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MICROSTRUCTURAL AND RHEOLOGICAL PROPERTIES OF PROTEIN NETWORKS FROM OVALBUMIN AND VICILIN

BY

SUSAN D. ARNTFIELD

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the degree of

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Food and Nutritional Sciences
University of Manitoba
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ABSTRACT

The protein interactions responsible for heat induced network formation were examined for ovalbumin and vicilin, a storage protein form *Vicia faba*. Dynamic rheological testing was used to follow network formation as well as to characterize the resulting network. Evaluation of the G moduli and tan δ values was necessary for characterization of both the type and strength of the network formed. Network microstructure, assessed using light microscopy, was related to the tan δ values determined rheologically. In some instances, increased G moduli were indicative of increased network intensity and therefore representative of network strength. The interactions responsible for network formation were evaluated by varying the protein environment prior to heating. Differential scanning calorimetry was used to evaluate which environments resulted in significant changes in protein conformation. There was a critical protein concentration required for network formation; in alkaline pH medium, this value was lower for ovalbumin (between 5 and 7.5 %) than vicilin (between 7.5 and 10%). Beyond this concentration, good network formation required a balance of attractive and repulsive forces. If attractive forces dominated, aggregation rather than good network formation resulted, while an excess of repulsive forces resulted in network solubilization. Hydrogen bonds and, to a lesser extent, hydrophobic interactions represented attractive forces in this balance and thus contributed to the overall strength of the network. Electrostatic charge supplied the repulsive

force for the balance and was critical in determining network type. For vicilin in all conditions and ovalbumin in aggregating conditions, electrostatic attractions also contributed to network strength. This response with vicilin implicated charge distribution rather than net charge as the controlling factor in network formation. Disulfide bond formation was not required for network formation as was demonstrated with vicilin. The better quality networks observed with ovalbumin, however, may be attributed to the significant impact of disulfide bonds on both the type and strength of the networks formed.

Conditions which resulted in significant changes in protein conformation also affected network structure. The conformation of the corpuscular or globular structures that associate to form networks has a significant impact on the availability of interactive residues and thus the type and strength of the networks that result.

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INTRODUCTION

The functionality of proteins in food systems is determined by the ability of these proteins to interact with components in that system. In many cases, this involves protein - protein interactions and the formation of a matrix which can entrap other food components. Heat induced protein networks represent one example of this type of matrix. The ability of a protein to form a well crosslinked network can be an important functional attribute. To effectively use networks of this type it is necessary to understand what interactions are involved in network formation and what role they have in developing that network. This type of information is particularly important if alternate protein sources are to be incorporated into existing food products.

There are many factors which can affect the protein - protein interactions responsible for network formation. These include such parameters as protein concentration, pH, the presences of salts, and heating conditions. Not only do these parameters determine the type of network associated with a given food product, but they can also provide information as to the type of interactions involved under these conditions. Although some information is available in the literature on heat induced networks for the more established network-forming proteins such as egg albumen (or ovalbumin) and soy protein, data for less well known plant proteins are limited. Furthermore, there are contradictions in the literature over the contribution of various interactions to those networks which have been studied. This is particularly true when

examining the role of disulfide bonds. One of the problems with research in this area has been the wide range of techniques used to evaluate network formation. The empirical nature of these techniques, particularly those used to determine rheological data, often makes it very difficult to compare data from different studies.

Present trends for product characterization favor more fundamental rheological tests, particularly when investigating the molecular basis for network development. In the current investigation, the interactions responsible for network formation with ovalbumin and vicilin have been examined. Purified proteins were used to improve control of surface properties with environmental manipulation. Ovalbumin was included as it represented an animal protein well known for its gel forming capacity. Vicilin is a storage protein from the legume Vicia faba, and was chosen as a novel plant protein with potential for incorporation into food systems. Dynamic rheology, a fundamental rheological technique, was used to follow network development as well as to characterize the final product. Rheological product characteristics were also compared to network microstructure determined using light microscopy.

By manipulation of the environment prior to heat setting the network, the involvement of electrostatic interactions, hydrophobic interactions, hydrogen bonds and disulfide bonds were investigated. Interactions common to both proteins were noted as were those interactions whose role seemed to differ depending on the protein source. These differences were used to explain the variations in network quality for the two proteins.

LITERATURE REVIEW

Role of Protein Networks in Food Systems

Functional properties of proteins include any physicochemical property affecting the processing and behavior of the protein in food systems, based on the quality of the final product (Kinsella, 1976). This definition encompasses a large number of properties ranging from solubility, to flavor binding, to network formation. In all cases, however, the manifestation of these properties is governed by the association of proteins with themselves and other food components. Network formation primarily involves protein-protein and protein-solvent interactions although the ability of protein networks to physically entrap other food components is an additional attribute.

Networks resulting from the interaction of food proteins have a number of forms depending on the balance between protein-protein and protein-solvent interactions (Hermansson, 1978, 1979; Schmidt, 1981). In order to clarify terminology with respect to these network types, the following definitions have been adopted (Hermansson, 1979):

Aggregation - Collective term for protein-protein interactions

Flocculation - Colloidal interaction between proteins

Coagulation - Random aggregation

Gelation - Continuous network with a certain degree of order.

Of these terms, the networks containing a degree of order (the products of gelation) are most often exploited in food products and processing. The properties of food protein gels are similar to other gelatinous material (e.g. polysaccharides) and can be described in terms of the

classical definition (Bezrukov, 1979). According to Flory (1974), the classical characteristics of a gelatinous state require that:

1. the system contain no less than two components
2. the system should exhibit certain mechanical properties of a solid
3. both the dispersed component and the dispersion medium should be continuously distributed throughout the system.

Overall, the gelatinous state may be considered as an intermediate state between dissolved and aggregated protein (Bezrukov, 1979). However, from both the definition and properties of a gelatinous state it is clear that a wide range of visibly different products can be included. In fact, Hegg et al. (1979) have described several networks in terms of dry matter (DM) content; these products include transparent gels (<5% DM), opaque gels (5-9% DM) and gel-like precipitates (9-13% DM). The specific gel type is instrumental in establishing the functional attributes that a protein imparts to a given food system.

Proteins capable of forming networks can be classified as either fibrous (e.g. collagen) or globular (e.g. ovalbumin). The conditions required to produce these networks vary with the protein and application. For the purpose of this review, however, the focus will be on those networks resulting from the heat denaturation of globular proteins. Reviews on the gelation of both fibrous and globular proteins can be found elsewhere (Ferry, 1948; Mitchell, 1976, 1980; Bezrukov, 1979; Clark and Lee-Tuffnell, 1986).

Mechanism for the Gelation of Globular Proteins

Due to the practical importance of protein networks, it is not

surprising that research has been focused in this area for a number of years. In a review of protein gelation in 1948, a two stage process for protein network formation was proposed (Ferry, 1948). The first stage involved at least partial protein unfolding into long polypeptide chains with association of the long chains into a network occurring during the second stage. It was also noted at this time, that denatured proteins form gels only under highly specific conditions in which there is a balance of attractive and repulsive forces between polypeptide chains.

A relationship between the rate of denaturation, the rate of association and the type of structure developed was also noted (Ferry, 1948). If the attractive forces between chains are low, then denaturation should proceed quickly relative to chain association, resulting in an accumulation of free denatured protein as an intermediate. Under these conditions, a fine gel network can result. An increase in attractive forces leads to gelation prior to a sufficient accumulation of free chains resulting in a coarse opaque gel. As attractive forces are further increased only a precipitate is formed.

There is a certain degree of validity in this approach even today. The relationship between denaturation and aggregation temperature and the quality of the aggregates have been investigated for the egg proteins ovalbumin and conalbumin (Hegg _et al._, 1978, 1979). By comparing protein unfolding determined with differential scanning calorimetry (DSC) to protein aggregation determined by a decrease in solubility, it was shown that when the aggregation temperature, T_A (10% decreased in protein solubility), preceded the onset denaturation

temperature, T_D, by 6-7°C, only a precipitate resulted. As the difference in these temperatures decreased the quality of the gel improved so that for transparent gels, the T_A was equal to or greater than the T_D value. Although this does not support the requirement for denaturation prior to network formation, it does demonstrate that a retardation in the aggregation process relative to denaturation results in improved gel structure.

Using a similar approach to study the formation of protein networks from albumen and whey protein concentrate (WPC), DSC data were compared to the development of rheologically significant structures assessed using small amplitude oscillatory testing (Beveridge et al., 1985). For the mixed protein system, the onset of structure development coincided with the temperature associated with maximum heat flow on the DSC curve (Td) for the most prominent protein in the mixture; this represented endotherms corresponding to ovalbumin and β-lactoglobulin for albumen and WPC, respectively. In this situation, the development of rheologically significant structure did not begin until most of the protein or a specific protein species had been denatured.

In a similar investigation using isolated proteins, ovalbumin and vicilin (from Vicia faba), it was shown that this relationship between Td and structure development varied with the pH of the system (Arntfield et al., 1989a). With both proteins, at pH values close to the isoelectric point (i.e. pH 5), the initial development of structure coincided with or slightly preceded the Td value, resulting in poor network formation as assessed by the rheological properties. At higher pH values, where attractive forces were reduced by the increase in net

negative charge, protein unfolding was complete prior to the initial structure development; the rheological properties of the resulting network were also improved. This is in good agreement with the mechanism proposed by Ferry (1948) for optimum gel formation. The need for a balance of attractive and repulsive forces was also apparent from the work on vicilin and ovalbumin (Arntfield et al., 1989a). Conditions which delayed structure development in relation to the Td value did not necessarily result in good network formation as was seen when various anions were incorporated into the system.

There are areas, however, where the formation of protein networks as a result of crosslinking of denatured protein chains has been questioned. There is increasing evidence to suggest that aggregation and network formation is due to the association of partially unfolded protein molecules; this approach is referred to as a corpuscular picture of globular protein gelation (Clark and Lee-Tuffnell, 1986). From a theoretical viewpoint, it has been shown that the random aggregation of spherical particles into strands can lead to gel formation (Tombs, 1974). As the attractive and repulsive forces on the protein limit the extent of aggregation, the gelation phenomenon can be viewed as a kind of limited aggregation process. Larger random aggregates and increased randomness of aggregation both result in shorter strands and coagulation rather than gelation. In addition, the calculated protein requirement for this type of aggregation is 10-12 times greater than that produced by chain interactions, thus accounting for the high concentrations of globular proteins required for gelation (7-10%) compared to some of the gelling polysaccharides (1% or less).

In using this theoretical approach, it was assumed that the properties at any point on the surface of the molecule were the same. Proteins, however, have distinct surface properties with areas differing in charge density and degree of hydrophobicity. This distinct surface, however, should only improve network formation through directed orientation during aggregation (Bezrukov, 1979).

Since the introduction of this concept, there have been a number of articles to support its validity. The formation of soluble aggregates from albumen has been reported under conditions which do not produce coagulation or gelation (Ma and Holme, 1982). Similarly, with glycinin, soluble aggregates have been observed prior to network formation (Nakamura et al., 1984, 1985; Mori et al., 1986). Using transmission electron microscopy (TEM), the soluble aggregates were found to be in the form of strands, having a thickness similar to the diameter of the native protein molecule. The gelled network produced with further heating seemed to be formed from these strands. As a result, it has been suggested that an extra step should be included in Ferry's (1948) scheme for network formation to account for this soluble aggregate (Ma and Holme, 1982; Nakamura et al., 1984). The revised scheme is as follows:

$$\text{Native} \rightarrow \text{Denatured} \rightarrow \text{Soluble Aggregate} \rightarrow \text{Gel or Coagulum}$$

The existence of beaded or stranded gel structures has also been noted using scanning electron microscopy (SEM) for gels from whey protein concentrate (Beveridge et al., 1983), glycinin (Hermansson, 1985) and egg white (Woodward and Cotterill, 1986).

The structure of the molecules within the aggregated portion of

the strands has also been investigated. Denatured ovalbumin molecules in dilute solutions have been shown to be only slightly larger than the native molecule and retain a significant portion of secondary structure using optical rotation dispersion (ORD) and circular dichroism (CD) measurements (Egelandsdal, 1986). In infra-red spectroscopy studies with bovine serum albumin (BSA), it was found that, although secondary structure content remained constant, the β-sheet content in the gels was high despite a low level of β-sheet and high level of α-helix in the native molecule (Clark and Lee-Tuffnell, 1986). It was suggested that β-sheet development during gelation is an intramolecular process and that altered surface structure resulting from this formation enhanced aggregation through other bonding mechanisms. Evidence of partial refolding during the thermal gelation process, as judged by DSC and CD measurements, has also been reported for the 11S soy protein (glycinin) (Damodaran, 1988). The extent of refolding has been related to the ultimate properties of the gels. As was the case with BSA, this refolding may, in part, account for the high levels of secondary structure in the protein following gelation.

Examination of soy glycinin using TEM showed the formation of hollow strands consisting of subunits associated in a circular arrangement (Hermansson, 1985). Dissociation of the quaternary structure and reassociation of subunits into strands was thought to be the mechanism responsible for the formation of these gels. With soy conglycinin gel, thicker and somewhat less regular strands resulted, suggesting a more complex mode of aggregation than the "string of beads" seen for glycinin. An association of protein molecules into a

double spiral arrangement has been proposed (Hermansson, 1985).

Overall, the idea that the gelation of globular proteins occurs through the formation of strands of aggregates is as acceptable today as when first proposed. Ferry's (1948) model involving the association of polypeptide chains should not be discounted entirely and may be a factor in systems involving extreme denaturing conditions (e.g. chemical denaturation) similar to those used to generate the original model (Clark and Lee-Tuffnell, 1986). In contrast, the gelation of fibrous protein more closely resembles the model proposed by Ferry (1948). For example, myosin gelation involves aggregation of the globular head segment of the myosin molecule but network formation results primarily from interaction of the unfolded tail segment (Samejima et al., 1981). Information on aggregation of fibrous food proteins, primarily form muscle tissue, can be found in a number of papers and review articles (Ziegler and Acton, 1984; Burgarella et al., 1985a, b; Egelandsdal et al., 1985; Wicker et al., 1986; Foegeding et al., 1986a, b, 1987; Kim et al., 1986; Morita et al., 1987).

Methods for the Characterization of Protein Networks

An important aspect of evaluating the influence of environmental manipulations on network formation is the technique used to characterize the properties of the network. There are a number of different properties which can be examined and the choice will depend on the type of information desired. Techniques suitable for this purpose will be considered from three different perspectives reflecting the type of information that can be obtained.

First, basic physicochemical properties have been examined. Visual

appearance and solubility have been used to test the existence of protein interactions, while gel solubility and gel melting temperatures have been used to characterize the resulting product. Secondly, the textural or rheological properties are of prime importance considering the criterion of solid-like behavior as a characteristic of a gelatinous state. Assessment of rheological properties, however, has been approached from two distinct angles. Empirical methods, intended to duplicate specific processes or consumer response (i.e. mastication), have seen widespread use. The value of these tests is that they correlate to end product use and are thus indices of product quality. A second approach involves the use of fundamental rheological techniques. Although these techniques have little in common with consumer evaluation, they are the methods of choice in understanding the mechanisms responsible for material behavior. The third approach has been to actually examine the structure developed using light scattering or electron microscopy.

For all techniques, both principles and examples will be discussed in order to give some insight in the current status of protein network research and the problems inherent in data interpretation associated with the techniques which have been used to date.

PHYSICOCHEMICAL PROPERTIES

Due to the visual changes that occur when proteins aggregate, a subjective evaluation of the physical appearance has been used to assess the temperature at which aggregation takes place (Hegg and Löfqvist, 1974) as well as the quality of the final product (Hegg et al., 1978, 1979; Voutsinas et al., 1983; Hasegawa et al., 1985,

Morris et al., 1988). The criteria for subjective quality evaluation vary considerably. In some cases, the consistency and firmness of the product are emphasized (Voutsinas et al., 1983; Hasegawa et al., 1985), while in other cases, clarity (opaque versus transparent) is the main concern (Hegg et al., 1978, 1979; Morris et al., 1988). Despite the subjective nature of this technique, these properties, particularly gel clarity can be of great significance in the ultimate product use. Transparent gels are essential for a number of food products.

The next logical approach has been to add a degree of objectivity to the assessment of appearance. In order to follow aggregation as a function of temperature, the visual assessment has been replaced with a turbidity measurement. Changes in optical density at 370 nm (Samejima et al., 1969; Morita et al., 1987), 600 nm (Egelandsdal et al., 1985), 660 nm (Foegeding et al., 1986a, b) and 700 nm (Hermansson, 1982a) have all been used to monitor protein aggregation. A further refinement of the approach has been the estimation of aggregate molecular weight using low-angle laser light scattering techniques (Kato et al., 1983; Kato and Takagi, 1987). This technique, however, gives no indication as to the nature of the interactions involved. Turbidity measurements have also been used to evaluate the degree of transparency in the resulting network. Using arachin (peanut storage protein) and a wavelength of 400 nm, Kella and Poola (1985) equated the turbidity value with the extent of protein-protein interaction. In work with ovalbumin (Kitabatake and Doi, 1985; Hatta et al., 1986; Doi et al., 1987) and soy globulins (Nakamura et al., 1986), the turbidity measurements did not correlate well with gel hardness but merely represented an

additional gel characteristic important to the consumer - transparency.

An alternative to measuring changes in turbidity is to separate the aggregated protein by centrifugation and measure the soluble protein remaining in the supernatant either spectrophotometrically or colorimetrically. This type of evaluation has been applied to study both coagulation at protein concentrations below the critical value for network formation (Nakamura et all, 1978; Varunsatian et al., 1983; Hayakawa and Nakai, 1985; Ma and Harwalkar, 1987) and gelation at higher protein concentrations (Lakshmi and Nandi, 1979; Watanabe et al., 1986). Although this technique gives an indication of the amount of protein involved in aggregation, it does not reflect the nature or strength of the interactions.

An extension of this concept has been to examine the contents of the soluble fraction to ascertain the relative participation of the various fractions (proteins) in a preparation of soy globulins (Bikbov et al., 1985a, b). The components in this sol-fraction have been inversely related to the elasticity developed in the gel network.

The amount of liquid associated with a given network is also considered to be an important characteristic. The water holding capacity (WHC), the parameter generally used, is calculated from the quantity of liquid released from a network following centrifugation. The centrifugation conditions, however, are highly variable ranging from 450 x g (Hashizume and Sato, 1988) to 30,000 x g (Hegg et al., 1978, 1979; Hegg, 1982) with various speeds in between (Samejima et al., 1969; Hermansson and Lucisano, 1982). The techniques using lower centrifugation speeds (≤790 x g) appear to be more reliable as

structure breakdown can occur at higher speeds (Hermansson and Lucisano, 1982). A similar, alternate test, giving a value of water retention index, can be obtained by pressing the sample between filter paper and measuring the wetted area (Ball, 1987). The values for this procedure should be comparable to the low-speed centrifugation. No correlations have been found between WHC and the structural properties of the network (Hermansson and Lucisano, 1982). Nevertheless, the WHC, or the % DM calculated from it, has been used as an indicator of the visible characteristics of the network; the lower the % DM without becoming soluble, the closer the network is to a transparent gel (Hegg et al., 1978, 1979). Furthermore, the WHC represents a parameter that can influence consumer acceptance.

To a great extent, the macroscopic properties used to characterize gels are influenced by the nature of the protein-protein associations responsible for the gel structure. Gel solubility in solvents which attack a particular type of linkage has been used in this respect. Aqueous urea and guanidine hydrochloride are expected to break down hydrogen bonds and hydrophobic interactions, while reducing agents such as mercaptoethanol (ME), cysteine hydrochloride or dithiothreitol (DTT) are required to attack covalent disulfide linkages (Clark and Lee-Tuffnell, 1986). The requirement for a reducing agent to dissolve the gel network has been used as evidence of disulfide involvement in network formation for myosin, fibrinogen and myosin-fibrinogen gels (Foegeding et al., 1987) as well as gels from milk proteins (Hashizume and Sato, 1988). The data for ovalbumin gels are somewhat less conclusive with disulfide reducers required for gel solubility in some cases (van Kleef

et al., 1978; van Kleef, 1986) but not all (Bikbov et al., 1986). A similar controversy exists for soy proteins with reports both for (Furukawa and Ohta, 1982) and against (van Kleef, 1986; Bikbov et al., 1986) the presence of disulfide linkages. As a result, the value of this technique is questionable. The major criticism of this approach is that the fragments produced on dissolution of a network are not always characterized. Often, only a small fraction of the bonds need to be broken to disrupt the network (Clark and Lee-Tuffnell, 1986); solubility in urea alone is not sufficient evidence for the absence of covalent bonds.

Melting behavior has also been used to examine cross-linking behavior. Gels formed through direct covalent bonds should not melt. The thermoreversibility of gels is, therefore, considered to be an indicator of physical, noncovalent attractions. Using the penetration of a gelled protein system by a steel ball as an index of melting, temperatures associated with the thermoreversibility of arachin (Kella and Poola, 1985), soybean (Catsimpoolas and Meyer, 1970; Babajimopoulos et al., 1983; Bikbov et al., 1986) and lysozyme (Clark and Lee-Tuffnell, 1986) have been reported. Problems inherent in gel solubility measurements are also of concern in gel melting studies. Thermoreversibility does not preclude the possibility of covalent bonds as a few broken fragments may be all that is necessary for liquefaction (Clark and Lee-Tuffnell, 1986). Once again, characterization of the melted fragments should be a factor with this technique.

Attempts have also been made to characterize protein gels based on thermal diffusivity (Kong et al., 1980) or thermal conductivity (Kong

et al., 1982). The values calculated assume a homogeneous system, do not account for water bound to the protein, and are influenced by protein species and the presence of fat. Although these parameters appear to have little value in terms of consumer acceptance or fundamental structure formation, they do provide a tool for the prediction of heat penetration for processing purposes.

RHEOLOGICAL PROPERTIES

In order to discuss the various techniques used to evaluate textural properties, it is necessary to first review some of the basic principles associated with rheological measurements. This represents only a superficial review as books on this subject are available elsewhere (Bourne, 1982; Prentice, 1984; Moskowitz, 1986). Rheology has been defined as the science concerned with the deformation and flow of matter and relates stress (applied force/area) and strain (deformation/original size) in materials. In the simplest terms, rheological behavior has two forms, corresponding to an ideal solid and an ideal liquid; in both cases, it is assumed that the samples are homogenous and isotropic. For an ideal solid, strain is directly proportional to the stress with the proportionality constant representing the characteristic elastic modulus. A viscous material, or ideal liquid, shows no elastic recovery, and flows in response to an applied stress. In this instance, a time factor must be included and the stress is proportional to the rate of strain and the proportionality constant is the viscosity. With food products, rheological behavior most often lies somewhere between these two; as a result, techniques are required which can measure both types of

behavior and thus describe the viscoelastic nature of the product. By definition, protein gels have significant solid-like behavior and in some instances this behavior has approached that of an ideal solid (e.g. ovalbumin in 6 M urea - van Kleef et al., 1978).

Techniques for assessing viscoelastic properties are in abundance; however, all essentially involve creating a stress (or strain) and monitoring the resulting strain (or stress). A force can be applied in a direction perpendicular to a surface, in which case normal stress and deformation are involved. These forces can be tensile (away from the surface) or compressive (toward the surface). Alternately, the force can be applied across a surface resulting in a shear stress.

The division of rheological tests into empirical and fundamental largely reflects the way these basic principles are addressed. Fundamental tests utilize only a small deformation applied on a single plane. This prevents sample destruction and allows determination of elastic and viscous components which should be comparable regardless of the equipment used and particular tests employed. As empirical tests are designed as index tests, large deformations often involving both normal and shear forces are encountered. This results in tests which are dependent on sample geometry and results which are of value only when compared to samples tested in a similar fashion.

Empirical Tests

A number of different tests, and a range of equipment designed specifically for these tests exist (Patel and Fry, 1982). For the purposes of this review, however, the focus will be on the basic principles of tests used to investigate gel structure during the past

decade. Variations in equipment and sample geometry will be reflected in the variability of results but will not be discussed in detail.

