrease in cross-linking was responsible for the increased tan δ value.

On the basis of the data obtained during cooling, the differences in the characteristics of the resulting network could be attributed to changes that occurred during the final phase of the cooling regime (Figure 4B). There was a decrease in structure development with increasing ME concentration such that the values at 25 and 50 mM ME
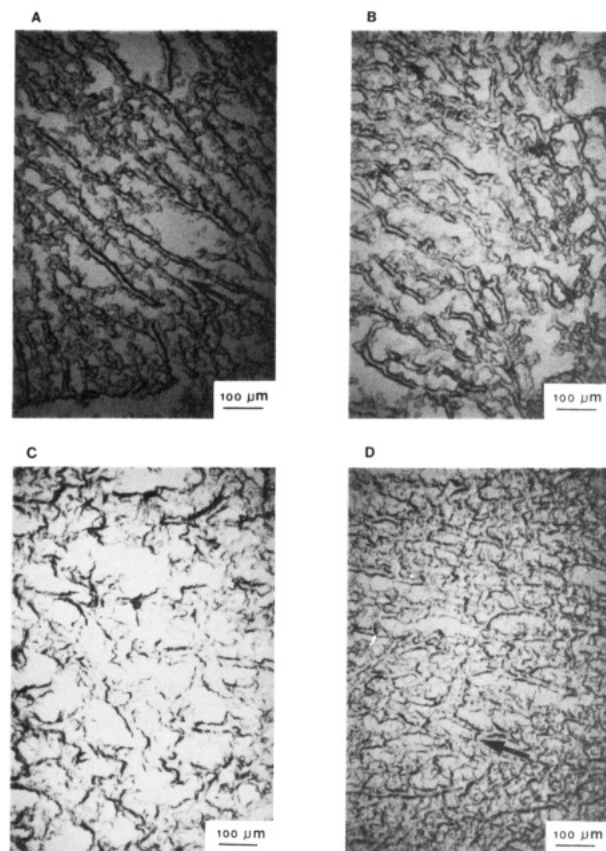
were significantly different. In fact, at these high ME concentrations, the values for changes in the G moduli were positive, indicative of structural breakdown. While ME did not alter the conformation of the native ovalbumin according to the thermal properties, it appeared to have influenced the participation of disulfide bonds in heat-induced networks prepared from ovalbumin. In the presence of sufficiently high ME concentrations, both gel strength and the degree of cross-linking in the gel (and hence the tan δ values) were impaired. Furthermore, this influence was associated with changes during the final stages of cooling rather than the initial network formation.

The influence of high levels of ME (50 mM) on gel structure during the final stage of cooling was also seen in the changes in tan δ as a function of cooling temperature (Figure 2), where tan δ increased significantly at temperatures below 40 °C. This indicated a loss in network integrity at this late stage in the cooling process.

As was the case with CysHCl, the addition of ME had no impact on the rheological properties of heat-induced vicilin networks (Table IV). Changes in microstructure were also minimal (not shown) with a slight thickening of protein strands in 25 mM ME. As expected from the similarities in the final network characteristics, no significant differences were detected in structure development during either phase of the cooling regime. Overall, these data did not support the participation of disulfide bonds as a factor in determining the rheological properties of heat-induced vicilin networks.