Shear Measurements: The use of steady-shear measurements based on the rotation of a concentric cylinder is well established for the assessment of apparent viscosity in protein dispersions (Circle et al., 1964; Fleming et al., 1975; Gill and Tung, 1976; Babajimopoulos et al., 1983; Bau et al., 1985). For more solid-like samples, this same technique has been adapted through the use of T-spindles and a Helipath stand (Circle et al., 1964; Ehninger and Pratt, 1974; Fleming et al., 1975). Due to the use of a single rotation speed for these measurements, values obtained are reported as "apparent" value representing T-spindle resistance. Although this technique has not been used in recent years, its use did recognize the need for nondestructive measurement to compensate for time dependent behavior and provided a reasonable technique for comparing the effects of sample environment on gel viscosity. An alternate use of shear measurements has been to determine the breaking strength by monitoring the force required for a razor edge to penetrate a gel surface (Hayakawa and Nakamura, 1986).

Shear measurements have also been used as the basis of several types of thermal scanning rigidity monitors (TSRM) used for monitoring changes in rheological properties as a function of temperature. Both rotating cylinders (Montejano et al., 1983; Wu et al., 1985a; Vigdorth and Ball, 1988) and moving plates (Burgarella et al., 1985a) have been used to generate the shear force. The value of these systems results from operating them in a cyclic fashion involving only small deformations. In this way, the sample is not influenced by the

measurement and can thus be evaluated in a scanning mode over the desired temperature range. The equipment is such that a single force-deformation curve is recorded. An apparent rigidity modulus (G), representing the proportionality constant between stress and strain in shear, and an apparent viscosity value are obtained. In this respect, the TSRM approaches the fundamental techniques; however calculation of the rigidity modulus and viscosity assumes a Kelvin model of rheological behavior, in which the stress is shared between the viscous and elastic components (Wu et al., 1985a). Although the model may not apply for all gel structures, it is a reasonable assumption to aid in the interpretation of this type of data.

A variation on the use of shear measurements is torsion failure testing. As the name implies, this technique involves monitoring the force required to rupture the gel network in a rotational fashion and has been described in detail (Hamann, 1983). Essentially, a dumbbell-shaped sample is prepared and attached (i.e. glued) to plates on an instrument capable of rotational motion. Shear strain and stress at rupture can then be calculated based on the geometry, controlled strain rate and resulting stress. Although this is an empirical test, the gel rupture strength has been related to the degree of crosslinking in the gel as well as the molecular weight of the interacting particles (Mitchell, 1976). Furthermore, if gel rupture strength is the parameter required, torsional tests are preferable to compression as the strains required for the compression may be so large that failure does not occur (Hamann, 1983). For some egg white gels, however, rupture strengths using the two testing techniques were comparable. In

addition, for fragile gels, handling and gluing may present problems. An alternative to preparing dumbbell-shaped test samples is to use cylindrical gels or gels that have been heat set between concentric cylinders (van Kleef, 1986; Heertje and van Kleef, 1986). For the latter, the gel rupture strength is a result of simple shear rather than torsion. Nevertheless, torsion failure tests have been used to characterize gels from a number of proteins including egg white (Montejano et al., 1983, 1984, 1985; Vigdorth and Ball, 1988) and fish (Montejano et al., 1985; Wu et al., 1985b; Kim et al., 1986). In fact, for gels from both these sources, the shear strain at failure has been highly correlated to textural parameters evaluated by a trained sensory panel (Montejano et al., 1985).

Tensile Measurements: Of the techniques which involve normal forces, tensile measurements are the least encountered. This is primarily due to problems in sample handling and no obvious relation to consumer response as is the case with compression. A major concern is in loading the sample for testing. Test pieces can be cut into a dumbbell shape as was the case for torsion measurements, or a strip can be made from a sample gelled between two plates. The test piece is then either glued or clamped into the instrument (Heertje and van Kleef, 1986; van Kleef, 1986). In either case a strong network is required to survive this loading procedure. However, strong gels do not guarantee good results. Other problems, such as slippage on the clamps can occur. To overcome the handling of fragile gels such as those from whey proteins, a mold has been designed that produces a dumbbell-shaped gel for tensile measurements (Langley et al., 1986; Green et al., 1986).

Problems of sample drying have been overcome by wetting with liquid paraffin (Heertje and van Kleef, 1986; van Kleef, 1986). Information from tensile measurements include the stress - extension (strain) relationship at small deformation (used to calculated elasticity modulus) as well as the force at failure, or tensile strength.

Compressive Measurements: Due to the compressive action involved in chewing, compressive tests are popular as a measure of normal force. There is considerable variation, however, in how the test is performed and the type of information that is obtained. Like the tensile measurements, both the initial slope of the force-deformation curve (elasticity) and the stress at fracture (hardness) have been used to characterize protein gels (Egelandsdal, 1980; Hermansson, 1982a, b; Montejano et al., 1984; Green et al., 1986). The elasticity modulus with this technique can also be referred to as Young's modulus (if in the linear elastic region), but terms such as rigidity (usually applied to the stress-strain proportionality constant with shear force), deformability, stiffness and initial tangent have also been used. The linear portion of the stress-strain relationship, however, is quite narrow due to changes in the cross sectional area (with cylindrical sample) and influence of viscous component during deformation (Calzada and Peleg, 1978). This problem can be overcome to a certain extent by measuring this change, calculating Poisson's ratio (lateral strain/ axial strain) and incorporating this value into the calculation of stress and strain (Montejano et al., 1984; Niwa et al., 1987). Despite these concerns, compressive tests are widely used, often without regard for this linear region.

An alternate approach to examining behavior under compression is to compress a sample in a reciprocating motion that imitates the action of the jaw. A number of parameters have been calculated from the resulting force-time curve to give a texture profile analysis (TPA); these parameters include hardness, fracturability, cohesiveness, adhesiveness, springiness, guminess and chewiness (Bourne, 1978; Mulvihill and Kinsella, 1988). The advantage of this technique can be seen in the use of descriptors to which the consumer can readily relate. The descriptor which has best correlated with consumer response is gel hardness, the most commonly reported parameter in compression testing (Montejano et al., 1985). Unfortunately, the types of structures and interactions which are responsible for these descriptors have not been elucidated despite the fact that this approach has been used to characterize gels from a number of protein sources including whey (Burgarella et al., 1985a; Mulvihill and Kinsella, 1988), egg whites (Burgarella et al., 1985a) and fish (Lanier et al., 1982; Burgarella et al., 1985a; Montejano et al., 1985).

Penetrative Measurements: The most popular of the empirical tests for gel characterization has been the penetration type measurement. This method, which is basically any system in which the force required to penetrate a gel is measured, appears to best duplicate the combined compressing and shearing actions involved in chewing. Probes used for this test have included circular ones (Kohnhorst and Mangino, 1985), and plate-like ones, where the action is primarily of a shearing nature (Beveridge et al., 1980; Yasui et al., 1979;, Morita et al., 1987), but the most commonly used are cylindrical. The most often reported

parameter with this technique is hardness or firmness, representing the force required to penetrate to a certain depth (Hashizume et al., 1975; Shimada and Matsushita, 1980; Nakamura et al., 1985; Utsumi and Kinsella, 1985; Hatta et al., 1986; Hirose et al., 1986; Mori et al., 1986; Nakamura et al., 1986). A texture profile analysis similar to that used with compression has also been applied to penetration measurements (Kitabatake et al., 1985). Other methods of interpreting penetration data have been to report the initial slope of the force-deformation curve as hardness or gel strength (Egelandsdal, 1984; Egelandsdal et al., 1985), the force required to rupture the gel as gel strength (Hayakawa and Nakai, 1985; Hermansson, 1982b; Hermansson and Lucisano, 1982) or the area under the curve at a set penetration depth as work (Foegeding et al., 1986a, b; Burgarella et al., 1985b). Penetration measurements with minimal deformation have been used as a variation of the TSRM so as to allow multiple measurements of the same sample (Wu et al., 1985; Kim et al., 1986). Generally, data from compression and penetration studies are in agreement, though the technique which monitors structural differences most effectively will vary with the type of network and sample conditions (Hermansson, 1982b).

Closely related to the penetration measurement in that both compressive and shear forces are involved, are the methods of extrusion and back extrusion. In extrusion, the compression of the gel prior to being forced through a small orifice has been used to calculate an apparent modulus of rigidity (Gossett et al., 1983a). The back extrusion technique is very similar to penetration, except the diameter

of the probe used is only slightly smaller than the diameter of the container holding the sample. Calculated values (e.g. hardness) are similar to those found with penetration except the influence of the shearing component should be greater with the back extrusion technique. This technique has been used for plasma protein (Hickson et al., 1982), egg proteins (Hickson et al., 1982; Holt et al., 1984) and oat globulins (Ma et al., 1988).

Others: One further method should be mentioned in the discussion of empirical techniques in that it has been specifically developed to monitor the coagulation (gelation) process as a function of time. This is a non destructive method and monitors the force exerted by the gel on a probe in the sample as coagulation proceeds (Gossett et al., 1983b, 1984). Although a number of properties, including thermal expansion and surface tension, may contribute to this force, it has been used successfully to follow structure development for gelation, enzyme-coagulated milk and egg albumen at a single temperature.

The empirical tests have been of value in investigations of protein networks and protein network formation. The properties measured, however, are usually dependent on the parameters used during testing. In addition to variations in sample size and parameters being monitored, the rate of deformation is also critical. With viscoelastic materials, there is often some time dependent behavior which is seldom addressed in empirical measurements. As a result, there is also a need for more fundamental techniques to elucidate structure and molecular interactions, rather that just the anticipated consumer response.

Fundamental Tests

To determine the fundamental viscoelastic properties of protein gels, techniques more common to polymer science have been employed, including static tests for the measurement of creep compliance and stress relaxation as well as a dynamic approach using small amplitude oscillatory testing (Mitchell, 1980). The principles behind these techniques have been described (Ferry, 1980; Peleg, 1987) and will be discussed only in sufficient detail to show how they can be applied to protein gels.

Creep Compliance: In a creep test, a constant stress is applied and the strain is recorded as a function of time. Either shear (Swartzel et al., 1980) or normal stress (Kamata et al., 1988) can be used in this respect. The results are generally expressed as the creep compliance (J(t)) where:

$$J(t) = \text{strain } (t) / \text{stress}$$

Creep curves can also be described by viscoelastic models, which allow calculation of the contribution of both elastic and viscous components (Kamata et al., 1988). The difficulty with this technique is in maintaining constant stress. Although computer controlled instrumentation with this capability is currently available, the use of creep measurement to analyze food protein gels has been somewhat limited to date.

A variation on this, based on essentially the same principles, involves measuring the volume displacement (rather than deformation) produced throughout a gel when air pressure is applied (Saunders and Ward, 1954; Stainby et al., 1984). The volume change is monitored using

an index liquid in the capillary arm of a U-tube. By monitoring the displacement as a function of time, a shear modulus can be derived. Problems of gel slippage due to syneresis have been overcome by coating the inner walls of the tube in which the gel is set with glass granules (Stainby et al., 1984).

Stress Relaxation: A stress relaxation test can be considered the opposite of the creep test in that a constant deformation is imposed and the change in force recorded as a function of time. The stress relaxation value (G(t)) can then be defined as:

$$G(t) = \text{stress } (t) \ / \ \text{strain}$$

For an ideal elastic solid the stress relaxation value is inversely proportional to creep compliance; this, however, is not the case for viscoelastic gels.

One critical factor that must be observed is that the imposed strain be in the linear viscoelastic region. In other words, the change in stress with time should be independent of the strain. As a result, strains of 5% (Bikbov et al., 1985a), 10% (Sone et al., 1983) and 20% (Hermansson, 1982a) have been used. The type of strain is not a critical factor as evidenced by the use of tensile (Furukawa et al., 1979), compressive (Hermansson, 1982a) and spherical indentor penetration techniques (Schmandke et al., 1981; Bikbov et al., 1985a). Stress relaxation curves have also been used to calculate elasticity (or shear modulus) values by comparing data at a set time (Schmandke et al., 1981; Sone et al., 1983; Bikbov et al., 1985a) or by measuring the recoverable height following stress removal (Hermansson, 1982a), a somewhat empirical use of this type of data.

Dynamic Behavior: Viscoelastic properties can be characterized by dynamic experiments in which a sinusoidally oscillating strain is applied to the material (Mitchell, 1980). An imposed small amplitude sinusoidal strain (γ) can be written as a function of time (t):

$$\gamma = \gamma_o \sin(\omega t)$$

where γ_o is maximum strain amplitude and ω is oscillatory frequency in radians/sec. Similarly, the strain (or shear) rate ($\dot{\gamma}$) is the first derivative of the strain with respect to time:

$$\dot{\gamma} = \omega\gamma_o \cos(\omega t)$$

For an elastic solid the induced stress will be in phase and, therefore, directly proportional to strain and can be described by the following relation:

$$\sigma = k\gamma = k\gamma_o \sin(\omega t)$$

where k is the proportionality constant. A viscous or Newtonian fluid, on the other hand will be 90° out of phase and proportional to the strain rate as follows:

$$\sigma = \eta\dot{\gamma} = \eta\omega\gamma_o \cos(\omega t)$$

where η is the proportionality constant. For viscoelastic material, a phase shift between 0 and 90° is obtained and the strain can be written as a composite function:

$$\sigma = \gamma_o (G' \sin \omega t + G'' \cos \omega t)$$

where G' is the dynamic shear storage modulus and G" is the loss modulus. Thus G' represents the energy recoverable for each deformation cycle and G" the energy dissipated. The energy lost compared to the energy stored may then be defined as the loss angle (δ) or loss tangent where:

$$\tan \delta = G'' / G'$$

One additional parameter, the complex modulus (G^*) defined as:

$$|G^*| = G'^2 + G''^2$$

has been used as a measure of total stiffness so that the contribution of both the viscous and elastic components are included (Bohlin et al., 1984).

Like the fundamental static measurements, the magnitude of the strain should be in the linear viscoelastic region and this region should be evaluated by analyzing the sample at various strains (amplitudes of oscillation). With the increasing availability of commercial, computer controlled rheometers capable of this type of measurement, the use of dynamic measurements in the analysis of food protein gels in increasing.

Dynamic measurements have been applied for two distinctly different applications. Due to the small strain associated with these measurements, there is no sample destruction and hence, multiple measurements can be made and recorded as a function of time or temperature. This approach has been particularly beneficial in relating protein conformational changes to the development of structure, and thus provides a basis for examining the mechanism of structure development. Normally, either G' (te Nijenhuis, 1981; Beveridge et al., 1984, 1985; Goldsmith and Toledo, 1985a; van Kleef, 1986; Arntfield et al., 1989a) or G^* (Bohlin et al., 1984; Paulsson et al., 1986) values are reported, though changes in loss tangent are also of value (Goldsmith and Toledo, 1985a). Plots of the input amplitude and resulting torque amplitude have also been used to show changes in

behavior as a function of temperature (Beveridge and Timbers, 1985), though it is somewhat difficult to compare these data to those reported using more traditional parameters.

In addition, the dynamic rheological parameters (G', G", loss tangent) can be evaluated as a function of the frequency of oscillation (Gill and Tung, 1978; van Kleef _et al_., 1978; Clark and Lee-Tuffnell, 1986; van Kleef, 1986; Dejmek, 1987; Paulson and Tung, 1989). For an elastic solid, G' is independent of frequency. The dependency on frequency, therefore, can be used to characterize the viscoelastic nature of the material. For viscoelastic material, a linear relationship usually exists between log G' (or log G") and log ω, so that data comparison is facilitated. In systems showing minimal frequency dependence, single point values can be compared directly.

The advantage of using these fundamental properties is that the values obtained should compare regardless of the sample size and instrumentation used. As a result, comparison of data from different studies is possible. Comparable data of this nature, however, are limited at this time.

STRUCTURAL PROPERTIES - MICROSCOPY

Microscopic examination provides an additional tool for characterization of protein networks. In this respect, light microscopy and electron microscopy (both transmission electron microscopy (TEM) and scanning electron microscopy (SEM)) have been used.

Light Microscopy

The use of light microscopy to examine food gels has been minimal

in the last decade presumably because of the general trend toward the use of the more sophisticated electron microscopic techniques that is prevalent in the area of food microscopy. Light microscopy does, however, provide a relatively simple tool for examining long range structures. The low magnifications involved make the technique less subject to artifact production during sample preparation. Furthermore, because of the two dimensional nature of the image obtained, it has been used to estimate the coarseness of a network simply by counting network strands intersecting an imposed grid (Green et al., 1986). Despite the apparent lack of interest in this technique, its potential should not be discounted when examining protein gel networks.

Transmission Electron Microscopy

Sample preparation for TEM requires fairly drastic procedures in order to give an image. To get thin sections for TEM, samples are first fixed with glutaraldehyde and/or osmium tetroxide, stained with uranyl acetate, dehydrated with ethanol and propylene oxide and embedded in epon resin (Clark et al., 1981; Hermansson and Buchheim, 1981; Nakamura et al., 1984; Hermansson, 1985; Heertje and van Kleef, 1986; Mori et al., 1986; van Kleef, 1986). Although artifacts can originate at any point in the procedure, the fixation step has been particularly criticized in that glutaraldehyde tends to promote protein aggregation and thus distorts the gel structure (Hermansson and Buchheim, 1981).

An alternative approach involves the use of freeze etching or freeze fracture replicate techniques (Harwalkar and Kalab, 1980; Hermansson and Buchheim, 1981; Schmandke et al., 1981; Hermansson, 1985; Bikbov et al., 1985). The initial step in this procedure involves

rapidly freezing the sample; however, sample size is sufficiently small to ensure rapid freezing without ice crystal formation. Samples are then dried under vacuum and shadow-cast to give a replicate. The original sample can be discarded and the replicate viewed microscopically. In view of the complexity of the procedure, it is not surprising that some artifacts result. In fact, free protein aggregates, observed with the freeze fracture technique, have sometimes been dismissed as artifacts (Bikbov et al., 1986). With an awareness of the potential for this type of problem and careful sample handling, this powerful technique can be of value in studying protein gels (Hermansson and Buchheim, 1981).

The value of TEM for characterizing protein gels is in its ability to probe molecular interactions (Clark and Lee-Tuffnell, 1986). In fact, it is this technique which played a major role in establishing the corpuscular or "string of beads" theory for network formation (Tombs, 1974). The potential for measuring both strand width and degree of branching render this a valuable tool for investigating the mechanism of interaction in network development as well as characterization of the resulting structure (Clark et al., 1981; Hermansson, 1985; Mori et al., 1986; van Kleef, 1986; Morita et al., 1987; Mulvihill and Kinsella, 1988).

Scanning Electron Microscopy

Although SEM cannot attain the high resolution available with TEM, it is a popular technique for examining the long range structural features of protein gels due to the three dimensional image which results. As with TEM, sample preparation is critical to avoid or minimize

artifacts and thus produce meaningful results. In its simplest form, it is necessary to dry the sample and coat it with a conducting metal (usually gold) in order to get an image with SEM. The large sample size (compared to TEM) and high moisture content in the gels have presented problems in the drying step. One approach has been to freeze dry the sample after rapid freezing in liquid nitrogen. This may (Kalab and Harwalkar, 1973; Furukawa et al., 1979; Fuke et al., 1985; van Kleef, 1986) or may not (Beveridge et al., 1980) be preceded by fixation in glutaraldehyde. Alternately, samples have been prepared by fixation using glutaraldehyde and/or osmium tetroxide, dehydrated with a graded series of ethanol solutions and dried in liquid CO_2 using a critical point dryer (Gill and Tung, 1978; Yasui et al., 1979; Hermansson, 1982a; Beveridge et al., 1983, 1984; Green et al., 1986; Woodward and Cotterill, 1985, 1986; Oe et al., 1987). The benefits of critical point drying in avoiding artifacts such as those resulting from ice crystal formation in the freeze drying procedure have been demonstrated clearly in several comparative studies (Woodward and Cotterill, 1985; Heertje and van Kleef, 1986). As with TEM, the fixative used can also be an area for concern. While Heertje and van Kleef (1986) have found glutaraldehyde to effectively maintain gel structure, Woodward and Cotterill (1985) reported a 50% shrinkage in the structure when glutaraldehyde was used alone. A further fixation with osmium tetroxide and uranyl acetate was required to stabilize the gels against shrinkage. Hermansson and Buchheim (1981) preferred to use osmium tetroxide alone as they felt that glutaraldehyde induced protein aggregation.

A variation on the SEM technique is the use of a cryo-SEM system in which the gel can be viewed at low temperatures in a hydrated state, thus avoiding any problems due to the drying step (Sone et al., 1983; Green et al., 1986). However, ice crystal formation during freezing can still lead to artifacts; this can be reduced to a certain extent by the inclusion of a cryoprotectant such as 30% sucrose (Davis and Gordon, 1984).

Overall, it is clear that SEM of protein gels is subject to artifact production and therefore sample preparation is critical. Nevertheless, under proper condition, the structures associated with coagulation and gelation and the intermediates between these extremes can be visualized. The relationship between information of this type plus rheological and physical properties provide the information that will make it possible to further the understanding of protein networks.

Factors Influencing Network Formation

A critical factor in the gelation phenomenon is the balance between attractive and repulsive forces resulting from protein denaturation (Ferry, 1948). As a result there is a great deal of interest in the interactions between protein molecules responsible for network formation. Of particular relevance to the heat induced gelation of globular protein are the structurization of protein owing to covalent interaction of their functional groups and selection of physicochemical conditions which limit protein solubility (Clark and Lee-Tuffnell, 1986). As a result, both covalent (primarily disulfide bond formation) and noncovalent interactions (including ionic plus hydrogen bonding and hydrophobic interactions) are involved either

directly or indirectly in network formation. These interactions perform different roles in the gel structure. One theoretical proposal for the role of these interaction is as follows (Schmidt, 1981):

Covalent Bonds	- bridging, ordering.
Hydrogen Bonds	- bridging, stabilizing.
Hydrophobic Interactions	- strand thickening, strengthening, stabilizing.
Ionic Bonds	- solvent interactions, salt links.

Evidence to support these roles is not always easy to obtain. The involvement of one or all of these interactions is dependent on the protein in question and the solvent environment to which the protein is exposed. By examining different proteins in different solvents, the interactions responsible can be assessed and their roles in protein gelation determined. Parameters that will be considered include protein source, protein concentration, heating regime, pH, salts, denaturants and disulfide reducing agents.

PROTEIN SOURCE

Sources of food proteins capable of network formation are varied and quite extensive. The quality of the network as well as the conditions required for network formation are also highly variable and governed by the protein being used as well as other environmental factors.

Muscle proteins are known for gel forming capacity (Wicker et al., 1986). The fibrous myofibrillar proteins, associated with the contractile elements in muscle, are responsible for this behavior. Investigations of myosin, a major protein in this group, have lead to

the suggestion that the mechanism for gelation for the fibrous proteins more closely resembles the interacting polypeptide chain model proposed by Ferry (1948) than is the case for the globular proteins (Samejima et al., 1969). The influence of this different mode of network formation is reflected in the superior gelling capacity (i.e. much lower protein concentration required for good gelation).

Blood plasma proteins are used as functional ingredients in meat products because of their gelling properties (Hermansson, 1982a, b; Hermansson and Lucisano, 1982; Hickson et al., 1982). Although blood plasma contains a number of different proteins, the two which demonstrate good gelling properties in an isolated form are albumin and fibrinogen (Foegeding et al., 1986a, b, 1987; Yasuda et al., 1986).

Milk proteins can also form gels. Heat-induced gels from dried skim milk (protein concentration 14-17%) contain casein micelles linked by short thin ionic bridges; calcium is a major factor in this linking process (Kalab and Harwalkar, 1973, 1974). Similar gels can be prepared with whole milk if the protein content is greater than 15% (Bezrukov, 1979). This heat induced gel formation is not always desirable and has been implicated as a factor in the age gelation of UHT sterilized milk, a storage defect which limits shelf-life (Swartzel et al., 1980; Manji and Kakuda, 1988).

Whey proteins, milk proteins remaining after casein removal, are used in food products mainly because of their gelling properties (Schmidt, 1981; Sone et al., 1983; Green et al., 1986; Mulvihill and Kinsella, 1987). Gel quality, however, is highly dependent on the isolation procedure (Mulvihill and Kinsella, 1987). Of the proteins in

a whey protein concentrate (WPC), bovine serum albumin (BSA) has the best gelling properties in that it gives a very elastic gel and will form a network at a concentration as low as 2%. The minimum protein concentration required for the gelation of β - lactoglobulin, on the other hand, is about 5% (Hegg, 1982; Paulsson et al., 1986). Furthermore, the pH range for which a gel can be obtained is narrower than for BSA and the gel itself has a significant viscous component. The α - lactalbumin does not form heat induced gels even at concentrations up to 20% (Paulsson et al., 1986). As β - lactoglobulin is the main protein in whey (up to 50%), its behavior is of prime importance to the functionality of WPC (Mulvihill and Kinsella, 1988); in fact, gel strength has been related to the β - lactoglobulin content (Langley et al., 1986), while α - lactalbumin acts as a diluent and diminishes gel quality (DeWit et al., 1986). The gelation properties of BSA are also of interest in studying basic gelation mechanisms (Clark and Lee-Tuffnell, 1986); however, their contribution to whey protein gelation is minimal due to the fact that BSA represents a relatively low proportion of the protein.

Perhaps the most well known group of food grade proteins capable of gel formation are those in egg albumen. As a result, many investigations with these proteins have focused on practical uses for albumen as well as fundamental investigations into network development (Beveridge et al., 1980; Shimada and Matsushita, 1980; Hickson et al., 1982; Ma and Holme, 1982; Gossett et al., 1984; Holt et al., 1984; Montejano et al., 1984; Goldsmith and Toledo, 1985a, b; Woodward and Cotterill, 1986). Of the constituent proteins, ovalbumin exhibits the

best gelling properties and is probably responsible for these same properties in albumen. Under conditions in which only pH and NaCl concentration are varied, conalbumin and lysozyme produce only aggregates rather than gel-like networks as assessed by a %DM rating (Hegg et al., 1978; Hegg, 1982). Based on SEM results, however, some network development for conalbumin at pH 8 - 8.8 in phosphate buffer has been reported (Oe et al., 1987). Gels from lysozyme have also been reported in buffers containing NaCl and dithiothreitol (Hayakawa and Nakamura, 1986). In studying these lysozyme gels, any heat induced aggregation was termed a gel and conditions optimized to give maximum breaking strength; breaking strengths of 0.0 dyn/cm^2 in some buffers would suggest that gels were not always formed. The discrepancies in these studies may simply reflect the different criteria used to confirm gelation. The importance of ovalbumin to albumen gelation is unquestioned. Due to its abundance and relative ease in isolation (relatively low cost), ovalbumin is an obvious choice as a pure protein for investigation into the gelation of globular food proteins (Hegg and Löfqvist, 1974; Nakamura et al., 1978; van Kleef et al., 1978; Hegg et al., 1979; Egelandsdal, 1980, 1984, 1986; Hayakawa and Nakai, 1985; Heerjte and van Kleef, 1986; van Kleef, 1986; Doi et al., 1987; Morris et al., 1988; Arntfield et al., 1989a).

With a trend toward increased utilization of plant proteins, it is not surprising that the potential for gel formation from these proteins has also received considerable attention. In this respect, a number of plant proteins have been investigated including arachin from peanuts (Tombs, 1974; Kella and Poola, 1985), sesame globulins (Lakshmi and

Nandi, 1979; Hasegawa et al., 1985), oat globulins (Ma and Harwalkar, 1987; Ma et al., 1988), rapeseed or canola proteins (Gill and Tung, 1978; Voutsinas et al., 1983; Paulson and Tung, 1989) as well as proteins from pulse crops such as field peas and fababean (Fleming et al., 1974; Schmandke et al., 1981; Voutsinas et al., 1983). The most popular proteins for this type of investigation, however, are those from soybean. Both soy protein isolates (Circle et al., 1964; Fleming et al., 1974; Hermansson, 1978, 1979; Furukawa et al., 1979; Furukawa and Ohta, 1982; Bau et al., 1985; Beveridge et al., 1985) and the purified storage proteins, glycinin (11S) and conglycinin (7S), have been studied (Utsumi et al., 1983; Babajimopoulos et al., 1983; Nakamura et al., 1984, 1985, 1986; Bikbov et al., 1985a, 1986; Hermansson, 1985; Utsumi and Kinsella, 1985; Mori et al., 1986; Damodaran, 1988; Kamata et al., 1988).

The use of plant proteins and in particular, plant protein isolates has been highly influenced by protein isolation procedures. Traditionally, plant protein isolates are prepared by extracting in either alkaline or acidic conditions and precipitating at the isoelectric point. This can lead to severe protein denaturation as evidenced by a lack of endotherms during DSC analysis (Arntfield and Murray, 1981). This change in protein conformation prior to heating may have a significant impact on unfolding during heating and the resulting network formation. This was demonstrated in the work of Beveridge et al. (1985) in which there was significant structure development (high G' values) in a soy protein isolate prior to heating. The spherical structures observed in the heat induced products may be a

product of this aggregation (Beveridge et al., 1984). More gentle isolation procedures such as the micellization technique of Murray et al. (1978, 1981) exist, and should be considered if native proteins are desired as starting material. Protein unfolding during the heating step can then be maximized to improve gel structure. Furthermore, this emphasizes the need to be aware of protein conformation in evaluating protein structure development.

PROTEIN CONCENTRATION

Protein concentration is a critical factor in the formation of extensive networks. There is a minimum protein concentration required for gelation which ranges from 2% for BSA (Paulsson et al., 1986) up to 8-10% for whey proteins (Mulvihill and Kinsella, 1987) and 14% for casein based milk gels (Kalab and Harwalkar, 1974). This critical concentration appears to depend on the individual proteins rather than the protein source as seen by the variation in the requirements for proteins from various plant sources; concentrations of 5% have been reported for oat globulins (Ma et al., 1988) and rapeseed (Gill and Tung, 1976) and 3.75% (Nakamura et al., 1979) and 6% (Bikbov et al., 1985a) for soy proteins. Of the soy proteins, conglycinin requires 7.5% protein to gel while for glycinin the protein requirement is only 2.5% (Nakamura et al., 1979). Overall, the critical concentration for gelation of globular protein is an order of magnitude higher than for other biopolymer systems such as gelatin or polysaccharides (Clark and Lee-Tuffnell, 1986); a factor important to the corpuscular theory for gelation.

Even below this critical concentration, an increase in the solids

content resulted in increased viscosity for heated dispersions for both egg (Shimada and Matsushita, 1980; Egelandsdal, 1986; Nakamura et al., 1986)and plant proteins (Circle et al., 1964). An increase in protein concentration above the critical level increased gel viscosity (Ehninger and Pratt, 1974; Bau et al., 1985) or hardness (Beveridge et al., 1980, Mulvihill and Kinsella, 1987; Ma et al., 1988) as well as the G' (storage modulus) obtained with more fundamental studies (Beveridge et al., 1984; Bikbov et al., 1985a; van Kleef, 1986). This dependence of gel structure on concentration has been described as a linear (Shimada and Matsushita, 1980), logarithmic (Beveridge et al., 1980) or power law ($G' = AC^{4.2}$ where A is a constant and C the concentration) relationship (van Kleef, 1986). The increase in G' is associated with an increase in crosslinking within the gel. Melting behavior supports this interpretation of increased crosslinking as there is a logarithmic relationship between concentration and melting temperature of soy protein gels (Babajimopoulos et al., 1983). Gel transparency has also been related to protein concentration; however increased protein concentration has resulted in increased turbidity for egg gels (Doi et al., 1987) but decreased turbidity for soy gels (Nakamura et al., 1986). Decreased water binding capacity is also associated with increased protein concentration in blood plasma gels (Hermansson, 1982a, b; Hermansson and Lucisano, 1982).

From a more fundamental perspective, attempts have been made to get a direct comparison of gels from different sources and thus obtain a master concentration dependence. This is done by scaling G' to unity at a concentration of 15% (i.e. G'(c)/G'(15)) and plotting it as a

function of the reduced concentration of the polymer (C/Co) where Co is the critical concentration required for gelation. By using this scaling technique, the relationships between G' and concentration for gelatin, agar and BSA at pH 7 have been shown to fall on the same curve (Clark and Lee-Tuffnell, 1986). This curve fits the theoretical prediction based on the relationship:

$$p = (Kf/M)C$$

where p is the extent of conversion to a network, K is the affinity for crosslinking between functional groups, f the number of functional groups, M the molecular weight and C the concentration. The G' value or yield value (G) (Bikbov *et al.*, 1985a) obtained from rheological measurements reflects the extent of conversion and have been related to concentration through this equation. In creating a master equation, however, values for K, f and M are clearly defined. Of particular concern in this respect is the high f value and its validity when applied to compact globular proteins (Clark and Lee-Tuffnell, 1986). As an alternative, therefore, a series of master curves has been proposed in which both f and C/Co are variables. The limited amount of fundamental data available at this time means there are still questions about the value of this theoretical treatment. In view of the number of different interactions associated with network formation, how can a single K value describe the affinity for all? How does the power law relationship observed by van Kleef (1986) fit into this theory? More data of a fundamental nature are needed in this area.

TEMPERATURE EFFECTS

The heating temperature used for gel formation has a major

influence on the textural quality of the network; ideal temperatures are determined primarily by the denaturation temperature of the protein. At least some protein unfolding is necessary to initiate the gelation process, but beyond this the extent of network formation is related to the completeness of denaturation (Bikbov et al., 1985a, 1986). As a result, when heating at a constant temperature below that required for complete denaturation, there is generally an increase in network formation with increasing temperature as detected by changes in gel hardness (Circle, 1964; Furukawa et al., 1979; Beveridge et al., 1980; Hermansson, 1982a; Woodward and Cotterill, 1986; Ma and Harwalkar, 1987, 1988), elasticity (Holt et al., 1984) and even more fundamental parameters such as G' values (Beveridge et al., 1984) and microstructure (Hermansson, 1985). For this reason, optimum gelation temperatures are closely related to the denaturation temperatures, thus accounting for egg albumen having an optimum gelation temperature at approximately the Td value for ovalbumin of 80°C (Beveridge et al., 1984; Holt et al., 1984, Bikbov et al., 1986), while the optimum temperature for oat globulins is closer to its Td value of 100°C (Ma and Harwalkar, 1988)

Interestingly, at heating temperatures greater than the Td values, there tends to be a decrease in hardness values (Hashizume et al., 1975; Furukawa et al., 1979; Hermansson, 1982a; Holt et al., 1984; Bikbov et al., 1986). At very high temperatures (> 115-120°C), complete network degradation results (Furukawa et al., 1979; Bikbov et al., 1985a). The initial decreases have been related to an increase in the size of aggregates in the network (Hermansson, 1982a) while the

degradation at high temperatures was attributed to amino acid destruction (Furukawa et al., 1979).

With constant temperature heating regimes the time of heating can also be a factor. Regardless of the temperature, there is an increase in gel strength with time until a maximum is reached, after which the gel strength levels off (Goldsmith and Toledo, 1985a; Nakamura et al., 1985). There is some indication that prolonged heating (> 1-2h) may result in a slight deterioration of gel structure (Hickson et al., 1982; Bikbov et al., 1985a). The time required to reach maximum gel strength decreases with increasing temperature; therefore, caution is required when comparing gel parameters at set times. Woodward and Cotterill (1986), for example, found differences in springiness and cohesiveness due to temperature after 10 minutes of heating which were not present at 30 and 50 min.

Examination of the rheological properties of a preset gel as a function of temperature has also been used as a technique to assess the types of interactions involved. Using a complex, five-component model to describe the creep behavior of soy protein gels, Kamata et al. (1988) interpreted a decrease in the plastic component as evidence of the importance of hydrophobic interactions to this network. As can be seen, temperature effects are important, both in terms of practical applications and fundamental investigations.

pH

As stated previously, the formation of a continuous network upon protein unfolding requires a balance between attractive and repulsive forces. The pH, and thus the charge on the protein molecule is of

fundamental importance in establishing this balance. Theoretically, at a pH close to the isoelectric point (IEP) the net charge is effectively neutralized and attractive forces predominate. In this situation, the tendency is toward massive aggregation and coagulum formation. As the pH is moved from the IEP, the net charge increases and repulsive forces balance attractive forces resulting in optimum conditions for gel formation. Beyond the optimal pH, repulsive forces may be sufficiently strong to impede protein interactions, once again giving a weak gel or even soluble protein.

Evidence to support this theory can be seen in the microstructure (SEM) of gels from egg (Woodward and Cotterill, 1986; van Kleef, 1986), soy protein (van Kleef, 1986) and rapeseed protein (Gill and Tung, 1978). At pH values of 5-6, large coarse aggregates are obtained while alkaline conditions produce finer strands and more homogenous matrices.

Physical properties follow a similar trends. The WHC of blood plasma proteins (Hermansson and Lucisano, 1982) and BSA (Yasuda _et_ _al_., 1986) are minimal at the IEP and increase at elevated pH values. Similarly, the turbidity or whiteness of the gel is maximal at the IEP and decreases with increasing pH (Beveridge _et_ _al_., 1980; Shimada and Matsushita, 1980; Yasuda _et_ _al_., 1986). In other words, the amount of water associated with the protein increases as the pH is moved from the IEP, resulting in a more transparent product. This is not surprising in view of the need for charged residues to optimize protein-water interactions. Nevertheless, as Hermansson (1982a) has noted, the interactions responsible for WHC values are not necessarily the same as those responsible for the textural properties.

How these different structures impact the rheological properties is not clear. One of the problems in trying to assess pH effects in terms of rheological behavior is the preponderance of conflicting data obtained using a variety of techniques and conditions. With blood plasma proteins, for example, there is no change in the initial slope in compression measurements over the pH range 6-9, but there is a gradual increase in the breaking strength as assessed with the penetration technique (Hermansson, 1982b). In the work of Beveridge et al. (1980) and Woodward and Cotterill (1986), shearing and penetration measurements indicated the hardest gels from egg albumen occurred at pH 5-5.5, around the IEP for ovalbumin; hardness values dropped to a minimum in the pH 6-7 range and increased slightly under alkaline conditions. In addition to the charge effect in the alkaline region, the increased potential for the disulfide interchange reaction at this pH has been cited as a possible factor for the observed increased in strength (Beveridge et al., 1980). Penetration measurements were also used by Egelandsdal (1980) and Doi et al. (1987); however, quite different results for egg albumen were obtained; hardness maxima were obtained at pH values of 3.5 and 6.5-7, with low values reported at the IEP. It is conceivable that the rate and depth of penetration determine the properties which are actually measured and account for this discrepancy. Under sufficiently large deformation, the extensive aggregation at the IEP may offer considerable resistance that is not detected at lower penetration depths. Tensile measurements concur with the results of Egelandsdal (1980) and Doi et al. (1987) in that lower breaking strengths were obtained at the IEP than at higher

pH values (Heertje and van Kleef, 1986). This may be a more realistic approach for assessing pH differences as the degree of compression of aggregated material is not a complicating factor. Added to this problem is the fact that pH effects cannot be isolated from other environmental factors. Of particular importance in this respect is the impact of added salt, which, as will be seen in the next section, has the effect of shifting the IEP.

In terms of fundamental rheological studies, van Kleef (1986) has shown that ovalbumin has a maximum G' value at pH 4 (pH 5 if NaCl is present) and is constant between pH values of 5 and 9. As the G' value is felt to represent the number of interactions, this was interpreted as a measure of the increase in protein-protein interactions in the vicinity of the IEP. With the glycinin storage protein, two G' maxima, at pH 4.6 and 6.4, were observed corresponding to the IEP of the acidic and basic subunits, respectively (van Kleef, 1986). A similar observation has been made for rapeseed protein in which both G' and G" are higher at pH 6 than pH 10 despite a coarser and lumpier product at the lower pH (Gill and Tung, 1978). Similarly, higher G moduli were observed for the opaque gels associated with succinylated canola protein at pH 5 compared to the translucent gels at higher pH values (Paulson and Tung, 1989). Loss tangent values for the opaque gels were also higher, indicating a less elastic character to the gels. The high elastic recovery for the coarser opaque material has been attributed to the elasticity of the aggregates within the system. Gill and Tung (1978) also had concerns over sample handling in that gels were preset and loaded into the rheometer (a procedure also used by van Kleef

(1986) and Paulson and Tung (1989)); the possibility of sample compression during loading was questioned, and it was suggested that this could be avoided by heat setting the gel in the rheometer.

Another aspect of this charge influence and its relation to pH can be seen in the results of protein charge modification studies. A number of compounds have been reacted with the ε-amino group on lysine residues to increase the net negative charge on the protein; these compounds include acetic anhydride (Schmandke et al., 1981); succinic anhydride (Nakamura et al., 1978; Ma and Holme, 1982; Gossett et al., 1983a; Montejano et al., 1984; Paulson and Tung, 1989) and oleic acid (Montejano et al., 1984; Vigdorth and Ball, 1988). The effect of this treatment is to increase the negative charge on the protein which essentially lowers the IEP so that gels previously obtained at alkaline pH values are formed in a more neutral pH range. Generally, charge modification of lysine residues at neutral pH values yields an increased gel strength based on torsional, shear and compressive measurements and G moduli (Schmandke et al., 1981; Montejano et al., 1984; Vigdorth and Ball, 1988; Paulson and Tung, 1989). A decrease in gelling ability at higher pH values due to charge repulsion can be countered by the inclusion of NaCl (Paulson and Tung, 1989). Alternately, the carboxyl groups can be modified with carbodiimide to increase the net positive charge (Ma and Holme, 1982). In this way the IEP or point of maximum aggregation is increased to a higher pH value. With this modification, clear gels for egg white have been obtained at pH 5, while turbidity is greatest around pH 8.

SALTS, SUGARS AND PROPYLENE GLYCOL

In examining the influence of salt on the gelation properties of globular proteins, both the pH and the salt concentration are factors. At low salt concentrations, small ions present in solution offer a degree of shielding to the fixed charges on the protein (Egelandsdal, 1980). In this way, salt addition is somewhat similar to charge modification in that it shifts the apparent IEP and hence the pH for optimum gelation (Hegg et al., 1979). The effect of salt will therefore be a factor at pH values normally responsible for good gelling properties (Hermansson, 1982a); salt effects above the IEP are more dramatic than those below the IEP possibly due to the differential shielding of the cations and anions (Hegg et al., 1979). In fact, with BSA, gel breaking strength increased upon the addition of 0.05-0.5 M $CaCl_2$ or $MgCl_2$ on the acidic side of the IEP but it decreased under similar conditions on the alkaline side (Yasuda et al., 1986).

At appropriate pH values, there appears to be an optimum salt concentration for network formation. It has been suggested that at very low concentrations, salts aid in solubilization prior to heating and provide a crosslink (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 of 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 produce the maximum G moduli (Paulson and Tung, 1989). Generally, salt levels greater than those giving maximum strength are examined and thus gel strength tends to decrease with increasing salt concentration.

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 fababean proteins (increase Td values) 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 Td 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 concentration where this effect becomes a factor is not clear. In stability studies, the transition from predominantly electrostatic to predominantly lyotropic conditions occurred at an

ionic strength of 0.5 (Arntfield et al., 1986). With lysozyme and salt concentrations up to 0.07 M, the salt concentration at which maximum breaking strength occurred varied with the type of salt but was not related to the position of the salt in the lyotropic series (Hayakawa and Nakamura, 1986). This may reflect differential influence on electrostatic rather than hydrophobic interactions. At a concentration of 0.5 M, the turbidity developed with egg protein in the alkaline pH region 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 $SO_4^{-2} < Cl^- < Br^- < SCN^-$ (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.

Salts in this series can be classified as stabilizing (e.g. Na_2SO_4 or NaCl) or destabilizing (e.g. NaSCN). In a comparison of NaCl and NaSCN, Utsumi and Kinsella (1985) found that soy protein gels in 0.5 M NaSCN had a hardness equivalent to those in 0.2 M NaCl. In a similar investigation, gels from 11S soy protein could not be formed in 0.5 M NaCl but could be formed in 0.5 M $NaClO_4$ (destabilizer) and 0.5 M NaSCN (Damodaran, 1988). This was related to a greater refolding of the molecule in NaCl, thus reducing the number of functional groups available for interaction, rather than a nonspecific influence on hydrophobic interactions.

To further complicate the use of salts in the lyotropic series as a tool for examining hydrophobic interactions, the binding of specific

anions must also be considered. Interactions between protein and SCN^- 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.

Sucrose has also been shown to stabilize proteins through preferential hydration which appears to enhance intramolecular hydrophobic interactions (Arakawa and Timasheff, 1982). A number of sugars have been used in this type of investigation including sucrose, dextrose, xylose and glucose (Ehninger and Pratt, 1974; Kella and Poola, 1985; Mulvihill and Kinsella, 1987), with all sugars having the same effect only to varying degrees. The influence of sucrose was the most pronounced (Kella and Poola, 1985). The implication of increased intramolecular hydrophobic interaction in terms of the rheological properties of gels is not clear due to the variety of techniques used for texture evaluation. Decreased gel viscosity with the addition of 10% sugar has been observed for both soy protein isolates (Ehninger and Pratt, 1974) and arachin (Kella and Poola, 1985); this was attributed to decreased protein aggregation. With WPC, on the other hand, sucrose addition resulted in an increase in gel firmness but a decrease in whiteness (Mulvihill and Kinsella, 1987) probably reflecting a change in gel structure. Extruded soy proteins (heating temperature 150-160°C) have also been shown to form better gels in 10% as opposed to 5% sucrose (Kitabatake et al., 1985). The reduced aggregation may be related to the improved gel characteristics.

Since propylene glycol (PG) favors the formation of intermolecular

hydrogen bonds, it has been used to assess the role of hydrogen bonds in protein gels. The addition of 20% PG increased the hardness of soy protein gels (Furukawa et al., 1979; Utsumi and Kinsella, 1985), suggesting hydrogen bonds have a major role in these networks.

Overall, from the use of these solutes, it can be seen that while the electrostatic profile is essential in establishing the repulsive forces for network formation, hydrogen bonds and perhaps hydrophobic interactions contribute to the necessary attractive forces.

DENATURANTS

Several protein denaturants have been used in studies on protein gelation including guanidine hydrochloride (GdnHCl), sodium dodecylsulfate (SDS) and urea. The approach used varied with the denaturant and the type of information sought. Guanidine hydrochloride represents a chemical alternative to heat denaturation in the initial step in the gelation process (Egelandsdal, 1984). In theory, heat denaturation does not completely unfold the protein and therefore does not optimize the exposure of functional groups necessary for network formation. The increase in gel strength for ovalbumin using this chemical denaturant was only partially explained by increased protein unfolding; control of the rate of aggregation through denaturant removal had more impact on the quality of the resulting network.

Low levels of SDS (1-20 mM) have been shown to have a protective effect against protein aggregation for conalbumin, ovalbumin (Hegg and Löfqvist, 1974; Hegg et al., 1978, 1979) and sesame α- globulin (Lakshmi and Nandi, 1979). Not only does SDS addition increase the temperature required to produce aggregation but it also shifts the pH

at which minimum aggregation occurs and actually extends the pH range over which a good gelled product can be obtained. Although the binding of SDS to the protein to increase in the net negative charge is a factor in this response, it cannot completely account for the magnitude of the change. It has been suggested that SDS acts as a bridge binding to a positively changed group (usually lysine) at one position and a hydrophobic region at another (Hegg and Löfqvist, 1974). Low levels of urea have also been shown to have a beneficial effect on gel formation. Rapeseed gels formed in 1 M urea had a higher elastic component and thus a lower loss tangent value than gels formed without additives (Gill and Tung, 1978).

Perhaps the most common use of denaturants is at high concentrations. A good example of this is the work of van Kleef et al. (1978) in which ovalbumin gels were prepared in 6 M urea. At this concentration, noncovalent interactions are suppressed and the resulting network should contain only covalent disulfide linkages. Interestingly, this unique system can be described in terms of classical rubber theory allowing calculation of the number of crosslinks per molecule (3.2 for 10% ovalbumin, and 4.2 for 20% ovalbumin). This gel system, however, is not typical of globular proteins and is better described by Ferry's (1948) interactive chain model that the corpuscular model. Not all proteins form gels under these conditions; soy protein isolates produce weak, sticky gels in 6 M urea (Furukawa et al., 1979).

The solubility of gels in high levels of denaturants has also been used to assess the role of covalent and noncovalent interactions in

protein networks; however, there is little agreement between studies. Sesame α- globulin and some soy protein isolates have been shown to be completely soluble in 6-8 M urea indicating the importance of noncovalent interaction in these systems (Lakshmi and Nandi, 1979; Bikbov et al., 1986; van Kleef, 1986). Other studies indicate soy proteins are only partially soluble in 6M urea and disulfide bond reduction is required for complete solubilization (Furukawa et al., 1979). A similar discrepancy exists for ovalbumin gels. While van Kleef (1986) found these gels were not soluble in 8M urea or 1% (35 mM) SDS, Hatta et al. (1986) found them to be completely soluble in 1% SDS but not 6M urea. For Bikbov et al. (1986), they were soluble in urea plus potassium rhodanide. 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.