Effect of N-Ethylmaleimide (NEM) on Network Properties. The effects of NEM were similar to those for ME. For ovalbumin, the G moduli in the presence of
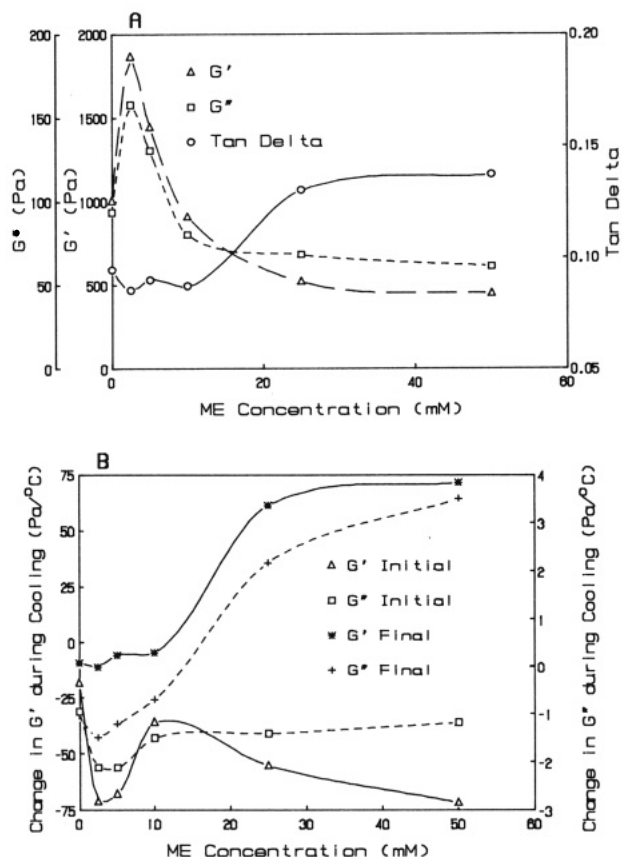
Figure 4. Effect of mercaptoethanol (ME) on (A) the rheological properties and (B) structure development (change in the G moduli) during the initial and final cooling phases for 10% ovalbumin in 0.15 M NaCl, pH 8.5.

low ME concentrations were higher than for the control, and both G moduli and tan δ values were fairly constant up to a concentration of 25 mM (Figure 6A). In 50 mM NEM, however, the network appeared to fall apart as evidenced by the extremely low G moduli (<20 Pa) and high tan δ value (0.473). The microstructure (not shown) of these networks was similar to that seen for ME. Reasonably well cross-linked networks were produced at low NEM concentrations, but with 50 mM NEM, only short protein strands with minimal cross-linking were observed. This was in agreement with the rheological data, where the high tan δ values were indicative of a lack of cross-linking within the network. As with ME, the dramatic change in network characteristics resulted from changes in structural development during the final cooling phase (Figure 6B). Furthermore, values for the changes in G' and G'' in the 50 mM NEM environment were positive, indicative of structural breakdown, as was the situation at high ME concentrations. Evidence of structural breakdown was also seen in the curve monitoring tan δ as a function of cooling temperature in 50 mM NEM (Figure 2), where there was a dramatic increase in the tan δ values during the latter stages of cooling.

As was the case with CysHCl and ME, vicilin networks were unaffected by the presence of NEM (Table V). Microstructure (not shown) was also similar up to a concentration of 50 mM NEM, where there were some aberrations in network structure.

DISCUSSION

The networks formed with the faba bean storage protein vicilin clearly demonstrate that disulfide bonds are not required for the production of heat-induced networks. This
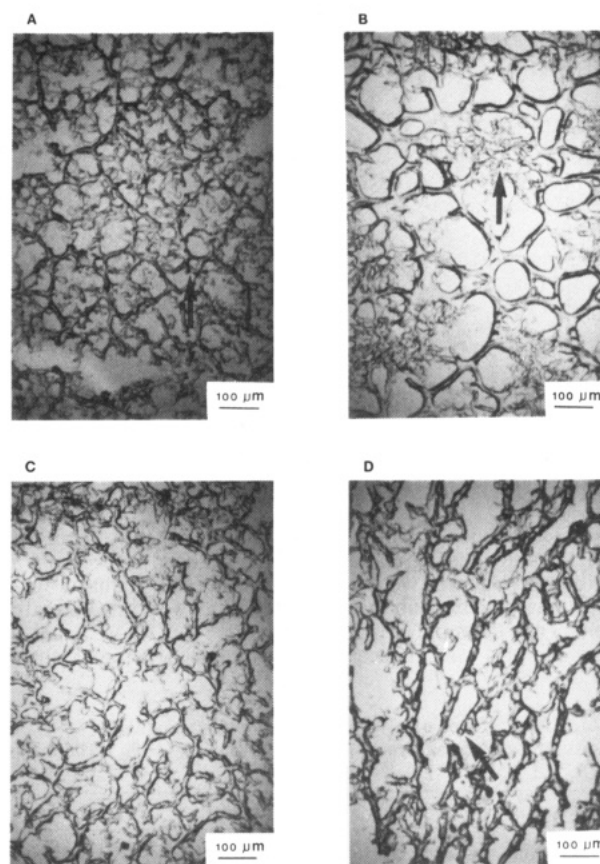

Figure 5. Photomicrographs showing the effect of mercaptoethanol on heat-induced networks for 10% ovalbumin in 0.15 M NaCl, pH 8.5. Mercaptoethanol concentrations were (A) 2.5 mM, where the cross-linking is indicated by the arrow, (B) 5 mM, where the arrow is pointing to an amorphous area, (C) 25 mM, and (D) 50 mM, where an example of the broken protein strands and diminished cross-linking is indicated by the arrow.

Table IV. Effect of Mercaptoethanol on the Rheological Properties of Heat-Set Vicilin (10% in 0.15 M NaCl, pH 8.5)[a]

concn, mM	G', Pa	G'', Pa	tan δ
0.0	1221 ± 91[a]	161 ± 5[a]	0.132 ± 0.006[b]
2.5	934 ± 21[a]	155 ± 7[a]	0.166 ± 0.004[ab]
5.0	903 ± 144[a]	158 ± 20[a]	0.176 ± 0.006[a]
10.0	956 ± 44[a]	163 ± 8[a]	0.170 ± 0.000[a]
25.0	1013 ± 162[a]	164 ± 23[a]	0.162 ± 0.004[ab]
50.0	1151 ± 80[a]	178 ± 8[a]	0.155 ± 0.003[b]

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

was evident with vicilin, where the addition of the three reagents had practically no effect on network characteristics. The lack of response to these reagents may be related to the level of sulfur-containing amino acids in the proteins. Ovalbumin, for example, has been shown to contain 4 mol of sulfhydryl (SH) groups and 1 mol of disulfide (SS) groups per mole of protein (Beveridge et al., 1974). Considering the molecular weight of ovalbumin is 65 000, this means approximately 2–3% of the amino acids are half-cystine residues. With vicilin, the level of sulfur-containing amino acids is not as clear. Simard and Boulet (1978) reported 0.4 mol of SH groups and 3.3 mol of SS groups per mole of protein (or approximately seven half-cystine residues), while Ismond (1984) found only three half-cystine residues per mole of protein when working with the same variety of faba bean as was used in this study. With a molecular weight of 150 000, this would correspond to 0.2% or 0.6% of the amino acid residues, depending on whose figures were used. In addition to the
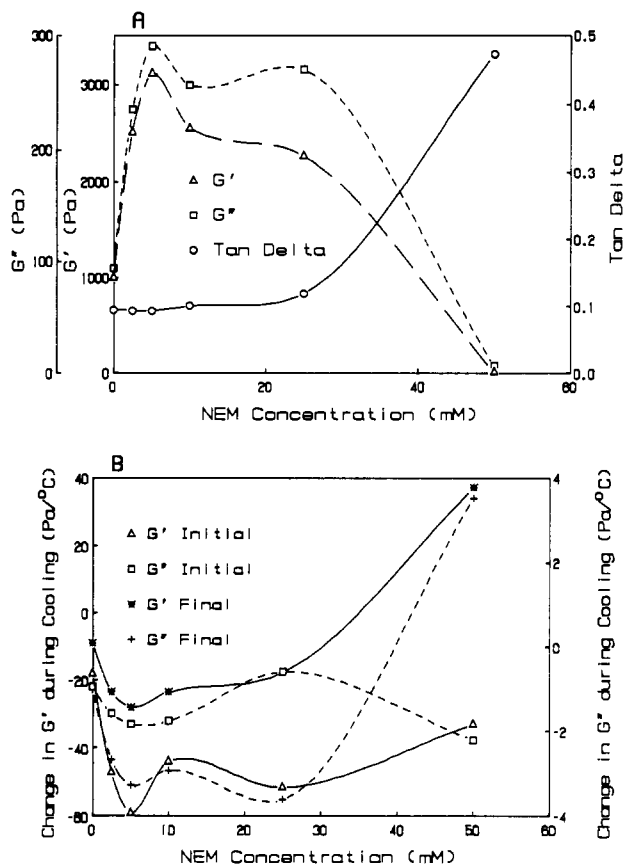
Figure 6. Effect of N-ethylmaleimide (NEM) on (A) the rheological properties and (B) structure development (change in the G moduli) during the initial and final cooling phases for 10% ovalbumin in 0.15 M NaCl, pH 8.5.