DISULFIDE REDUCING AGENTS

The importance of disulfide bond formation to protein networks has been an area of concern for a number of years. In early studies of soy protein gelation, the disulfide reducing agents sodium sulfite and free cysteine reduced the viscosity of both unheated and heated soy protein dispersions (Circle et al., 1964). As the proteins from whey and egg have a significant amount of cysteine and cystine residues, there is potential for intermolecular disulfide interactions. With plant proteins, there is considerable variability in the level of sulfhydryl groups even within a given protein isolate, so the potential for disulfide linkages is not as readily apparent. Data from various plant

proteins are also somewhat inconclusive. For example, the precipitation of oat and sesame proteins was unaffected by the inclusion of disulfide reducing agents (Lakshmi and Nandi, 1979; Ma and Harwalkar, 1988) while the elasticity of rapeseed gels was reduced (Gill and Tung, 1978). With soy proteins, the 11S globulin is more responsive to disulfide manipulation than the 7S globulin (Utsumi and Kinsella, 1985).

As was seen for the solubilization studies with urea, the requirement for disulfide reducing agents to completely solubilize both ovalbumin and soy protein gels is questionable with conflicting data for both proteins (Furukawa et al., 1979; Lakshmi and Nandi, 1979; Bikbov et al., 1986; Hatta et al., 1986; van Kleef, 1986). The use of a disulfide reducing agent alone (without denaturant), however, is not sufficient to dissolve either ovalbumin (Beveridge et al., 1980; Hatta et al., 1986) or soy protein gels (Furukawa et al., 1979). Regardless of the role of disulfide bonds, noncovalent interactions are a factor. Interestingly, in the resolubilization of ovalbumin gels with mercaptoethanol (ME), the degree of solubilization was greater in the alkaline region than in the acid region despite a higher gel strength at the lower pH (Egelandsdal, 1980). This implies that the disulfide bonds were not the main factor in determining gel strength. The importance of disulfide linkages may depend on the conditions under which the gel is formed and the primary structure of the protein.

One approach to examining the presence and role of disulfide interactions has been to monitor the free SH groups (those not involved in disulfide bonds) in the system. With this approach, the gel strength of ovalbumin gels was shown to be related to the level of SH groups but

the ability to coagulate was not (Hayakawa and Nakai, 1985). With whey protein, on the other hand, the level of SH groups was not a good predictor for gel strength (Kohnhorst and Mangino, 1985).

A more common technique for studying disulfide interactions is the inclusion of reducing agents, such as cysteine, ME, DTT and N'- ethylmaleimide (NEM), prior to heating. The effectiveness of these compounds varies; twice as much ME (1 SH group) as DTT (2 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, 1988). Gel hardness and torsional rigidity for both ovalbumin and soy proteins, however, tend to decrease with the inclusion of these disulfide reducing agents (Furukawa and Ohta, 1982; Hayakawa and Nakamura, 1986; Mori et al., 1986; Vigdorth and Ball, 1988). An exception to this trend is the study of Utsumi and Kinsella (1985) in which the hardness of the gels for the 11S and 7S globulins decreased with increasing ME concentration, but with a soy protein isolate only an initial decrease in hardness was observed and at ME concentrations greater than 0.01 M the trend was reversed so that increased values were obtained. A similar result was obtained with DTT. This was attributed to a cleavage of intermolecular disulfide bonds at low concentration and intramolecular disulfide bonds at higher concentrations. It was suggested that cleavage of intramolecular disulfide bonds may increase the exposure of functional groups and

allow subunit interaction between the 7S and 11S globulins. In some instances, low levels of reducing agents have been shown to actually increase gel hardness; maximum hardness for 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).

In addition to the effects on gel hardness, reducing agents also resulted in lower WHC values (Furukawa and Ohta, 1982; Yasuda et al., 1986). Interestingly, the changes in gel strength were not reflected in the microstructure (SEM) for albumen gels (Vigdorth and Ball, 1988). In terms of fundamental rheological properties, DTT decreases G′ 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 evidence obtained from gel solubility where canola gels were soluble in both 8 M urea and 6 M GdnHCl (Paulson and Tung, 1989).

From the data available in the literature, it appears that disulfide bonds may have a role in network formation. Despite conflicting data, the formation of disulfide bonds appeared to occur once the gel structure had been established and simply added strength to the structure. This would account for the changes in elasticity and gel hardness without affecting the initial coagulation or microstructure. With this in mind it would seem to be possible to form gels without disulfide bond involvement. This role for disulfide bonds is not in agreement with the bridging and ordering function during initial structure development proposed by Schmidt (1981). Conformation of the this role requires further, more fundamental investigations.

MATERIALS AND METHODS

Materials

Ovalbumin

Ovalbumin was obtained from Sigma Chemical Co. (Grade V, Lot 115F-8115) and used without further purification.

Vicilin

Vicilin was isolated from fababean (Vicia faba minor var. Diana). The initial step in the recovery of this protein was the preparation of a protein isolate following the procedure of Murray et al. (1978). This procedure essentially involves salt solubilization followed by precipitation through a reduction in ionic strength; these mild conditions do not significantly alter the native conformation of the fababean proteins (Murray et al., 1985). The resulting isolate is predominantly composed of the two main storage proteins of fababean, legumin and vicilin (Murray et al., 1981).

Of these, vicilin was chosen for this study because of its low Td value, despite representing a lower proportion of the total protein in the isolate. As the Td value for vicilin is comparable to that for ovalbumin, it would represent a reasonable substitute for ovalbumin in terms of the processing conditions required for thermal denaturation. For example, in 0.5 M NaCl, the Td for vicilin is 84.2°C compared to 86°C for ovalbumin. Furthermore, the Td value for vicilin in some environments is considerably lower than that for legumin, the other

major fababean storage protein; in 0.5 M NaCl the Td value for legumin is 103.5°C, 9.3° higher than vicilin (Arntfield et al., 1986). It was advantageous to heat set the protein in the rheometer and thus follow structure development during both heating and cooling. In this way, possible compression problems associated with loading preset gels as were observed in the work of Gill and Tung (1978) were avoided. However, the rheometer available for monitoring structure development had an upper temperature limit of 95°C, thus limiting environments where legumin network formation could be examined.

Vicilin was purified from the fababean isolate following the procedure of Ismond et al. (1985). This involved preferential solubilization in 0.2 M $NaC_2H_3O_2$, pH 7.5, followed by gel filtration on Sephacryl S-300. The vicilin produced with this techniques has been shown to be homogeneous based on analytical ultracentrifugation and isoelectric focusing data (Ismond, 1984).

Other Reagents

All other chemicals used were reagent grade (Fisher Scientific).

Methods

Sample Preparation

The different environments used to manipulate the potential for protein-protein interactions for both ovalbumin and vicilin are listed in Table 1. Except with SDS and urea, the same environments were examined for the two proteins. Variations between the two proteins, when used with these solutes, were due to poor network formation with vicilin, particularly at higher concentrations. Vicilin was therefore

TABLE 1. Environments used to assess structure development for ovalbumin and vicilin.

Variable	General Conditions	Values Examined
Concentration	0.15 M NaCl pH. 8.5	5, 7.5, 10, 12.5, 15 %
pH	10% 0.15 M NaCl	3, 4, 5, 6, 7, 8, 9
Sodium Dodecylsulfate	10% 0.15 M NaCl pH 8.5	2.5, 5, 7.5, 10, 20 25, 35, 50 mM[1]
$CaCl_2$	10% pH 8.5	.1, .2, .3, .4, .5 M
NaCl	10% pH 8.5	.1, .2, .3, .4, .5 M
Anions of Sodium Salts (.1 and .5 M)	10% pH 8.5	SCN^-, $C_2H_3O_2^-$, Br^-, Cl^-, SO_4^{-2}
Urea	10% pH 8.5	.05, .1, .25, .5, 1, 2, 4, 6, 8 M[1]
Cysteine HCl	10% pH 8.5 0.15 M NaCl	2.5, 5, 10, 25, 50 mM
Mercaptoethanol	10% pH 8.5 0.15 M NaCl	2.5, 5, 10, 25, 50 mM
N'-ethylmaleimide	10% pH 8.5 0.15 M NaCl	2.5, 5, 10, 25, 50 mM

[1] Not all values examined for both proteins

examined at lower (SDS) or fewer concentrations (urea). With all systems, except urea, there was always some salt included in the solvent to ensure proper dispersion or solubilization of the protein prior to heating. This was more a problem with vicilin where, in the absence of salt, a cohesive mass formed at the bottom of the beaker. In all cases, sample pH was adjusted to the desired value with rapid stirring using either 1 M NaOH or 1 M HCl. To ensure the pH was maintained, samples were equilibrated for 30 min and rechecked. Only when pH had been constant for 30 min were the analyses performed.

Calorimetry

Thermal properties for ovalbumin and vicilin were determined as a check for major conformational changes due to the environmental factors as described previously (Arntfield and Murray, 1981). The instrument used was a DuPont 9900 thermal analyzer with a 910 Differential Scanning Calorimeter Cell Base and a high pressure DSC Cell. Thermal curves were obtained using 10-15 μL of a protein dispersion and heating at a rate of 2°C/min with an empty pan as reference. The rate of 2°C/min was chosen as a compromise so that the same heating rate could be used for all parameters tested. The maximum controlled heating rates possible for the water baths used for microstructure sample preparation and rheological assessment was approximately 3°C/min; at lower heating rates (<1°C/min) background noise became a significant factor in the DSC analysis. Denaturation temperature (Td), measured at the point of maximum heat flow and enthalpy of denaturation (ΔH) were calculated instrumentally; the equations used for these calculations have been described previously (Arntfield and Murray, 1981).

Microstructure

The microstructure of the heat induced networks was examined for ovalbumin and vicilin in all experimental solvents. Heat induced networks were prepared by first placing 1 mL of dispersed protein in a small (1 cm diameter) capped vial. The sample was placed in a Haake F3 digital water bath equipped with a Haake PG 20 temperature programmer and heated to 95°C at a rate of 2°C/min. Samples were held at 95°C for 5 min to ensure that maximum temperature had been attained throughout and placed in ice to cool to room temperature.

Samples were cut into 7 μm thick sections using an American Optical Cryo - Cut II Microtome (Model 851C). The heat set sample was mounted on an object disc using an O.C.T. compound embedding medium (Fisher Scientific) and covered with the same embedding compound. Samples were frozen using the copper quick-freeze shelf located in the microtome. Freezing time was approximately 30 - 60 sec. The frozen sample was then sectioned and the sections recovered by mounting directly onto a warm slide. Microtome temperature was maintained between -25 and -30°C during sectioning for optimum results. All sections were prepared immediately following network formation.

Network structures were examined without staining and without a coverslip using a Zeiss Universal Research Microscope. This was possible due to the low magnifications used. A blue filter was used to improve contrast for all photomicrographs. Representative structures for each environment were photographed using a C35M Carl Zeiss automatic exposure 35 mm camera and Kodak Ektochrome 160 ASA film.

Rheology

Rheological parameters were assessed using a Bohlin VOR rheometer (Bohlin Reologi, Inc.) equipped with a programmable water bath. Protein samples at 25°C were placed between parallel plates (30 mm) in the rheometer and the gap of 1 mm set between the two plates. To avoid sample drying during heating, a masking tape wall similar to that described by Beveridge et al. (1984) was formed around the outer edge of the cylinder holding the lower plate. Paraffin oil, Saybolt viscosity 125/135 (Fisher Scientific), was added until it just covered the upper plate. The sample was then heated at a rate of 2°C/min to 95°C, held there for 2 min and cooled to 25°C at a rate of 2°C/min. Dynamic test characteristics were assessed every minute. Input strain amplitude for dynamic analysis was 0.02, a value found to be in the linear viscoelastic region for heat induced protein networks in preliminary experimentation. A frequency of 0.1 Hz was used for the thermal scans. The sensitivity of the measurement was determined by a torque bar calibrated to 93.2 gcm, attached to the upper plate. No correction was made for the change in gap width at different temperatures as was done by Beveridge et al. (1984). In preliminary experiments, the difference in the gap as a function of temperature up to 95°C was less than 5%. It is possible that the smaller plates on the Bohlin rheometer (3 cm) do not present as great a problem as was reported for the 7.5 cm plates on a Weissenberg rheogoniometer (Beveridge et al., 1984).

Dynamic test characteristics monitored included G', the storage modulus, and G", the loss modulus. These parameters were determined

instrumentally; the appropriate equations have been reported previously (Bohlin et al., 1984). The loss tangent or tan delta (tan $\delta = G''/G'$), a measure of the energy lost due to viscous flow compared to the energy stored due to elastic deformation in a single deformation cycle, was also calculated.

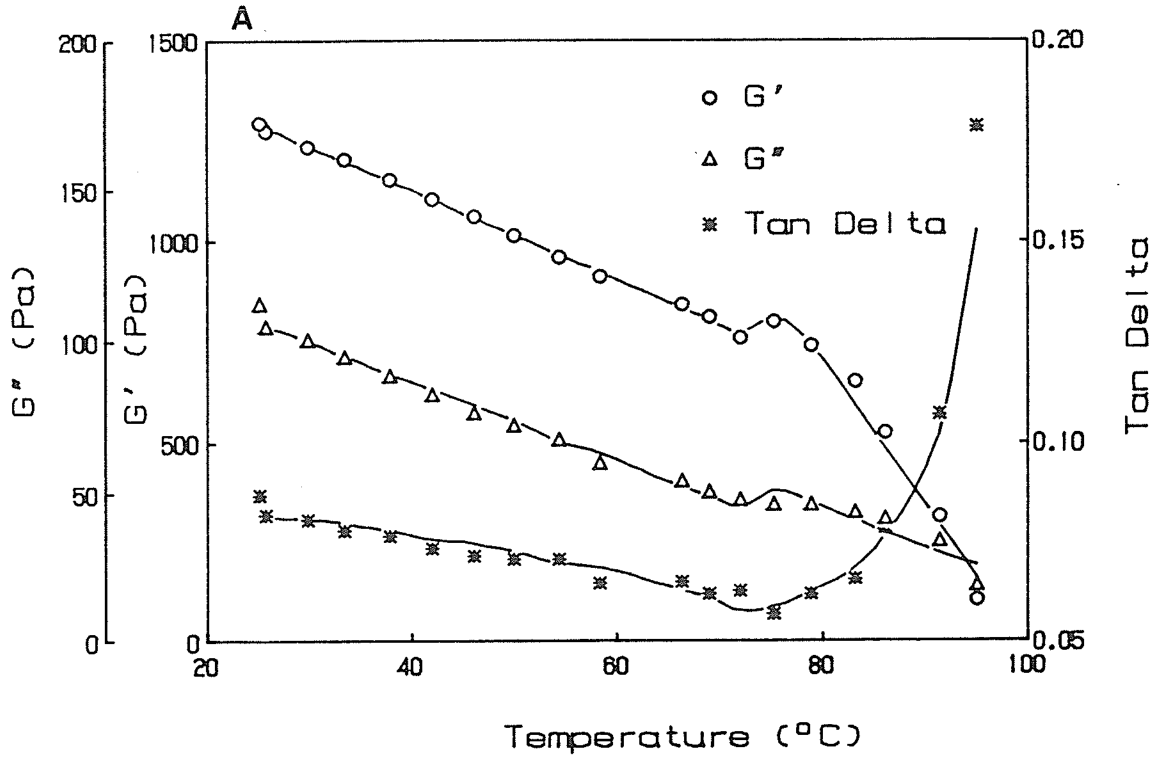
Comparison of the changes in the rheological parameters during the heating phase to DSC data has indicated the need for protein denaturation prior to structure development. The impact of the temperature difference between denaturation and structure development on the characteristics of the resulting network has been reported previously (Arntfield et al., 1989a). For most environments in this investigation, structure development did not begin until temperatures greater than 90°C, and the G moduli as a function of increasing temperature curves were basically all the same. As a result, the focus in this investigation has been on changes during the cooling phase and the characteristics of the final network.

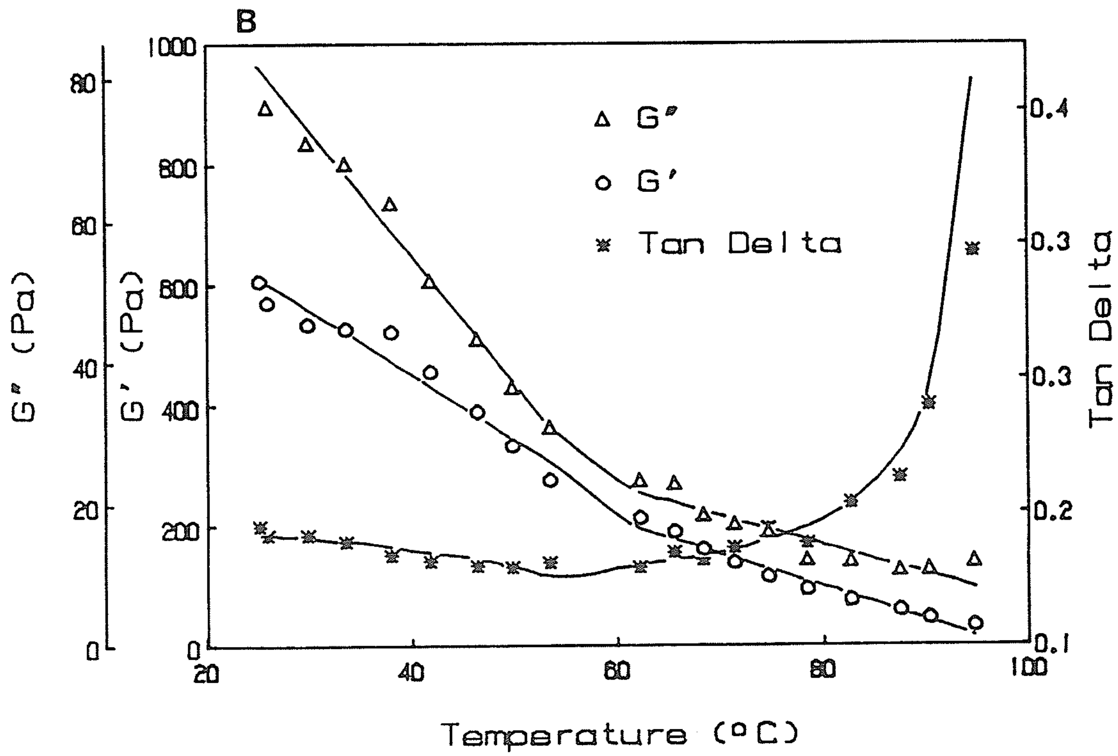
With a few exceptions, both the G' and G" moduli on the cooling curves had two distinct phases (Fig. 1). In some instances, the increase in the G moduli was greater during the initial cooling (Fig. 1A), while in other cases, greater increases in the G moduli were associated with the latter cooling phase (Fig. 1B). For some curves, the relationship between the G moduli and temperature could be described as an exponential or logarithmic one; however, the best correlations were usually attained when the curves were described as two distinct linear portions, or a biphasic linear relationship. The inflection point in this biphasic model was determined visually. Added

Figure 1. Changes in the rheological parameters of protein dispersions as a function of temperature during cooling. Symbols on the graphs represent the observed data. Lines for G' and G" represent the two linear phases for each curve calculated from the observed data. The curve for tan delta represents predicted values based on the biphasic models for G' and G".

A. 10% ovalbumin in 0.15 M Nacl, pH 9.

B. 10% vicilin in 0.15 M NaCl, pH 9.


A
G'
G″
Tan Delta
G″ (Pa)
G' (Pa)
Tan Delta
Temperature (°C)
200
150
100
50
0
1500
1000
500
0
0.20
0.15
0.10
0.05
20
40
60
80
100


B
G″
G'
Tan Delta
G″ (Pa)
G' (Pa)
Tan Delta
Temperature (°C)
80
60
40
20
0
1000
800
600
400
200
0
0.4
0.3
0.3
0.2
0.1
20
40
60
80
100

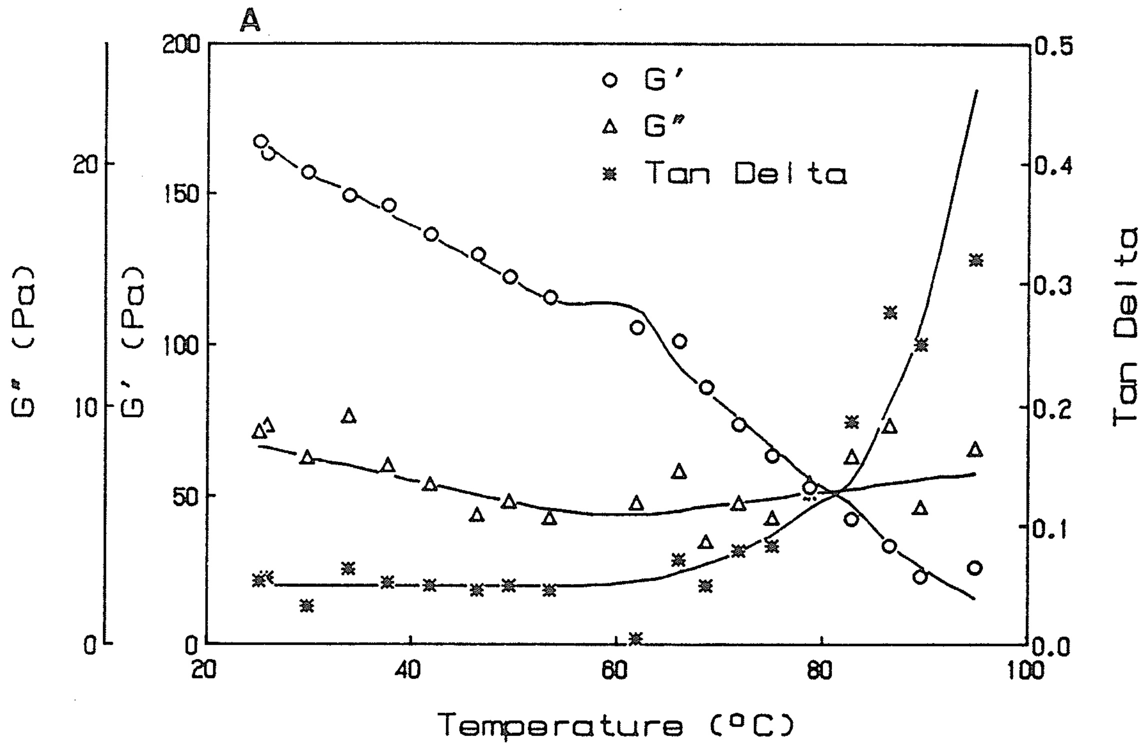
support for the use of this biphasic model was seen in the ability of tan δ values calculated from the linear regression values for G' and G" to describe the tan δ values calculated from the observed data. This was rarely the case with values obtained with the logarithmic and exponential models in that the initial decrease in tan δ was often lost in the prediction equations. Based on these observations, it was decided that the best approach for handling the cooling curve data was to compare the slopes of the linear portions of the cooling curves for both G' and G". Slopes for which an increase in the G moduli was observed with decreasing temperature were, therefore, reported as negative values. In this way the effect of various interactions could be examined in terms of their roles during the establishment of the network (initial phase) as well as in stabilizing the network (final phase).