Table V. Effect of N-Ethylmaleimide on the Rheological Properties of Heat-Set Vicillin (10% in 0.15 M NaCl, pH 8.5)[a]

concn, mM	G', Pa	G'', Pa	tan δ
0.0	1221 ± 91[a]	161 ± 5[a]	0.132 ± 0.006[a]
2.5	1247 ± 28[a]	174 ± 0[a]	0.140 ± 0.008[a]
5.0	1223 ± 126[a]	178 ± 23[a]	0.145 ± 0.004[a]
10.0	1109 ± 13[ab]	153 ± 2[ab]	0.138 ± 0.003[a]
25.0	806 ± 65[b]	111 ± 4[b]	0.138 ± 0.006[a]
50.0	1057 ± 146[ab]	150 ± 12[ab]	0.143 ± 0.008[a]

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

low relative concentration of half-cystine in vicilin, the work of Simard and Boulet (1978) indicated that the half-cystine residues existed in the disulfide form rather than the sulfhydryl form, a reverse of the situation seen with ovalbumin. Clearly, this limits the potential for disulfide bond formation as a necessary component in vicilin networks. A similar lack of disulfide bond involvement in network formation has been observed for other plant proteins including sesame (Lakshmi and Nandi, 1979), oat (Ma et al., 1988), and, in some instances, soybean (Bikbov et al., 1986; van Kleef, 1986). This lack of disulfide bond involvement is not characteristic of all plant protein networks; gels from rapeseed (Gill and Tung, 1978) and soy protein (Furukawa and Ohta, 1982; Utsumi and Kinsella, 1985) have been shown to contain disulfide bonds.

It should be noted, however, that the properties of the networks obtained with vicilin were not comparable to those from ovalbumin, where disulfide bonds were a factor. This was best demonstrated by the higher tan δ value of 0.132 for vicilin in 0.15 M NaCl (Table III) compared to 0.094 for ovalbumin in a similar environment. Differences

were also noted when the microstructures were compared, where strands in vicilin networks were not as well-defined and cross-linked (Figure 3A) as those in the ovalbumin network (Figure 5A). The lack of disulfide bond involvement may contribute to this difference. This possibility was supported by the similarities in microstructure for most vicilin networks (e.g., Figure 3B) and those obtained for ovalbumin at ME concentrations which appeared to interfere with disulfide bond formation (Figure 5D).

For ovalbumin, both ME and NEM at concentrations of 25 and 50 mM, respectively, lowered G moduli and raised tan δ values in the final network. This network deterioration was attributed to changes during the latter stages of cooling rather than during network formation. Although the concentrations of CysHCl examined did not alter final network characteristics, changes in the rheological properties during cooling were indicative of disulfide bond involvement. It is possible that binding of Cl⁻ to protein during thermal unfolding impacted the charge on the protein, and hence the contribution of electrostatic interactions to network formation was altered. In this situation, the decline in structure development that was noted during the latter stages of cooling did not result in network deterioration as was the case with the inclusion of ME and NEM.

The presence of disulfide bonds in ovalbumin networks has been noted previously (Beveridge et al., 1980; Hatta et al., 1986; van Kleef, 1986); however, the exact role of these bonds during network formation is unclear. By examining different concentrations of disulfide modifying agents and following changes in rheological characteristics during the formation of the networks, we obtained information that could help clarify this issue.