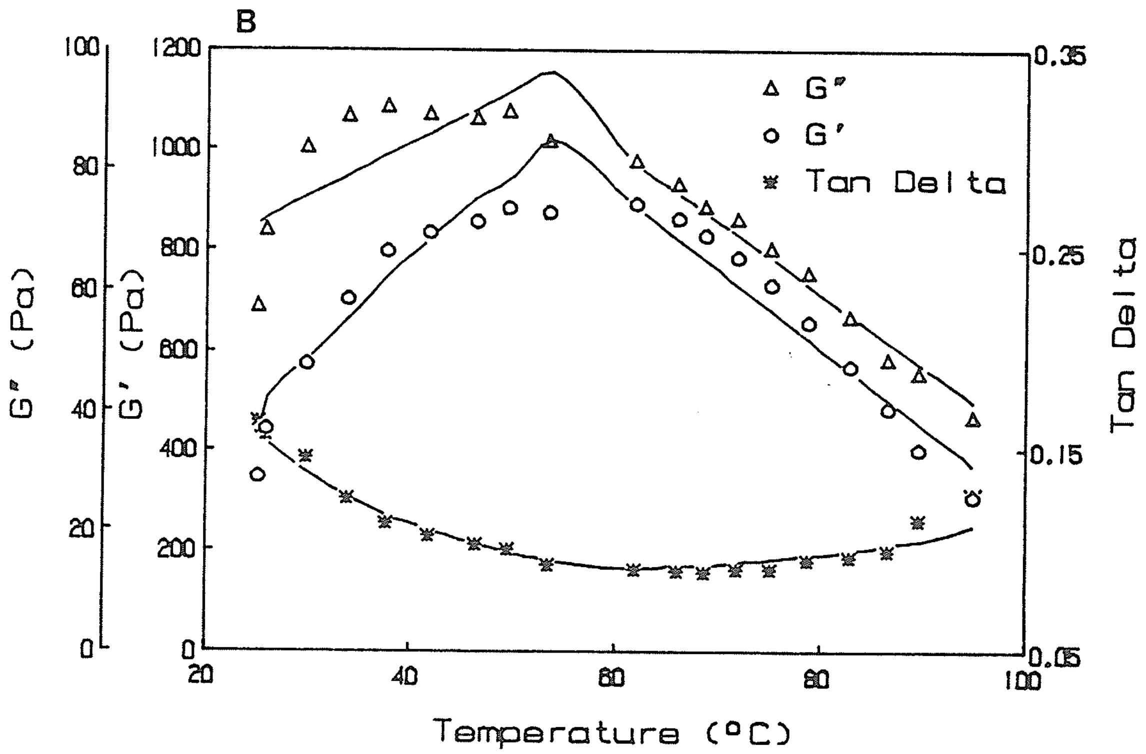
It should be noted that there was some variation as to how well curves were described by this biphasic model. For ovalbumin at pH 7, for example, no obvious inflection point could be seen in the G curves; hence a linear relationship was assumed and the initial and final slopes were the same (Appendix 2A). In this case, the tan δ values calculated from the linear relationship between the G moduli and temperature adequately described the observed tan δ values. Another problem resulted from the fact that in some instances, there was very little variation in the G" modulus during the cooling regime (Fig. 2A). In fact, the correlation between G" and temperature was not significant no matter what relationship was used to describe it. Nevertheless, the biphasic approach was still used, primarily because of the ability of

Figure 2. Changes in the rheological parameters of protein dispersions as a function of temperature during cooling. Symbols on the graphs represent the observed data. Lines for G' and G" represent the two linear phases for each curve calculated from the observed data. Curve for tan delta represents predicted values based on the biphasic models for G' and G".

A. 10% vicilin in 0.15 M Nacl, pH 3.

B. 10% vicilin in 0.15 M NaCl, pH 5.


A
G'
G"
Tan Delta
G" (Pa)
G' (Pa)
Tan Delta
Temperature (°C)
0
10
20
0
50
100
150
200
0.0
0.1
0.2
0.3
0.4
0.5
20
40
60
80
100

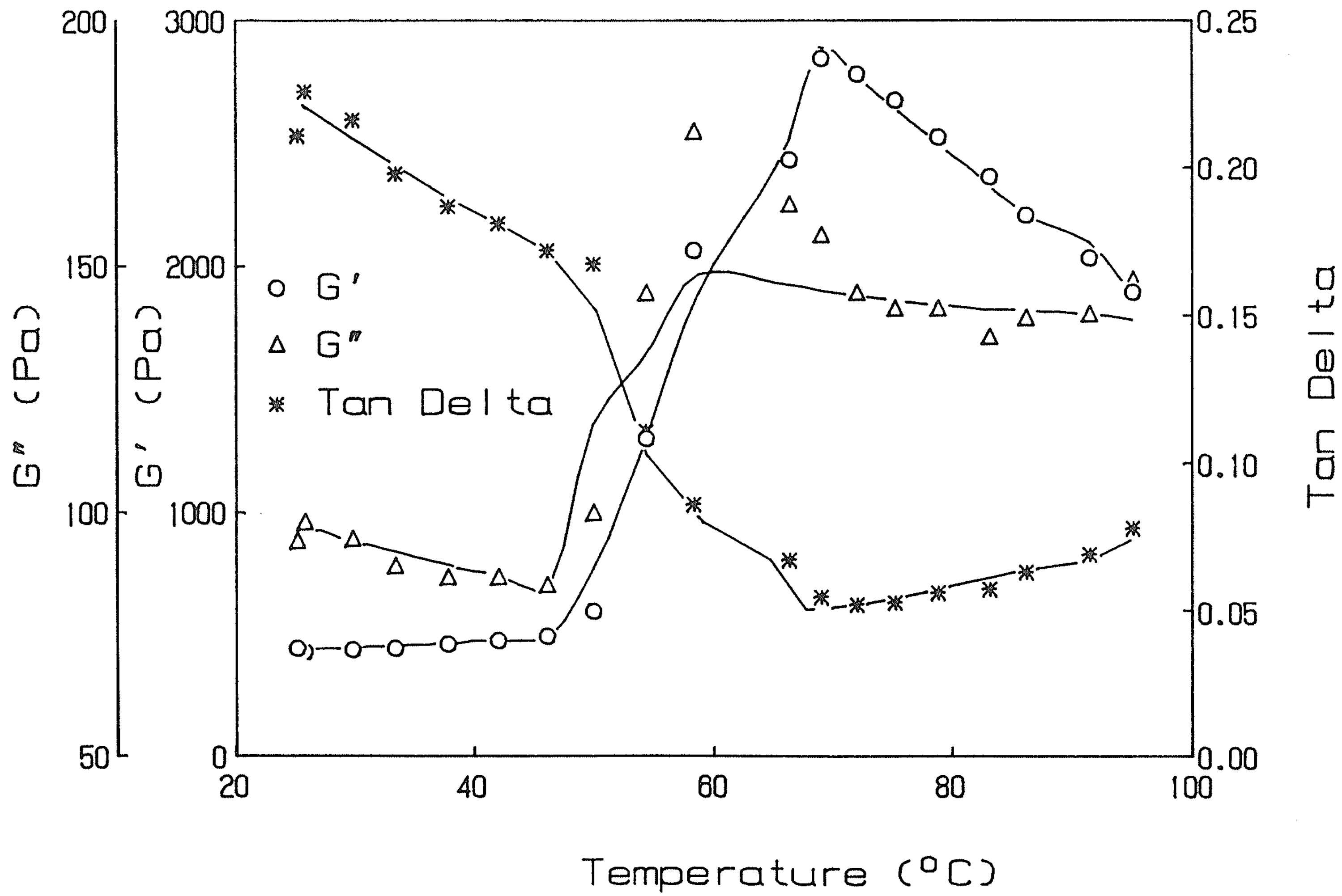

B
G"
G'
Tan Delta
G" (Pa)
G' (Pa)
Tan Delta
Temperature (°C)
0
20
40
60
80
100
0
200
400
600
800
1000
1200
0.05
0.15
0.25
0.35
20
40
60
80
100

this model to predict tan δ values. A similar problem was seen in curves which seemed to plateau in the middle of the cooling curve, resulting in poor correlations for both G′ and G″ during the final phase (Fig. 2B). Once again, the tan δ values based on this relationship were able to describe the observed variations in tan δ, thus justifying the use of this model.

The only environments for which the two phase approach was not appropriate for describing the changes in the G moduli as a function of temperature were for ovalbumin at pH 3 and pH 4; the curves for ovalbumin at pH 3 can be seen in Fig. 3. Under these conditions an initial increase in the G moduli was followed by a dramatic drop to much lower values, at which point the G moduli remained relatively constant. For these curves, a triphasic model seemed most appropriate and adequately predicted tan δ values. However, to compare these data with those from other environments only the first two phases of this model have been evaluated and are included in subsequent tables and figures as the initial and final slopes.

Characterization of the heat induced networks involved measuring dynamic properties as a function of oscillatory frequency (ω) at a constant temperature of 25°C, using the same strain amplitude and torque bar as during the heating and cooling regimes. For both ovalbumin and vicilin (10% protein, 0.15 M NaCl and pH 8.5), there was a linear relationship between the log of the G moduli and log ω (Fig. 4). The dependency of the G moduli on ω demonstrated the viscoelastic nature of the network formed; for an ideal elastic solid, G′ is independent of frequency. The tan δ values reflected the ratio of the G moduli and were,

Figure 3. Changes in the rheological parameters of 10% ovalbumin in 0.15 M NaCl, pH 3, as a function of temperature during cooling. Symbols on the graphs represent the observed data. Lines for G' and G" represent the two linear phases for each curve calculated from the observed data. Curve for tan delta represents predicted values based on the biphasic models for G' and G".

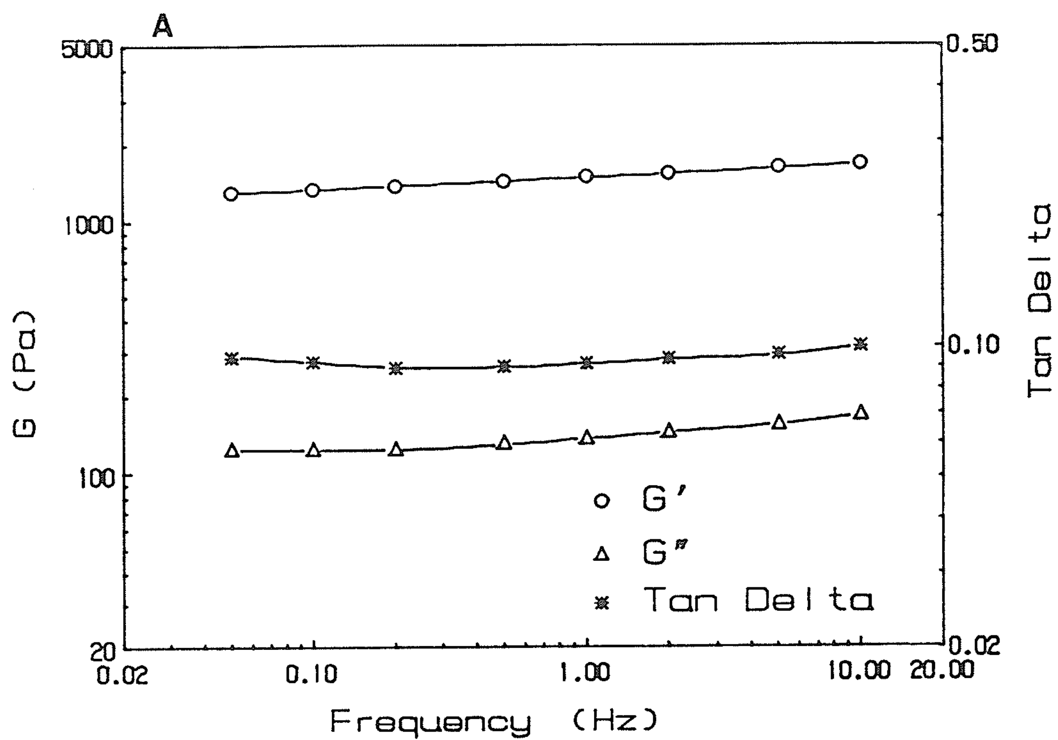


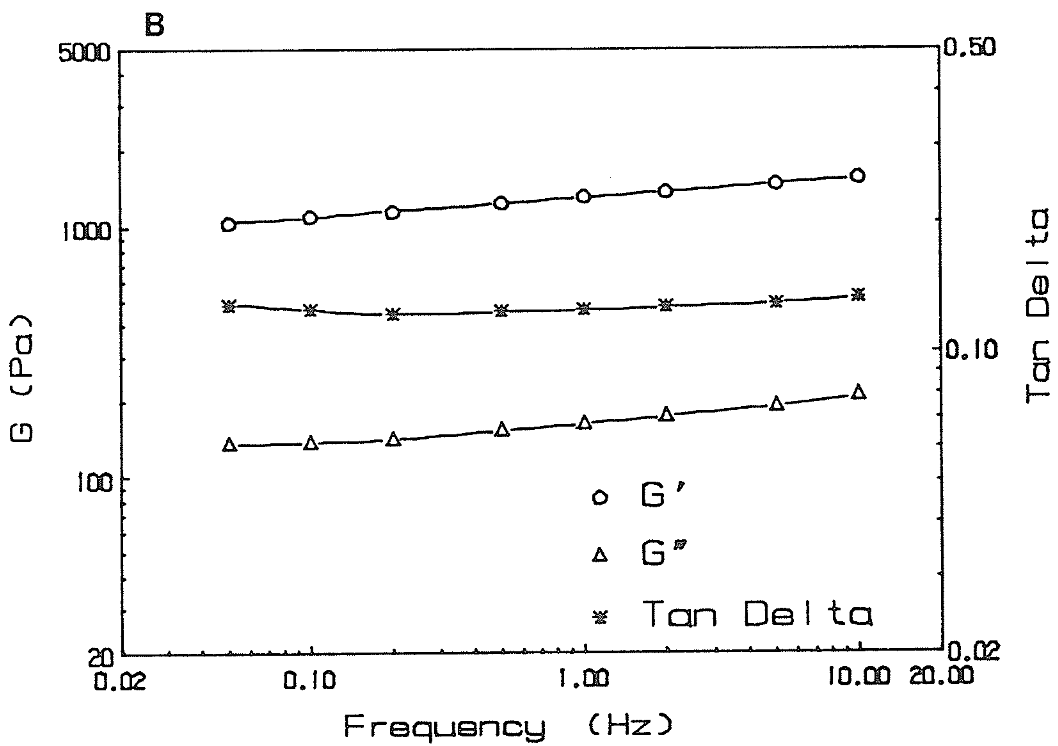
G″ (Pa)
G′ (Pa)
Tan Delta
200
150
100
50
3000
2000
1000
0
0.25
0.20
0.15
0.10
0.05
0.00
20
40
60
80
100
○ G′
△ G″
⋇ Tan Delta
Temperature (°C)

Figure 4. Changes in rheological parameters of heat induced protein networks as a function of oscillatory frequency.

A. 10% ovalbumin in 0.15 M NaCl, pH 8.5.

B. 10% vicilin in 0.15 M NaCl, pH 8.5.


A
5000
1000
100
20
G (Pa)
0.50
0.10
0.02
Tan Delta
G'
G"
Tan Delta
0.02
0.10
1.00
10.00
20.00
Frequency (Hz)


B
5000
1000
100
20
G (Pa)
0.50
0.10
0.02
Tan Delta
G'
G"
Tan Delta
0.02
0.10
1.00
10.00
20.00
Frequency (Hz)

in fact, less dependent on the frequency. As the slopes of these lines were relatively small, G moduli from regression analyses at a frequency of 1 Hz (i.e. regression intercept) were used for data comparison. The tan δ value for a given environment was calculated from these G moduli.

Statistical Analysis

All experimental conditions were examined in duplicate and the average values of these duplicates have been presented in all tables and figures. The time factor involved in preparing purified vicilin for use on this scale precluded any further replications. Statistical differences due to the various environments were determined using an Analysis of Variance in conjunction with a Duncan's Multiple Range Test. This was done using an IBM personal computer and a Number Crunching Statistical System (NCSS) software package. Statistical differences not given in tables in the text have been included in the appendices at the end for both rheological and calorimetric data.

RESULTS AND DISCUSSION

Protein Concentration

Protein concentration is of prime importance in establishing heat induced protein networks, primarily because a critical protein concentration (CPC) is required to produce a three dimensional structure. The CPC is considerably higher for globular proteins than fibrous proteins (Clark and Lee-Tuffnell, 1986). This factor favors aggregation of corpuscular structures rather than unfolded polypeptide chains as the mechanism for network formation with globular proteins. It was therefore necessary to examine the effect of concentration on network formation for ovalbumin and vicilin to ensure that the critical concentration had been reached in subsequent experiments.

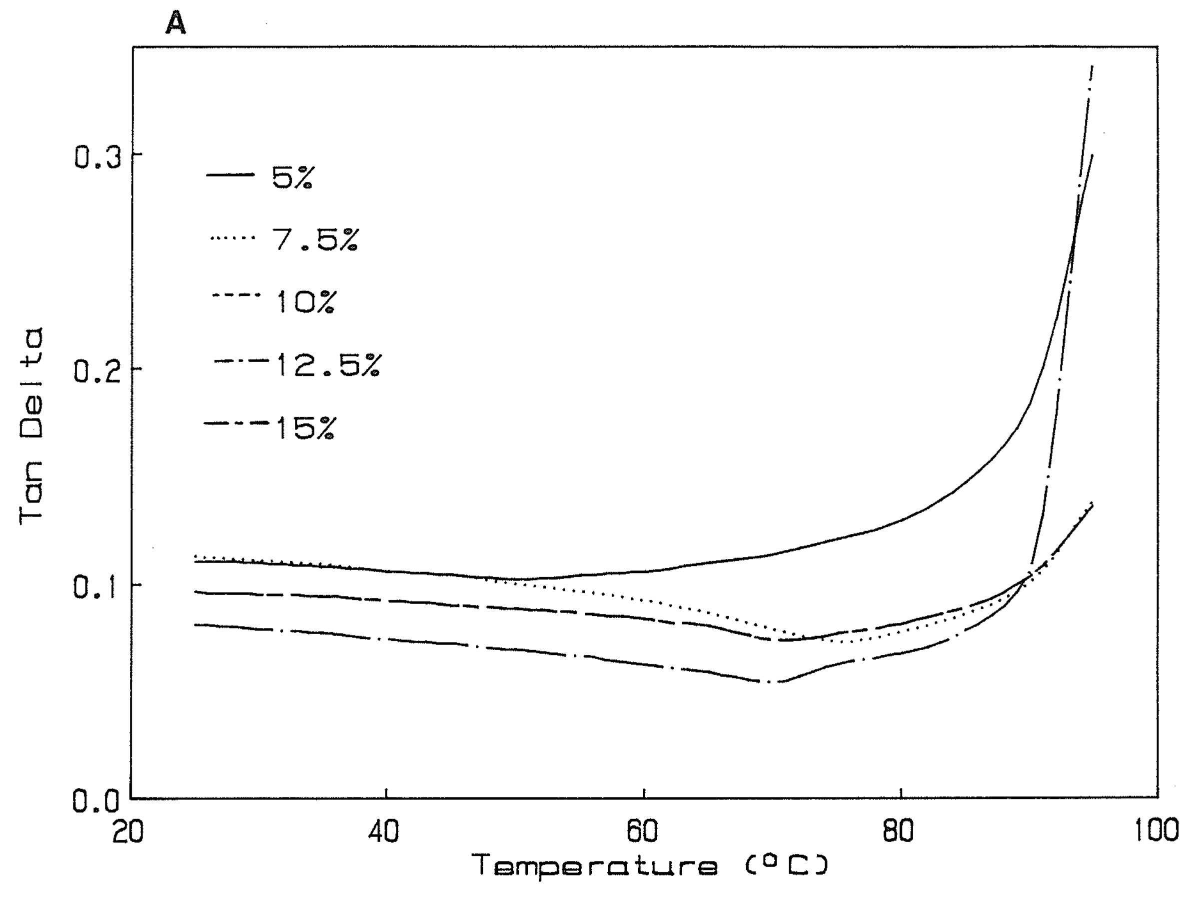
Changes during the Cooling Regime

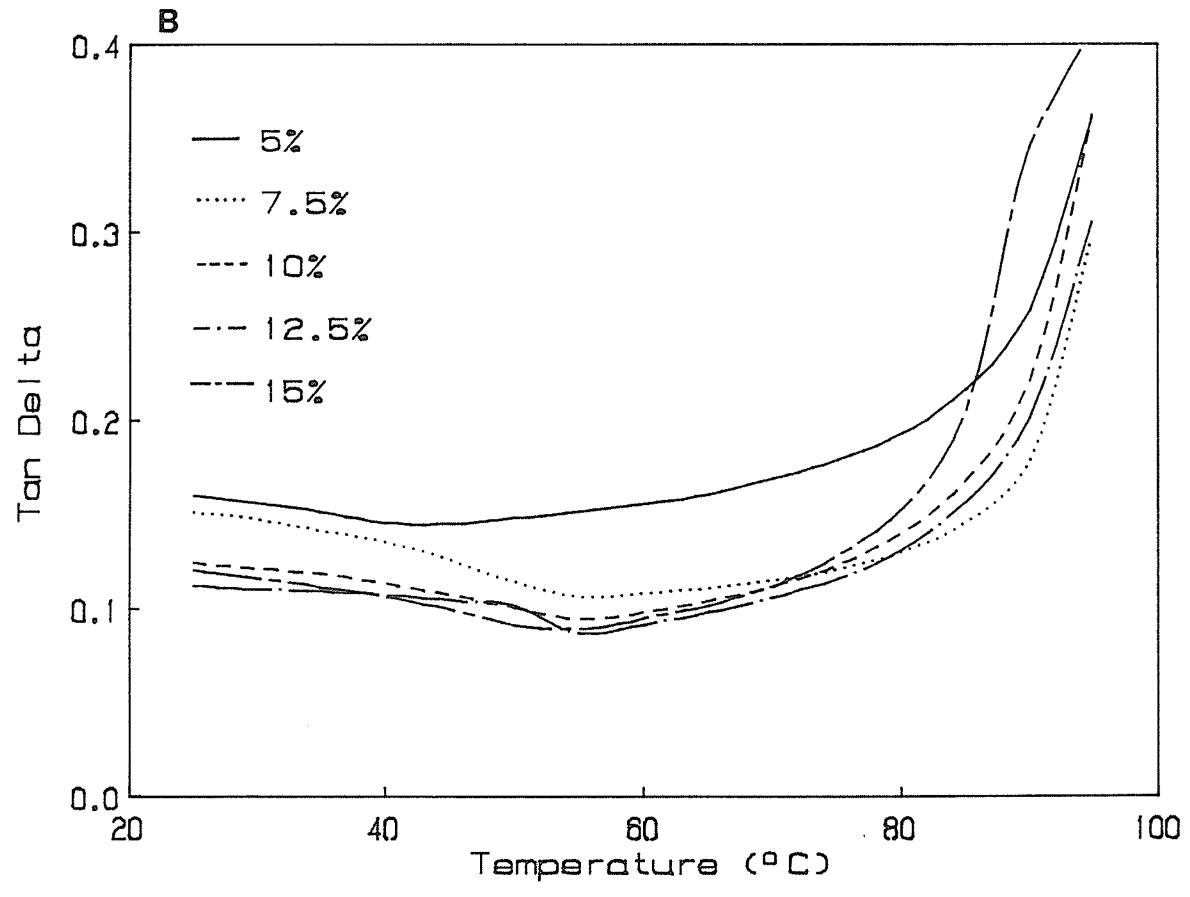
Ovalbumin: Structure development during cooling can be monitored by following tan δ as a function of temperature. With ovalbumin, there was an immediate decrease in tan δ at the onset of cooling (95°C), after which the tan δ values either leveled off or increased slightly (Fig. 5A). The extent of this initial decrease in tan δ was variable and showed no trend with respect to concentration; with 7.5, 10 and 12.5% protein, a decrease in tan δ occurred during the heating phase thus accounting for the low initial values during cooling. As a lower tan δ value is indicative of an increase in G' relative to G", it is reasonable to interpret this decrease as evidence of the establishment of a protein network. At all concentrations, therefore, the

Figure 5. Influence of protein concentration on structure development during cooling for heat induced protein networks. Tan delta curves were calculated using a biphasic linear model to describe changes in G' and G".

A. ovalbumin in 0.15 M NaCl, pH 8.5. Note that the curves for 10% and 15% overlap exactly and thus only the 15% curve is visible.

B. vicilin in 0.15 M NaCl, pH 8.5.


A
Tan Delta
0.0
0.1
0.2
0.3
20
40
60
80
100
Temperature (°C)
5%
7.5%
10%
12.5%
15%


B
Tan Delta
0.0
0.1
0.2
0.3
0.4
20
40
60
80
100
Temperature (°C)
5%
7.5%
10%
12.5%
15%

establishment of a protein network occurred quickly at temperatures around the maximum attained (95°C) either during the heating or cooling regimes. The nature of the network did not seem to change significantly during subsequent cooling.

Changes in both G' and G" contribute to the changes in tan δ associated with network development. In this respect, the G' modulus measures the elastic component of the structure and is therefore an indicator of the development of a three dimensional matrix. The G" modulus, on the other hand, measures the viscous component and may represent interactions which do not directly contribute to the three dimensional nature of the network. Although the changes in tan δ values were similar for the various protein concentrations, the rates of change in G' and G" moduli reflected the impact of varying protein levels (Table 2A). These rates of change represented the slopes of the two linear regression models describing the two phases (initial and final) of the curves monitoring G moduli as a function of cooling temperature. When a decrease in temperature resulted in increased G moduli, the rates of change were reported as negative values. An increase in ovalbumin concentration resulted in an increase in the magnitude of the change in G' and G" during both initial and final phases of the cooling regime. From these results, it appeared that protein-protein interactions continued during the entire cooling regime and the rate of interaction was highly dependent on the quantity of protein present. The fact that both G' and G" responded to the change in protein concentration was indicative of a situation in which not all the interactions were contributing to network development. In this way,

TABLE 2A. Effect of protein concentration on the rate of structure development during cooling from 95°C to 25°C at 2°C/min for ovalbumin in 0.15 M NaCl, pH 8.5.

Concentration	Initial Cooling Phase		Final Cooling Phase	
(%)	G' (Pa/°C)	G" (Pa/°C)	G' (Pa/°C)	G" (Pa/°C)
5	-1.2±0.1	-0.10±0.00	-0.6±0.1	-0.08±0.00[a]
7.5	-15.4±1.2[a]	-0.70±0.23[a]	-3.9±0.3	-0.47±0.04[a]
10	-17.8±0.9[a]	-0.94±0.05[ab]	-8.8±0.2	-1.30±0.32[b]
12.5	-25.2±1.7	-1.27±0.06[b]	-13.4±0.6	-1.46±0.09[b]
15	-42.5±3.9	-2.42±0.18	-25.9±1.0	-3.08±0.13

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

TABLE 2B. Effect of protein concentration on the rate of structure development during cooling from 95°C to 25°C at 2°C/min for vicilin in 0.15 M NaCl, pH 8.5.

Concentration	Initial Cooling Phase		Final Cooling Phase	
(%)	G' (Pa/°C)	G" (Pa/°C)	G' (Pa/°C)	G" (Pa/°C)
5.0	-1.6±0.1[a]	-0.18±0.02[a]	-2.8±0.2[a]	-0.58±0.08[a]
7.5	-3.1±0.8[ab]	-0.27±0.05[a]	-7.6±1.1[a]	-1.42±0.13[a]
10.0	-8.4±0.2[b]	-0.46±0.17[ab]	-17.8±2.2	-2.76±0.38
12.5	-17.9±3.0	-0.86±0.02[b]	-45.4±0.6	-5.92±0.28
15.0	-40.8±1.9	-2.05±0.18	-58.1±2.0	-9.50±0.61

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

the increased rates for G′ and G″ production at higher concentration did not substantially alter the nature of the network (tan δ value). It was noteworthy that the changes in both G′ and G″ were greater during the initial cooling phase than the final cooling phase for all concentrations.

Vicilin: As was the case with ovalbumin, the relationship between tan δ and temperature during cooling of vicilin was characterized by an initial sharp decrease; however, subsequent changes were relatively minor (Fig. 5B). The initial decease was not as sharp as with ovalbumin and a temperature of about 80°C was usually attained before values leveled off. At a protein concentration of 5%, the initial drop in tan δ was not as great as at higher concentrations and consequently the final tan δ value was higher. In this situation, the interactions during the establishment of the network did not produce a structure whose elastic component was comparable to that at higher protein concentrations. For 7.5% vicilin, the initial decrease in tan δ was closer to that obtained at higher concentrations but the resulting tan δ value was comparable to that for the 5% vicilin concentration.

As with ovalbumin, the impact of varying concentration was evident by the increased rates of change in G′ and G″ moduli during both the initial and final cooling phases (Table 2B). This reflected an increase in the rate of protein-protein interaction at concentrations above the CPC. Contrary to ovalbumin, however, the rate of change in G′ for vicilin was greater during the final cooling phase than the initial cooling phase. This difference appeared to be associated with a higher rate of interaction during the final phase than was seen for ovalbumin

rather than a lower rate during the initial phase. The changes in tan δ during cooling were not influenced by this difference in terms of the relative importance of initial and final phases for ovalbumin and vicilin as evidenced by the similarity in the shapes of the cooling curves. Overall, the impact of concentration on the changes which occurred during cooling was reflected in the changes in G′ and G" moduli, rather than tan δ values.

Network Characteristics

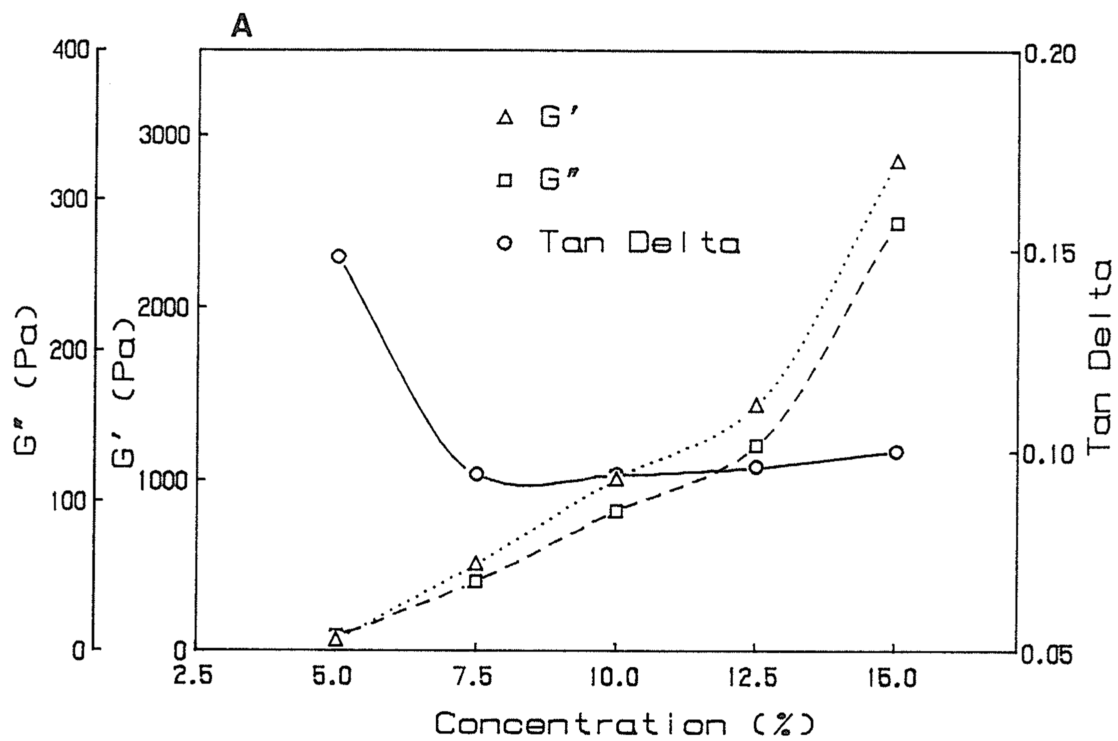
Ovalbumin: The influence of concentration on the rates of change for G′ and G" during the cooling of ovalbumin was also seen in the rheological characteristics of the resulting networks. Both G′ and G" increased with increasing concentration (Fig. 6A). Exact values, including significant differences, can be found in Appendix 1A. The nature of this relationship was such that it was best described as a second order polynomial (e.g. $G' = 466 - 172\ Conc + 21.6\ Conc^2$; $R = 0.9880$). A power law relationship, such as that used by van Kleef (1986) could also have been used to describe the data (e.g. $G' = 0.51\ Conc^{3.2}$); however, the correlation coefficient was slightly lower ($R = 0.9792$). Similar relationships were found for the G" modulus.

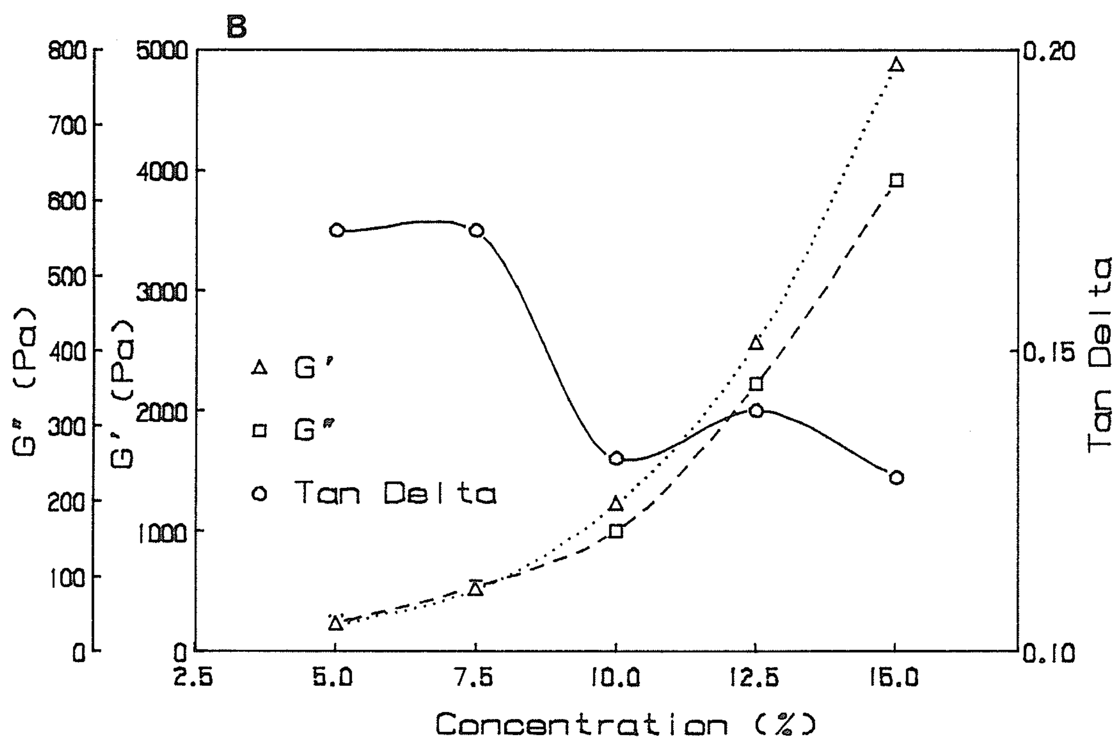
The response of tan δ values to varying protein concentrations was quite different. At concentrations of 7.5% and higher, the tan δ value was independent of concentration. The higher tan δ value with 5% protein indicated a less elastic structure as would be expected if the CPC for network formation had not been reached. Above the this concentration, however, it appeared that the type of network formed was unaffected by the amount of protein present. As a result, the CPC for

Figure 6. Effect of protein concentration on rheological properties of heat induced protein networks.

A. ovalbumin in 0.15 M NaCl, pH 8.5.

B. vicilin in 0.15 M NaCl, pH 8.5.


A
G″ (Pa)
G′ (Pa)
Tan Delta
△ G′
□ G″
○ Tan Delta
Concentration (%)
400
300
200
100
0
3000
2000
1000
0
0.20
0.15
0.10
0.05
2.5
5.0
7.5
10.0
12.5
15.0


B
G″ (Pa)
G′ (Pa)
Tan Delta
△ G′
□ G″
○ Tan Delta
Concentration (%)
800
700
600
500
400
300
200
100
0
5000
4000
3000
2000
1000
0
0.20
0.15
0.10
2.5
5.0
7.5
10.0
12.5
15.0

network formation with ovalbumin was between 5 and 7.5 %. It should be kept in mind that the tan δ value at a concentration of 5% was approximately 0.15 (Appendix 1A), considerably lower than at the onset of the cooling regime (0.30). Some degree of interaction and structure development resulted during cooling; however, the structure lacked the organized crosslinking necessary to give a three dimensional network similar to those found at a higher protein concentrations.

These differences were also reflected in the microstructure of the heat induced networks at various protein concentrations (Fig. 7). With 5% ovalbumin, strands of protein were visible but connections between strands required for a good two dimensional network were limited and large open spaces were visible. When using light microscopy it should be remembered that only two dimensions are photographed. Based on the examination of adjacent sections throughout the gel, a strong two dimensional network (in sections 7 μm thick) in light microscopy was thought to represent a strong three dimensional network in reality. The two dimensional network, visible at higher protein concentrations was indicative of good matrix formation. As the concentration increased, the same very interactive network persisted, except the intensity of the network increased reflecting the increased degree of interaction measured by the high G moduli.

Vicilin: The influence of protein concentration on the rheological characteristics of heat induced vicilin networks was similar to those for ovalbumin (Fig. 6B). The increase in G moduli with concentration could be described as either second order polynomial (e.g. $G' = 2102-612\ Conc + 53.5\ Conc^2$; $R = 0.9990$) or a power law relationship (e.g.

Figure 7. Photomicrographs showing the effect of protein concentration on heat induced networks for ovalbumin in 0.15 M NaCl, pH 8.5.

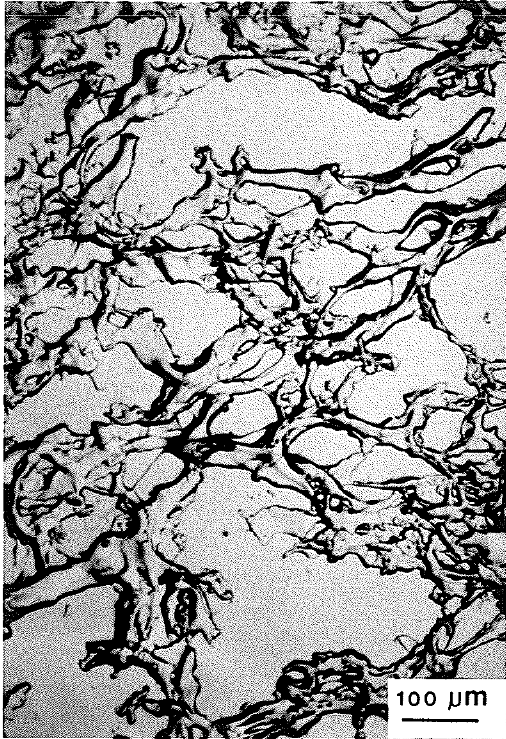
A. 5% protein

B. 10% protein

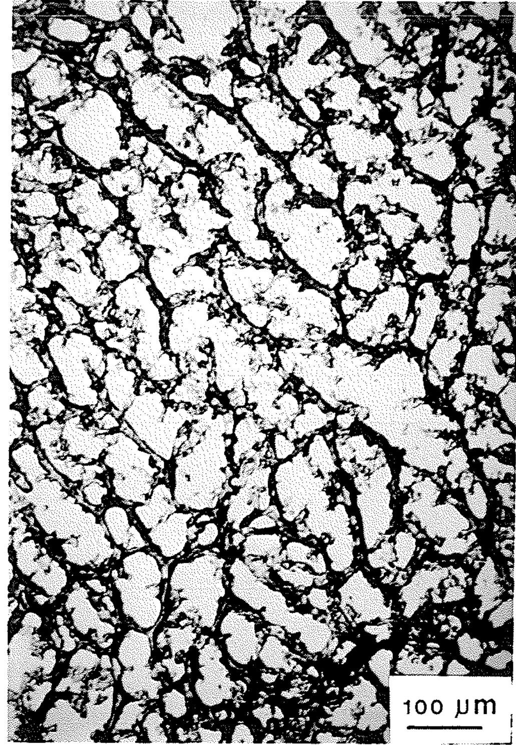
C. 12.5% protein

D. 15% protein

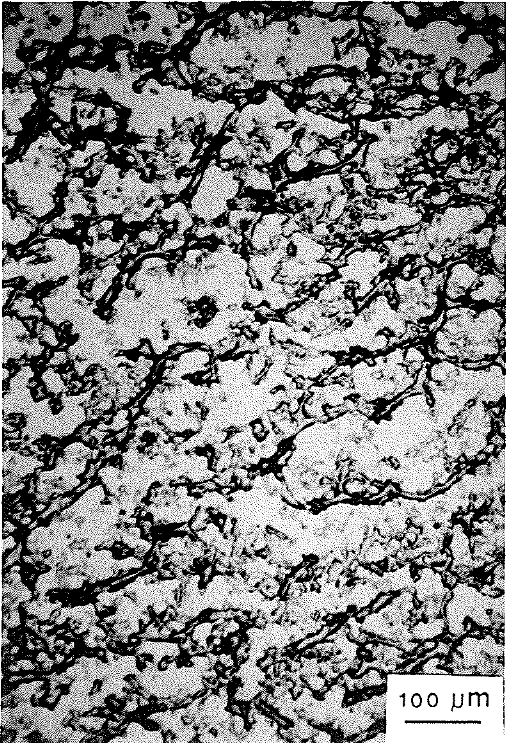
A



B



C



D

$G' = 2.17\ Conc^{2.8}$; R = 0.9933), though the correlation with the second order polynomial was higher. Interestingly, the concentration dependence was greater for vicilin than ovalbumin so that the G moduli at 15% protein were almost twice as high as those obtained with ovalbumin (Appendix 1B).

Tan δ values for the vicilin networks were described as two significantly different groups (Fig. 6B; Appendix 1B). At concentrations of 5 and 7.5%, the tan δ value was 0.17, but dropped to 0.14 or less at higher protein concentrations. This situation was comparable to that for ovalbumin and indicated the CPC for network formation for vicilin was between 7.5 and 10%. This observation was supported by the microscopic evaluation (Fig. 8). Below a concentration of 10%, the microstructure appeared as a sheet like mass with no indication of a rigid structure. In fact, at 5% protein, the product was so fluid it was impossible to section for microscopic analysis. At concentration of 10% and above, a well structured network was visible, the intensity of which increased with increasing concentration.

Protein Concentration in Relation to Network Characteristics

An increase in the G' modulus with increasing protein concentration was expected based on previous literature (Beveridge et al., 1984; Bikbov et al., 1985a; van Kleef, 1986). The relationships between these parameters, for ovalbumin and vicilin in this study, were similar to the power law relationship reported for ovalbumin by van Kleef (1986). The higher correlation obtained with the second order polynomial did not preclude the use of the power law. The nature of these relationships was in contrast to those reported previously for

Figure 8. Photomicrographs showing the effect of protein concentration on heat induced networks for vicilin in 0.15 M NaCl, pH 8.5.

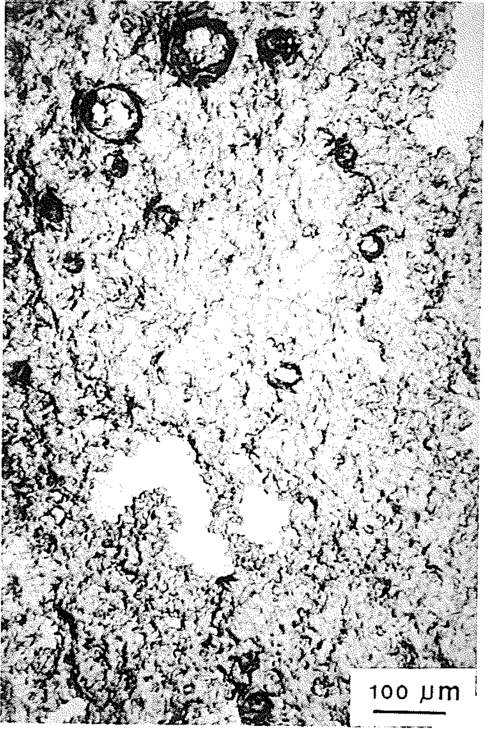
A. 7.5% protein

B. 10% protein

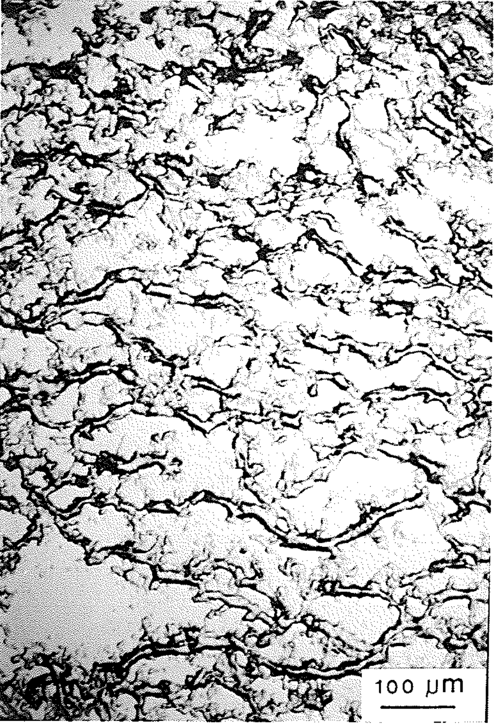
C. 12.5% protein

D. 15% protein

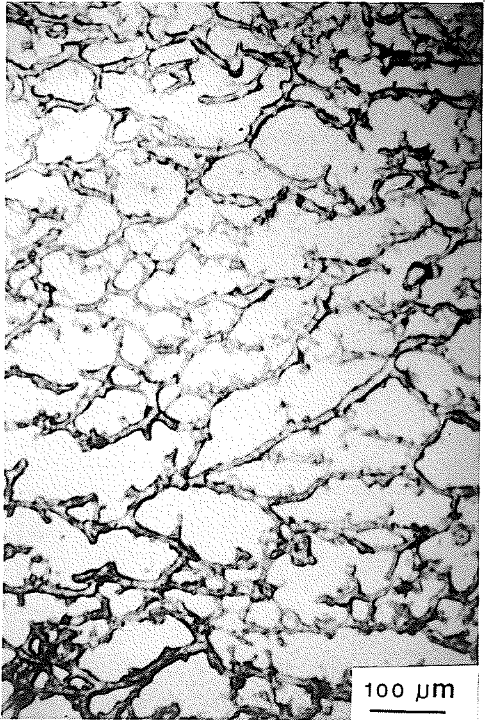
A



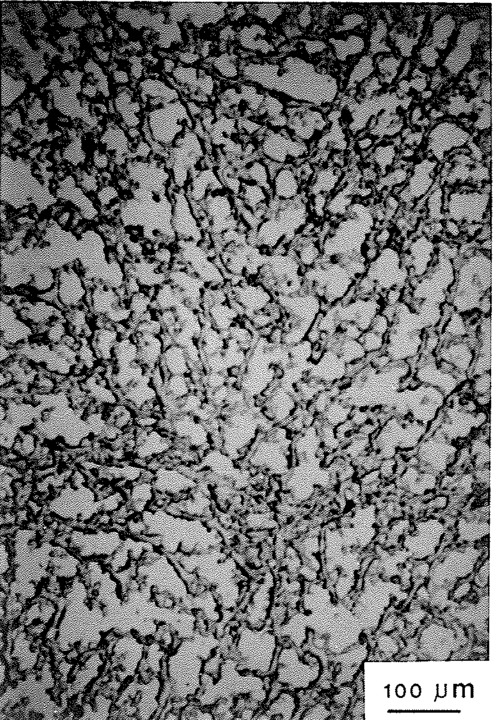
B



C



D



gelatin, BSA, agar and soy protein (Clark and Lee-Tuffnell, 1986), in which the G moduli increased rapidly near the CPC and varied more slowly thereafter. In that situation, a CPC was determined by extrapolation of the curve to a zero G value. These values were then used to describe the data in terms of a master dependence of the extent of network conversion on concentration (Clark and Lee-Tuffnell, 1986). The origins of this theory were based on weakly interacting systems and although this approach has been applied successfully to the studies on gelatin, BSA, agar and soy protein, the G moduli in this study could not be described in terms of this theory. In fact, changes in the G curves with concentration could not even be used to identify a CPC for network formation.

The increase in G′ modulus has been attributed to increased crosslinking within the network. While this is probably true in this investigation as well, the impact of increased crosslinking on network structure cannot be realized without also considering the impact on the G" modulus. Increases in G" appear to reflect increased protein-protein interactions without formation of an elastic structure. As the G" modulus increased in a similar fashion to the G′ modulus, the overall increase in G′ did not necessarily increase the relative elasticity of the resulting network. As a result, the type of structure that was obtained was best defined by the tan δ value, which reflected both the G′ and G" contributions. From the data obtained, it was apparent that the tan δ value could be used to determine the CPC for network formation. Below the CPC, higher tan δ values were obtained; once the CPC had been exceeded, the tan δ value was independent of concentration.