For all three reagents used in this study, their inclusion at low concentrations resulted in increased G' values or stronger networks. For CysHCl, this occurred at 25 mM, for ME at 2.5 mM, and for NEM at 5 mM. This ability of disulfide modifying agents to maximize gel hardness has been reported previously for WPC in cysteine (Schmidt, 1981) and lysozyme in DTT (Hayakawa and Nakamura, 1986). What is noteworthy in this investigation is that this increased network strength is primarily associated with changes during the initial cooling stage when the network is being formed. This was particularly evident with the ME, where the change in G' during the initial stage of cooling was much greater (-8.4 in 0 M ME compared to -71.2 in 2.5 mM ME) than during the final stage (-3.0 in 0 M ME compared to -10.2 in 2.5 M ME). To understand the impact of these lower concentrations of the disulfide reducing agents CysHCl and ME in network formation, it must be kept in mind that both intramolecular and intermolecular disulfide bonds may be involved. Although there was no evidence of conformational change in ovalbumin due to manipulation of intramolecular disulfide bonds when low levels of disulfide modifying agents were used, the unfolding of the molecule during the heat treatment was sufficient to expose the disulfide bonds and free sulfhydryl groups to these reducing agents. The disruption of intramolecular disulfide bonds during the heat treatment could therefore promote a rearrangement in the molecular structure involved in network formation such that intermolecular protein interactions were encouraged. These interactions could include both covalent (disulfide bonds) and noncovalent associations. Thus, the impact of disulfide bond disruption on the conformation of the associating protein structure, rather than a specific role for disulfide within the network, would explain the improved network strength at the low con-

centration of disulfide modifying agents CysHCl and ME. The NEM, on the other hand, simply reacts with thiol groups, preventing disulfide bond formation rather than reducing disulfide bonds. As ovalbumin contains 4 mol of SH/mol of protein (Beveridge et al., 1974), the exposure of these groups during thermal unfolding may have induced binding which would not have occurred with the native molecule (did not impact thermal properties determined by using DSC). As was the case with ME and CysHCl, binding to free sulfhydryl groups under these circumstances may have been sufficient to alter the conformation of associating structures and thus promote network formation.

At higher concentrations, however, the impact of all three reagents did not seem to be associated with the initial network formation but resulted in changes in network properties during the latter stages of cooling, when the network was being stabilized and strengthened. The impact of disulfide modifying agents, however, did not just affect network strength when ME and NEM were included. Changes in the type of network were evident in the higher tan δ values, indicative of decreased elasticity, as well as diminished cross-linking between strands in the microstructure. The type of network formed, therefore, was not determined entirely during the initial cooling phase. The cross-linking during cooling also impacted network type. This was not the case with CysHCl, where binding of Cl⁻ may have altered the charge profile of the associating structure such that the type of network was dependent on electrostatic interactions.

The fact that a high level of elasticity (low tan δ values) was observed at the onset of cooling yet deteriorated during cooling for both ME and NEM suggested some role for SH groups even during the initial network formation. The existence of a sulfhydryl group/disulfide bond interchange reaction has been demonstrated previously in the heat-induced gelation of whey protein (Shimada and Cheftel, 1989). The presence of a sulfhydryl/disulfide interchange reaction could contribute to the high elasticity observed at the onset of cooling for ovalbumin alone as well as when CysHCl and ME were included. The free SH groups naturally present in ovalbumin, as well as these two reagents, have the capacity to reduce SS bonds and could therefore promote this type of interchange. As cooling proceeded and temperatures became lower, the ability to form new SS links following reduction in the presence of high concentrations of ME (25 and 50 mM) may have been limited, thus accounting for the observed deterioration in network structure.

The inclusion of NEM was to block free SH groups and limit the formation of SS bonds, but at a concentration of 50 mM, it gave a similar response to that seen with ME (high initial elasticity and network deterioration upon cooling) at 25 and 50 mM. The mode of action of NEM precludes the possibility of promoting a sulfhydryl/disulfide interchange type reaction. Although the intent in including NEM was to simply block disulfide bond formation, it should be kept in mind that NEM is not specific for thiol groups, and at alkaline pH values (e.g., pH 8 and above), it has also been shown to react with α-amino groups and imidazole groups (Friedman, 1973). NEM binding of this nature could impact the charge on the protein structure, thus altering the type of network through a mechanism independent of any disulfide bond involvement. In this respect, NEM is not an ideal reagent for investigating disulfide bond involvement. Nevertheless, the fact that the nature of the changes during cooling in the presence of 50 mM NEM (structural breakdown

and loss of network integrity) was identical with those observed with ME at 25 and 50 mM does support the importance of disulfide bonds in a role of cross-linking and strengthening, during cooling of heat-induced ovalbumin networks.

Overall, it would appear that disulfide bonds are not necessary for network formation but when present they play a stabilizing or strengthening role in protein network formation. Initial establishment of heat-induced protein networks could be attributed to noncovalent protein-protein interactions.

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