Based on the variations in tan δ, the CPC of network formation with ovalbumin (between 5 and 7.5%) was found to be slightly lower than for vicilin (between 7.5 and 10%). In all other respects, the influence of protein concentration on rheological and microstructural properties was similar for both ovalbumin and vicilin, proteins from two very different sources.

The complimentation between rheological and microstructure data in this investigation was most interesting. Low tan δ values, indicative of strong elastic networks, were obtained for conditions where the microstructure was well crosslinked for both ovalbumin and vicilin (Figs. 7 and 8). The slightly higher tan δ value corresponded to a system with a more open matrix and not as well crosslinked (5% ovalbumin - Fig. 7A) or an unstructured mass (7.5% vicilin - Fig. 8A). The impact of increased G moduli was also evident in the photomicrographs. For a network whose basic structure remained the same, an increase in degree of interaction (increased G moduli) was reflected in a visible increase in network intensity. Based on the critical concentrations estimated at this stage, a concentration of 10% protein was chosen for both ovalbumin and vicilin to be used in all further investigations. At this concentration, networks can form if conditions are appropriate yet network intensity should be low enough to detect structural differences with light microscopy.

Charge

The charge on a protein in a given environment determines the potential for electrostatic interactions. As proteins are amphoteric molecules, under most conditions they will contain both positive and

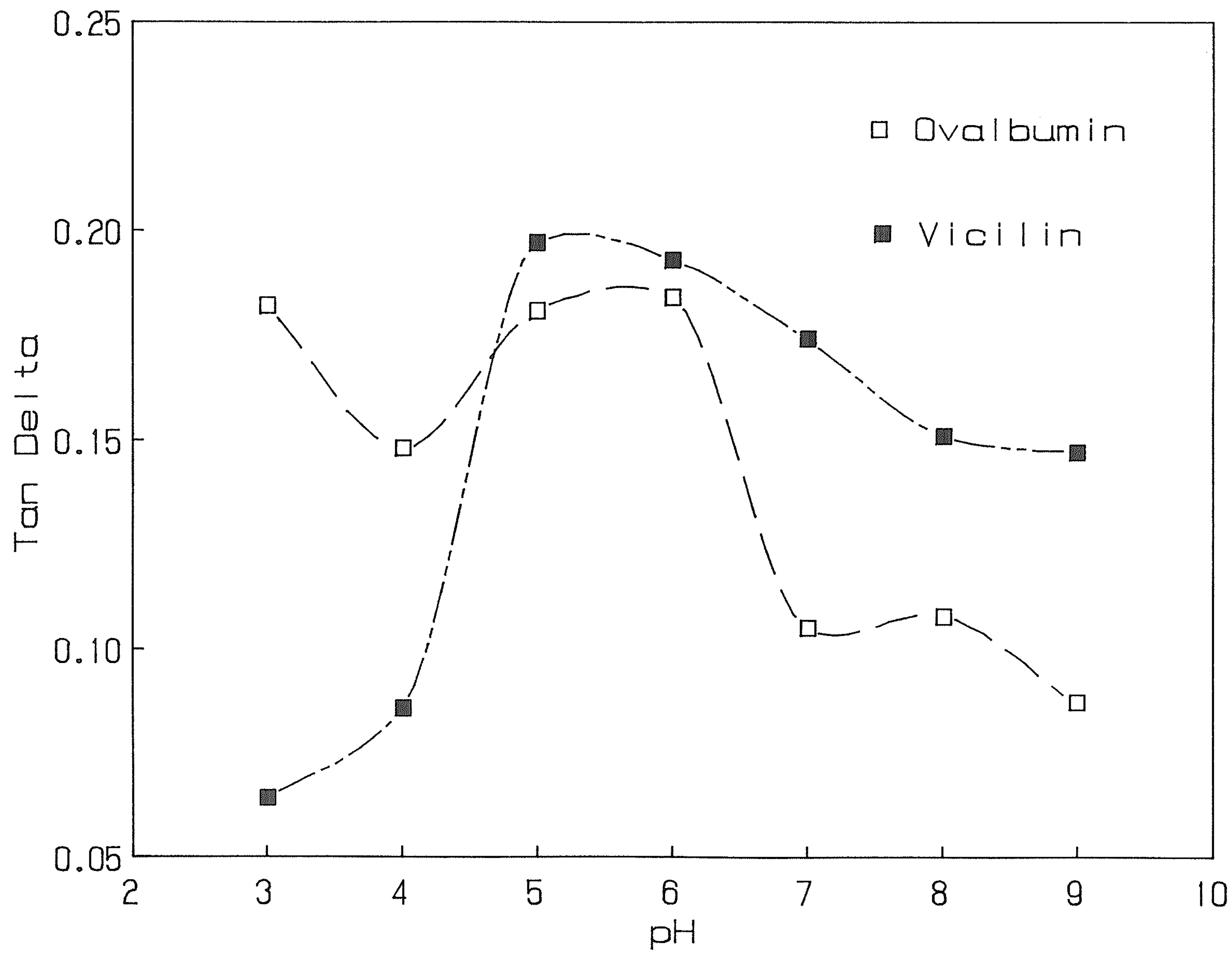
negative charges. It is the relative proportions and distribution of these charges that will impact the balance between attractive and repulsive forces required for network formation. In order to investigate the role of electrostatic interactions in heat induced network formation with ovalbumin and vicilin, two approaches were used. The first involved pH manipulation, based on the concept that there is no net charge at the isoionic point (or from a more practical approach the isoelectric point - IEP) and as the pH is moved from this point there will be an increase in the net positive (below IEP) or net negative (above IEP) charge. The second approach was to include solutes which interact with the protein and in so doing change the net charge. In this respect, SDS was included to increase the net negative charge. Calcium chloride has been shown to decrease protein stability through the binding of the calcium ion (Hegg et al., 1979; Arntfield et al., 1986) and thus was included to increase the net positive charge.

pH

Variations in pH influence the charge on the protein as well as its overall conformation. Conformational changes can be monitored using DSC, where reduced ΔH values are indicative of protein unfolding due to the environment rather than the heat treatment. In terms of the network structure resulting from heat denaturation, there may be a conformational influence on the globular structure that associates to form the network subsequent to the heat treatment. As a result, DSC data should not be overlooked when interpreting pH effects.

Ovalbumin: The tan δ values associated with heat induced ovalbumin networks showed a distinct pH effect (Fig. 9). At pH values of 3, 5 and

Figure 9. Effect of pH on the tan delta values for heat induced protein networks for 10% ovalbumin and 10% vicilin in 0.15 M NaCl.


0.25
0.20
0.15
0.10
0.05
Tan Delta
2
3
4
5
6
7
8
9
10
pH
Ovalbumin
Vicilin

6, tan δ values were high (>0.18). They decreased slightly at pH 4 and decreases in the alkaline pH region were even greater. Statistical differences are given in Appendix 2A.

Changes in G′ exhibited a distinct bimodal characteristic with low values at pH 5 and 6, around the isoelectric point (IEP = 4.6-4.9, Hegg et al., 1979), higher values as the pH was shifted away from the IEP and lower again at extremes of pH (Fig. 10A). A similar trend was observed for G"; however, differences were not significant due to the low values and high variability (Appendix 2A).

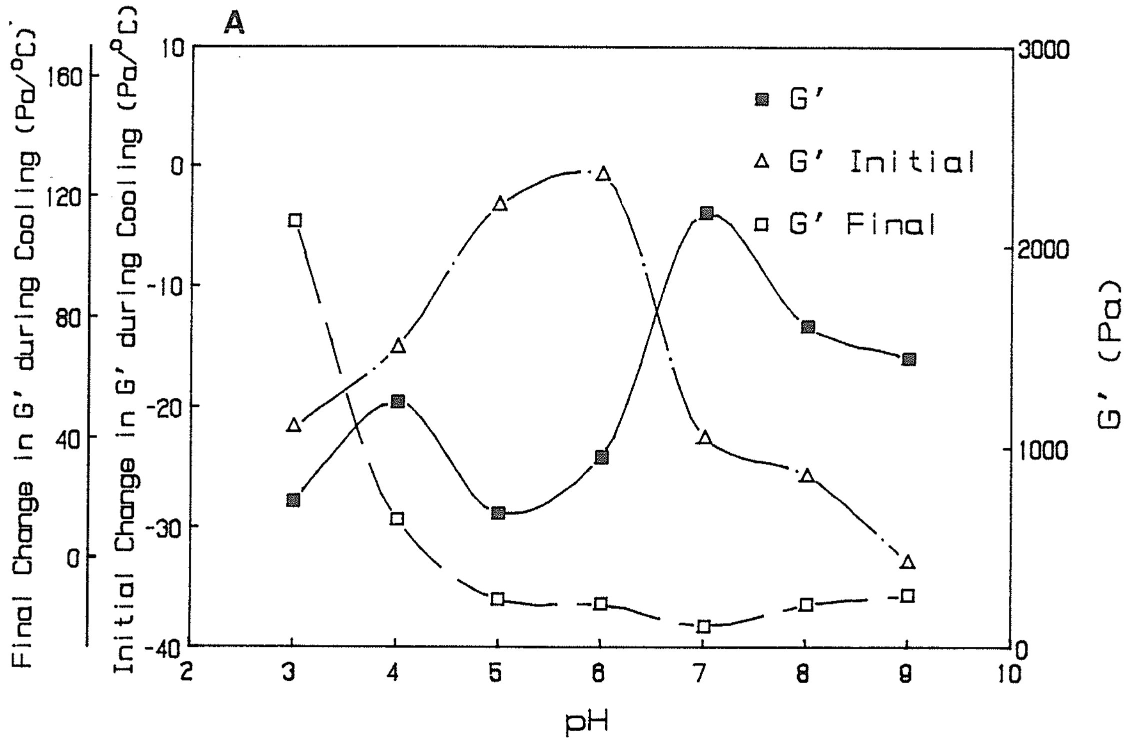
Differences in rheological characteristics were reflected in the microstructure (Fig. 11). The aggregated structure at pH 5 was indicative of a network lacking the crosslinking required for improved elasticity and thus accounted for the lower G′ and higher tan δ values. A similar network was observed at pH 6. The structure at pH 4 was highly aggregated though there was some evidence of alignment into strands which may have been responsible for the improved rheological characteristics. At pH 3, there was evidence of strand like structures; however, they were not well crosslinked. It is difficult to explain the better rheological characteristics for pH 4 compared to pH 3 based on the microstructure. It is possible that although these values were significantly different, these subtle changes at the higher tan δ values could not be detected as microstructural differences. Well crosslinked networks were seen in the alkaline pH regions where low tan δ values were obtained.

These results reflected the influence of charge on network structure rather than conformational changes. At pH values of 5 and 6,

Figure 10. Effect of pH on the rate of structure development (change in G') during the initial and final cooling phases in relation to the G' moduli for the resulting networks.

A. 10% ovalbumin in 0.15 M NaCl.

B. 10% vicilin in 0.15 M NaCl.


A
G'
G' Initial
G' Final
Final Change in G' during Cooling (Pa/°C)
Initial Change in G' during Cooling (Pa/°C)
G' (Pa)
pH
160
120
80
40
0
10
0
-10
-20
-30
-40
3000
2000
1000
0
2
3
4
5
6
7
8
9
10

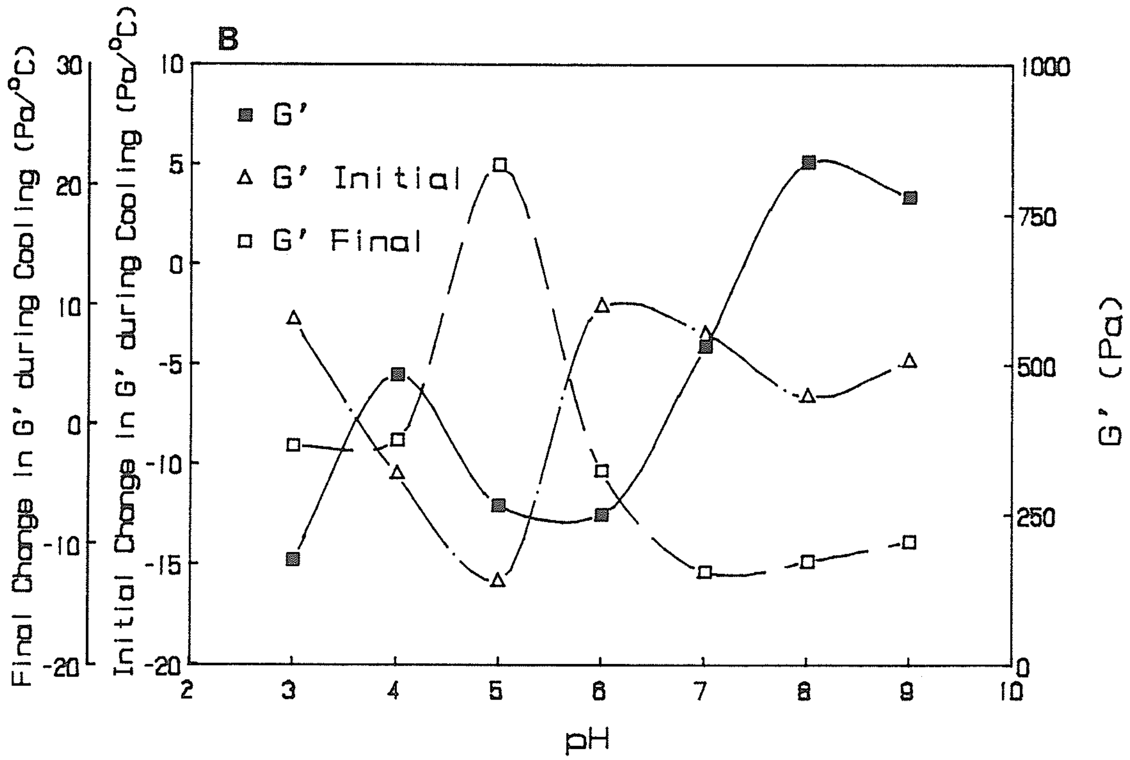

B
G'
G' Initial
G' Final
Final Change in G' during Cooling (Pa/°C)
Initial Change in G' during Cooling (Pa/°C)
G' (Pa)
pH
30
20
10
0
-10
-20
10
5
0
-5
-10
-15
-20
1000
750
500
250
0
2
3
4
5
6
7
8
9
10

Figure 11. Photomicrographs showing the effect of pH on heat induced networks for 10% ovalbumin in 0.15 M NaCl.

A. pH 3

B. pH 4

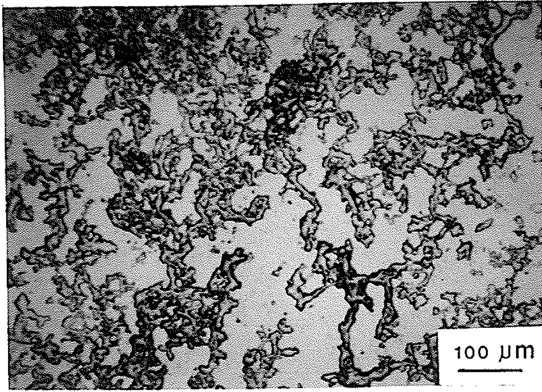
C. pH 5

D. pH 7

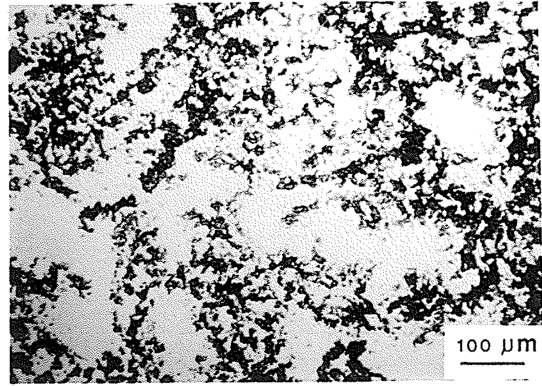
E. pH 8

F. pH 9

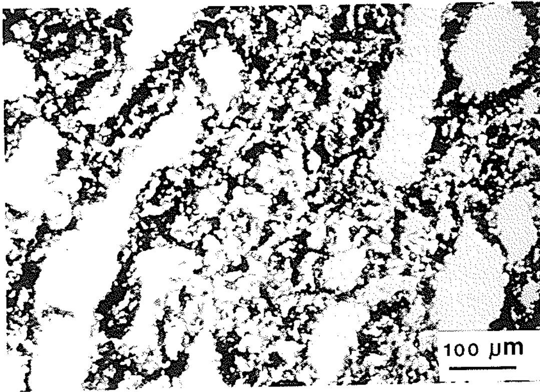
A



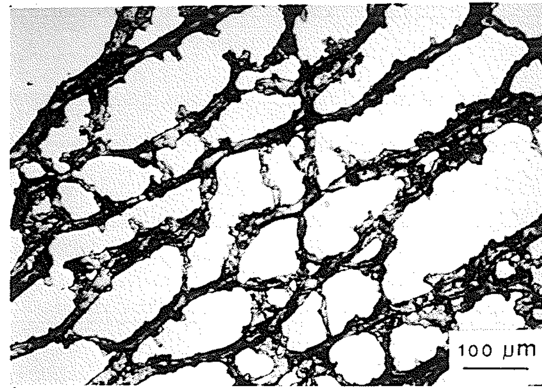
B



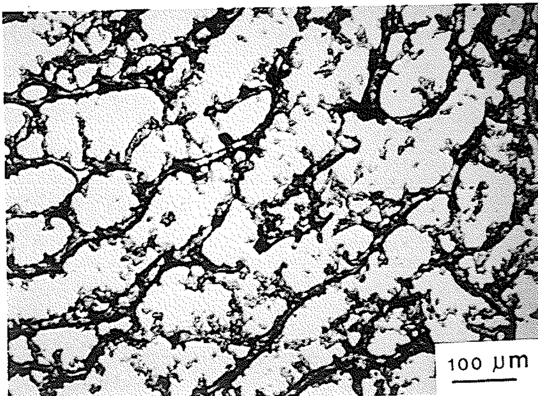
C



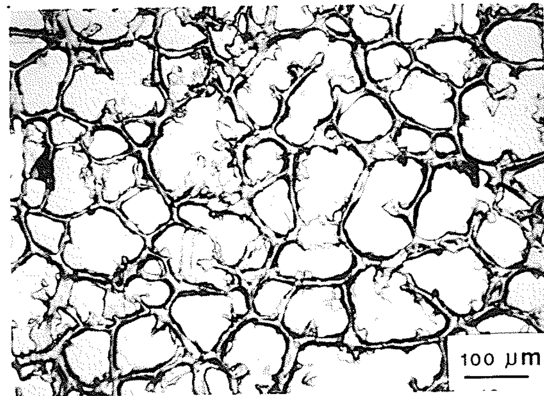
D



E



F



around the IEP for ovalbumin, the net charge is minimal. As a result attractive intermolecular electrostatic forces should be maximized. This lead to the formation of an aggregated product characterized by low G moduli and high tan δ values rather than a strong elastic network. Network characteristics were not greatly improved in acid conditions. At pH 4, there was an increase in the net positive charge of the protein resulting in slightly higher G' values and lower tan δ values than at the IEP. This improvement reflected the importance of repulsive positive electrostatic interactions in establishing the balance of forces necessary for improved network characteristics. The improvement, however, was not sufficient to alter the appearance of the network using light microscopy. At pH 3, this trend was reversed and rheological characteristics were poor, although there was some indication of protein strand formation. This reflected a shift in the attractive - repulsive balance past the optimum so that repulsive electrostatic forces dominated and network formation was inhibited. Interestingly, the response to acid conditions appeared to be dependent on charge variations only; significant denaturation of ovalbumin at both pH 3 (ΔH = 3.3 J/g protein) and pH 4 (ΔH = 6.9 J/g protein) compared to other pH values (mean ΔH for pH 5 to 9 = 16.0 J.g protein) did not seem to be a factor in establishing network characteristics (Appendix 16).

At alkaline pH values, a similar response was seen in terms of the G moduli (Fig. 10A). The high G' and G" moduli at pH 7 reflected conditions in which attractive and repulsive electrostatic forces were optimized for network formation. At higher pH values, the increase in

net negative charge was sufficient to reduce the extent of interaction and hence lower G moduli resulted. This bimodal trend in terms of pH response was similar to that observed for gel rigidity measurements on ovalbumin (Egelandsdal, 1980). These results, however, do not concur with those of van Kleef (1986) where the maximum G′ modulus for ovalbumin in salt was obtained at pH 5. The possibility of sample compression during loading, causing the aggregated material to behave in a more rigid fashion, may account for the high G′ modulus at pH 5 in the work of van Kleef (1986). The changes in the G′ modulus in the alkaline range were not reflected in the tan δ or microstructural data where similar structures were indicated at pH values of 7, 8 and nine. It is possible that the range for the attractive - repulsive balance necessary for a three dimensional network was greater at alkaline pH values, so that good networks were obtained throughout. Differences in the extent of the interactions were seen in the G moduli. In this respect, microstructural data more closely resembled the changes in tan δ; the variations in the G′ modulus at the higher pH levels could not be seen as intensity differences as was the case with varied protein concentration.

In order to examine the importance of the charge effect on both the establishment and stabilization of these ovalbumin networks, the rates of structure development (change in G′) during the initial and final cooling phases were compared to the G′ modulus for the resulting networks (Fig. 10A). A low rate of structure development at pH 5 and 6 during the initial cooling phase (values close to zero) indicated the charge profile was not appropriate for the establishment of a well

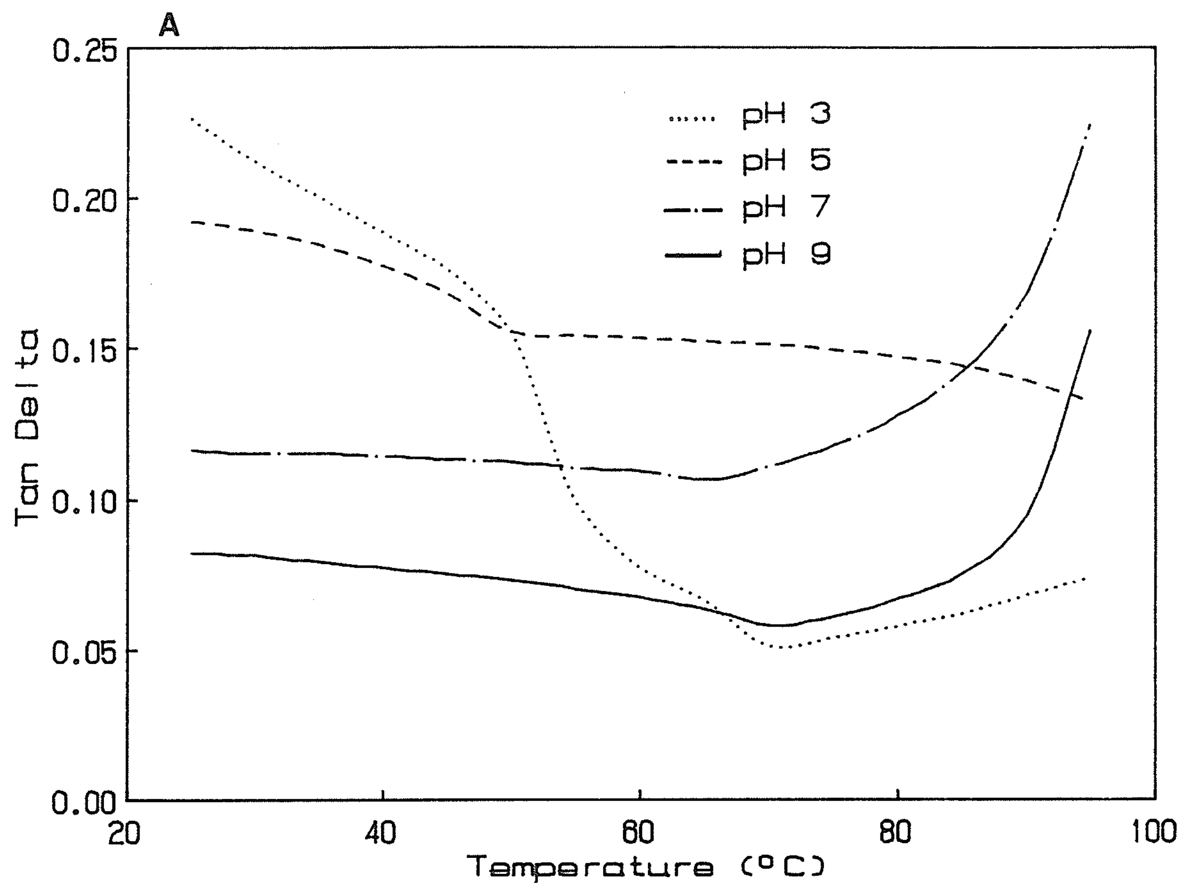
crosslinked network. As the pH was moved from the IEP, the rate of change in G' continued to improve but did not reflect the bimodal response for G' in the final product. In acidic conditions, this difference may have reflected changes during the final cooling phase. The positive values for the rate of change in G' at both pH 3 and pH 4 demonstrated a decrease in the extent of interaction during this phase. Exact values are given in Appendix 3A. The acidic conditions appeared to have disrupted the interactions established during heating and the initial cooling phase. It was therefore a combination of the rate of change during initial and final phases that was responsible for the network characteristics found at acidic pH values. This behavior in the alkaline range was not so apparent. The slight decrease in the rate of structure development during the final cooling phase at pH 8 and 9 was not significant (Appendix 3A), although it may have had some impact on the final structure. It was also possible that the differences in the alkaline region resulted during the heating phase and thus the rates of change in G' during cooling were not the determining factors.

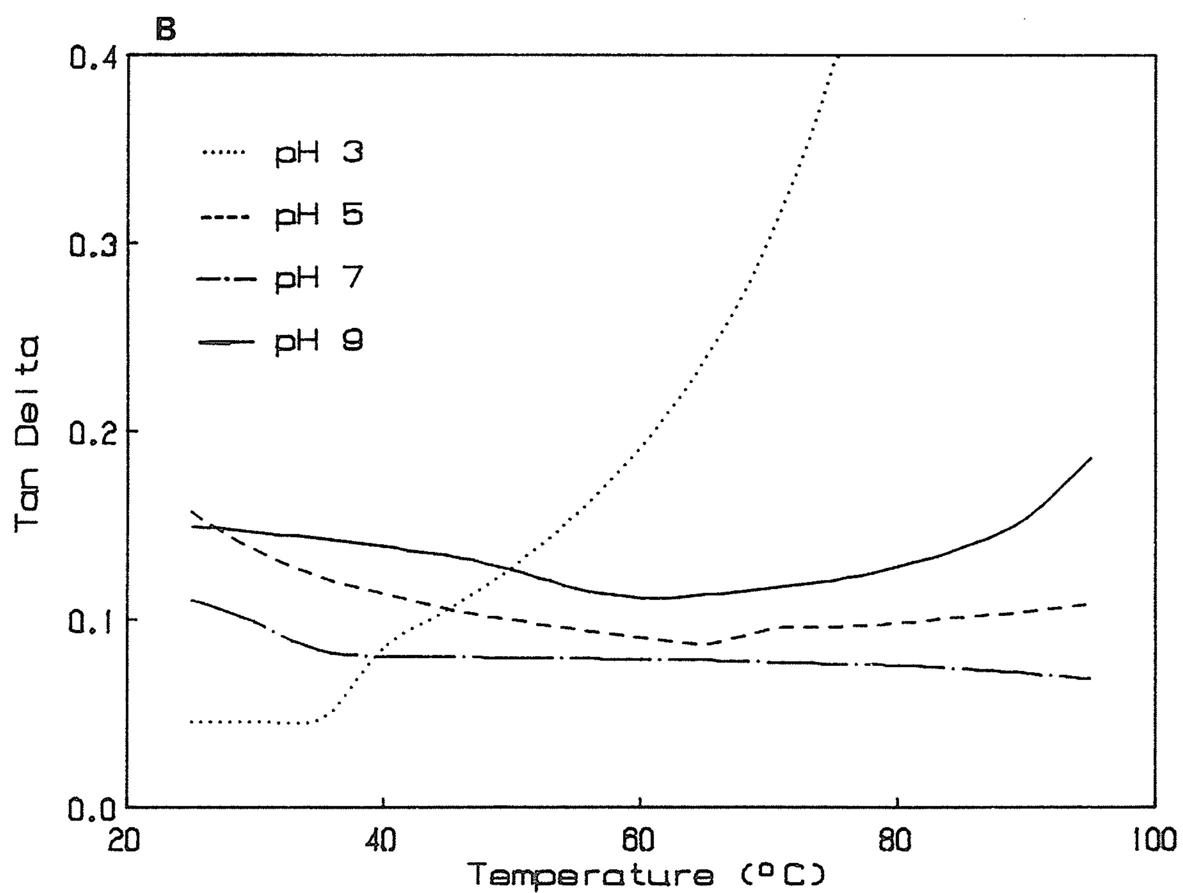
The influence of pH on the changes in structure during cooling were also reflected in the changes in tan δ as a function of temperature (Fig. 12A). At pH 5, there was very little change in the nature of the structure during cooling indicating the aggregated product had been established during the heating phase. This was, in part, due to the lower temperature associated with structure development during heating at pH 5 (Arntfield et al., 1989a). Similar curves, in which the only change in network type during cooling involved a slight reduction in the elastic component, were observed at

Figure 12. Influence of pH on structure development during cooling for heat induced protein networks. Tan delta curves were calculated using a biphasic linear model to describe changes in G' and G" for all curves except ovalbumin at pH 3 where a triphasic linear model was used.

A. 10% ovalbumin in 0.15 M NaCl.

B. 10% vicilin in 0.15 M NaCl.


A
0.25
0.20
0.15
0.10
0.05
0.00
Tan Delta
pH 3
pH 5
pH 7
pH 9
20
40
60
80
100
Temperature (°C)


B
0.4
0.3
0.2
0.1
0.0
Tan Delta
pH 3
pH 5
pH 7
pH 9
20
40
60
80
100
Temperature (°C)

pH 4 and pH 6 (not shown as there would have been too many lines for one figure). At pH 3, tan δ values were low at the onset of cooling due to structure development during heating. However, in this situation, the influence of an acidic medium resulted in disruption of this structure so that after cooling, tan δ values were high. This may explain why protein strands were evident in the microstructure yet rheological data were poor. The tan δ curves at pH 7, 8 (not shown) and 9 were essentially the same, only values at pH 9 were lower throughout the curve. These observations supported the concept that differences in network structure due to pH variations in the alkaline range probably occurred during heating, where the network was first established.

Vicilin: The influence of pH on vicilin networks was somewhat different than for ovalbumin. Tan δ values were again highest around the IEP (IEP for vicilin = 5.0, Ismond, 1984) but were considerably lower in the acid pH range and decreased only slightly at alkaline pH values (Fig. 9). A similar decrease in tan δ with increasing pH in the alkaline range has also been observed for canola, where the network went from an opaque to a transparent gel (Paulson and Tung, 1989). The tan δ values at pH 3 and 4 were lower than those for ovalbumin at any pH. The G' modulus, on the other hand, exhibited a bimodal response similar to that seen for ovalbumin, with G' maxima at pH 4 and 8 (Fig. 10B). A similar response was obtained for G" modulus, and unlike ovalbumin, the G" moduli at pH 7, 8 and 9 were significantly higher (Appendix 2B).

For most pH values, the variations in tan δ were apparent in the microstructure (Fig. 13). The structure at pH 5 (and also 6 though not

Figure 13. Photomicrographs showing the effect of pH on heat induced networks for 10% vicilin in 0.15 M NaCl.

A. pH 3

B. pH 4

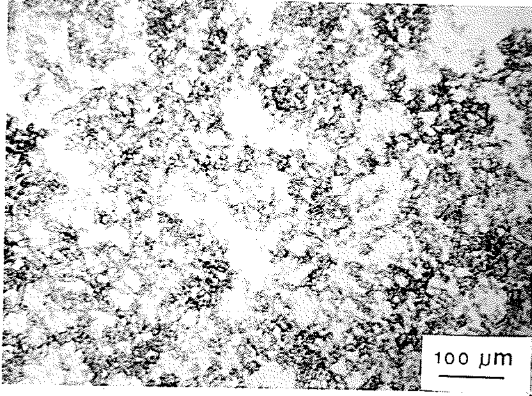
C. pH 5

D. pH 7

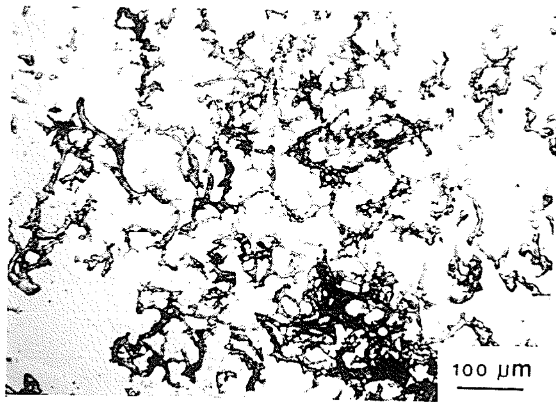
E. pH 8

F. pH 9

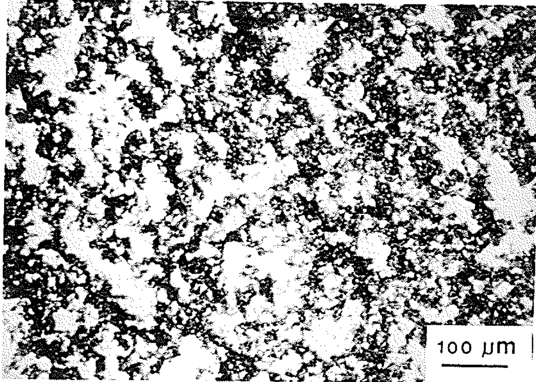
A



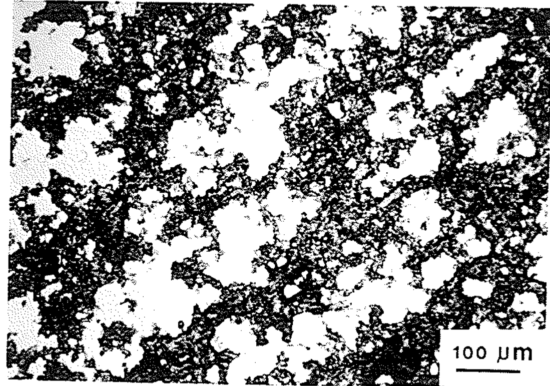
B



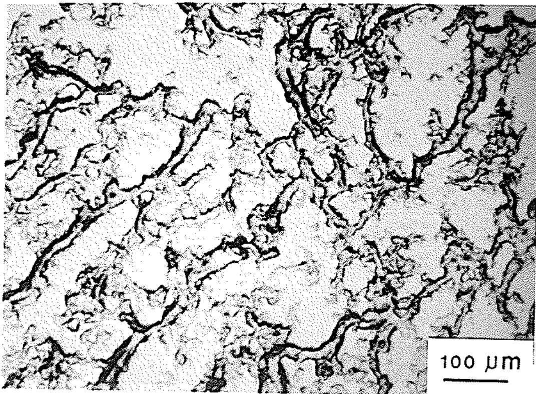
C



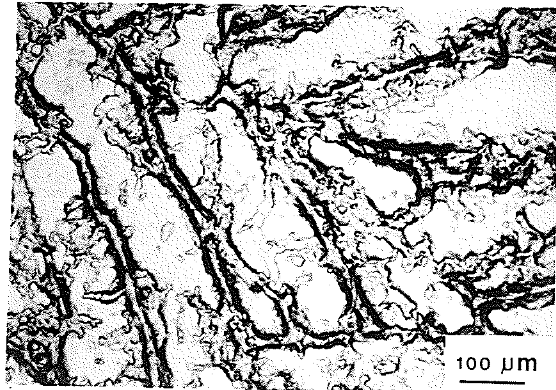
D



E



F



shown) was characterized by small aggregates as was seen with ovalbumin. At pH 4, the appearance of small, rather poorly connected strands indicated some progress toward network formation. The microstructure at pH 3 was difficult to interpret considering the rheological data. The low tan δ and low G moduli were indicative or a very open yet well structured network. The short strands and aggregated masses obtained did not support this concept. It should be noted, however, that the network formed at pH 3 was very weak and difficult to handle when sectioning for microstructural analysis. The possibility of network distortion or collapse during sectioning could not be discounted. Indications of a crosslinked network could be seen at pH 7; however, the protein strands were more clearly defined at pH 8 and 9, reflecting the decreases observed for tan δ values. Even these networks, however, were not as well crosslinked as those obtained for ovalbumin, a phenomenon which was also apparent in the rheological data.

The bimodal response to pH for vicilin can be explained in terms of the influence of charge on the degree on interaction. At pH values sufficiently distant from the IEP, the repulsive electrostatic forces (either predominantly positive or negative) were most appropriate for attaining the balance of forces necessary for the formation of elastic protein networks. As the pH was moved further from the IEP, repulsive forces became dominant such that the degree of interaction decreased. This response was similar to that for ovalbumin. In an investigation of pH effects on rapeseed proteins, it was shown that G' for heat induced gels was lower at pH 10 than pH 6 (Gill and Tung, 1978); the high G'

modulus at pH 6 was attributed to the elasticity of the aggregates at this pH value. This result would not disagree with the current observations if the repulsive forces at pH 10 were sufficient to lower the G' modulus below that at pH 6. In the work for Paulson and Tung (1989), unmodified canola protein could not form networks at pH values below 9.5. For succinylated (5.2% succinic anhydride) canola protein in 0.35 M or 0.7 M NaCl, maximum G' moduli were observed at pH 6.5, although the values at pH 5 were higher than those in the more alkaline environment. At a higher degree of succinylation, this maximum was shifted to pH 5.0. These results supported the work in the current investigation in that a maximum G' was obtained at a pH removed from the IEP; but they also supported the high G' values seen by Gill and Tung (1978) at pH values closer to the IEP. The possibility of sample compression during loading being responsible for the high modulus at these pH values for both these investigations (Gill and Tung, 1978; Paulson and Tung, 1989), as was suggested for the ovalbumin data of van Kleef (1986), should not be discounted.

Based on the fact that this bimodal response was not observed for the tan δ data, it is possible that conformational changes in the protein may have contributed to the type of network formed. In this respect, ΔH values at pH 9 (7.4 J/g protein), pH 4 (5.0 J/g protein) and pH 3 (no endotherm) were significantly reduced compared to other pH values (mean for all other pH values = 13.2 J/g protein), indicating a certain degree of unfolding in these environments (Appendix 16). With vicilin, interactions between these unfolded molecules seemed to give a more elastic network than at other pH values. This was particularly

true at pH 3, where extensive protein denaturation may have produced a network in which the interaction of unfolded polypeptide chains rather than globular structures may have been responsible for network formation. This would represent a system such as that described by Ferry's (1948) original theory on network formation.

As was the case with ovalbumin, the G' modulus in the final network resulted from contributions during both the initial and final cooling phases; however, the relationship was complex (Fig. 10B, Appendix 3B). At pH 5, for example, the rate of change during the initial phase of cooling was high. During the final cooling phase, there was a positive correlation between temperature and the G' modulus, indicative of structural breakdown. This combination was responsible for the low final G' modulus. At pH 6, on the other hand, the initial change in G' was poor but structure development during the final stage was improved, such that in the final product, the G' modulus was comparable to the that at pH 5. The high G moduli in the alkaline pH region seemed to be associated with a relatively high rate of structure development during the final cooling phase. The complexity of this relationship may be related to the compounded effect of altering both conformation and charge characteristics by pH manipulation.

Examination of the variations in tan δ as a function of temperature did little to clarify this complex relationship (Fig. 12B). For pH 5 and 7, networks appeared to have been established during heating and any changes during cooling were detrimental to the established network. The curve for pH 9 was similar except there was

evidence of structure improvement at the onset of cooling. Changes in tan δ for pH 3 were rather interesting. As this pH environment had resulted in protein unfolding, there was significant structure present prior to the heat treatment as indicated by the high G' modulus (Arntfield et al., 1989a). The rheological characteristics of this structure did not change substantially during the heat treatment. This structure, however was characterized by a high viscous component; hence tan δ values were high at the onset of cooling. The rearrangement into a more elastic structure occurred throughout most of the cooling phase and only at temperatures below 40°C did the tan δ values level off. In this respect, the behavior responsible for network formation at pH 3 was quite different from that at other pH values. Although not presented, curves at pH 4, 6 and 8 were comparable to those at pH 5, 7 and 9 except that at pH 8 there was indication of some structure establishment similar to that seen in the concentration study.

Sodium Dodecylsulfate (SDS)

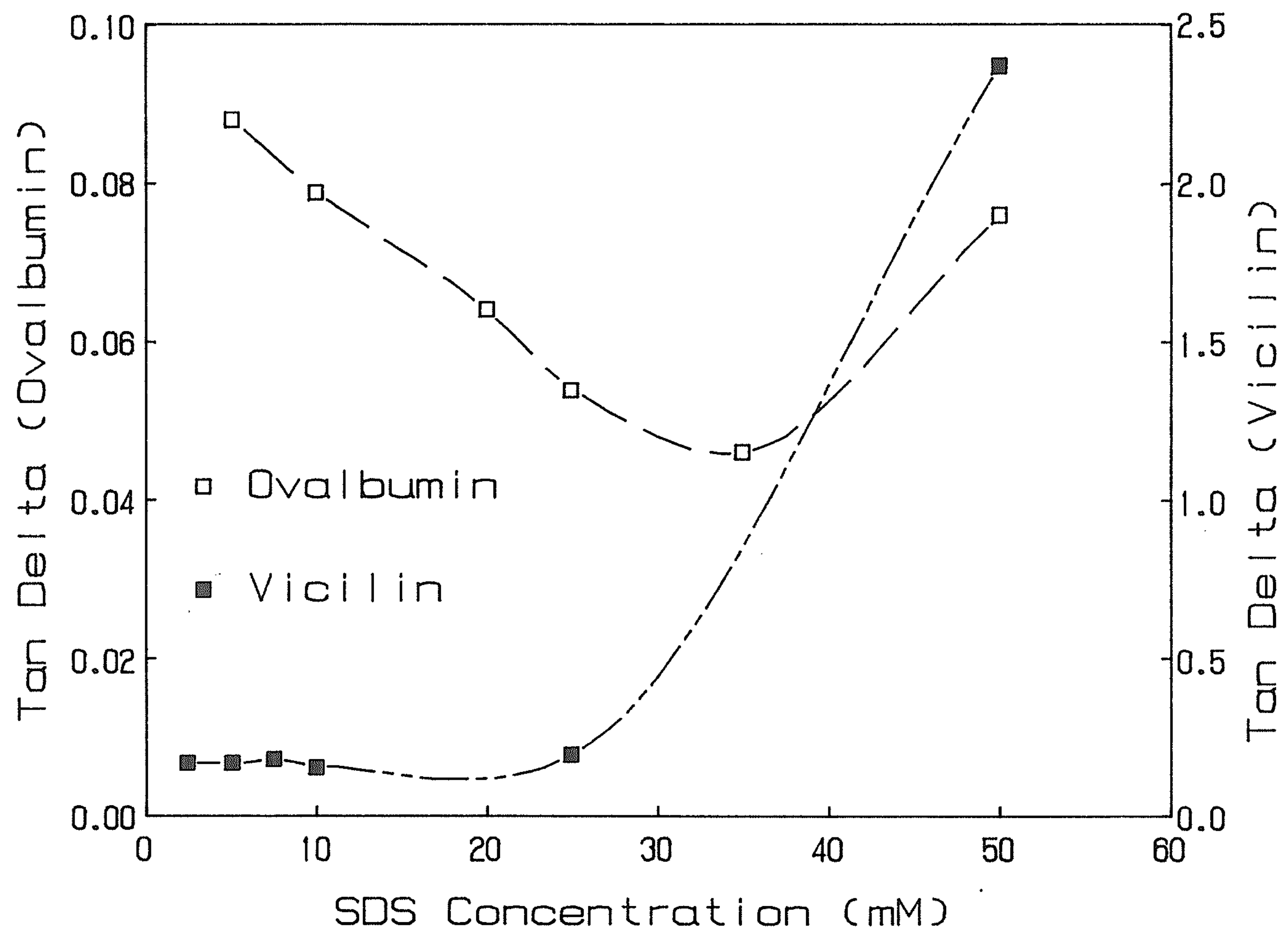
The inclusion of SDS when preparing heat induced protein networks is of both theoretical and practical significance. The systematic increase in the net negative charge should further our understanding of the importance of electrostatic interactions in determining network characteristics. On a more practical level, the inclusion of low levels of SDS has been shown to stabilize both animal and plant proteins and thus protect them from precipitation (Hegg and Löfqvist, 1974; Hegg et al., 1978, 1979; Lakshmi and Nandi, 1979; Harwalkar and Ma, 1987; Arntfield et al., 1989b). In fact, SDS is commonly included in dried egg albumen to improve whipping characteristics (Bergquist, 1977). A

similar improvement in network characteristics could be of economic importance. A pH value of 8.5 has been used throughout as both ovalbumin and vicilin have been shown to form reasonable networks at this pH; this should facilitate detection of changes due to the added solute.

Ovalbumin: The addition of SDS up to a concentration of 35 mM resulted in a gradual decrease in the tan δ values for ovalbumin (Fig. 14; Appendix 4A). At 50 mM SDS, this trend was reversed and the tan δ value increased. Within this concentration range, however, all tan δ values were less than 0.1, indicative of good elastic networks. Furthermore, the microstructure for these networks tended to support this observation (Fig. 15). At low SDS levels (5 mM), the stranded network was interspersed with small sections of aggregated material. As the concentration increased, the intensity of the network increased (10 mM), due to increased crosslinking, and the aggregated portions disappeared (25 mM). Structures similar to those shown for 25 mM were obtained at 20 and 35 mM SDS. The increase in tan δ at 50 mM SDS corresponded to a thickening of the strands in the protein network. The net increase in negative charge may have increased protein solubility and the thickened strands may simply represent highly hydrated protein. The high tan δ values, resulting from a relatively high viscous component in the network, could also be rationalized in terms of this protein hydration.

The improvement in network structure was not demonstrated in the G moduli and there was a gradual decrease in both G' and G" with increasing concentration (Fig. 16A; Appendix 4A). In consideration of

Figure 14. Effect of sodium dodecylsulfate on the tan delta values for heat induced protein networks from 10% ovalbumin and 10% vicilin in 0.15 M NaCl, pH 8.5.



Tan Delta (Ovalbumin)
Tan Delta (Vicilin)
SDS Concentration (mM)
Ovalbumin
Vicilin
0.00
0.02
0.04
0.06
0.08
0.10
0.0
0.5
1.0
1.5
2.0
2.5
0
10
20
30
40
50
60

Figure 15. Photomicrographs showing the effect of sodium dodecylsulfate on heat induced networks for 10% ovalbumin in 0.15 M NaCl, pH 8.5.

A. 5 mM

B. 10 mM

C. 25 mM

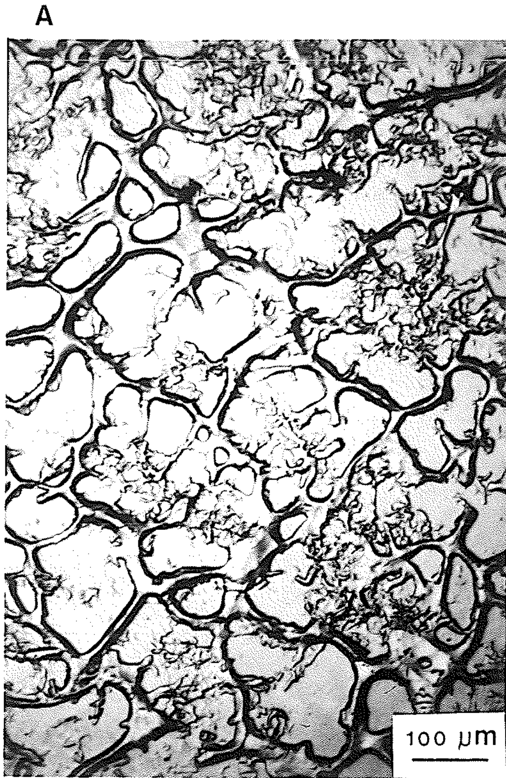
D. 50 mM

Figure 16. Effect of sodium dodecylsulfate on the rate of structure development (change in G') during the initial and final cooling phases in relation to the G' moduli for the resulting networks. Curves represent the second order polynomial relationship for each data set.

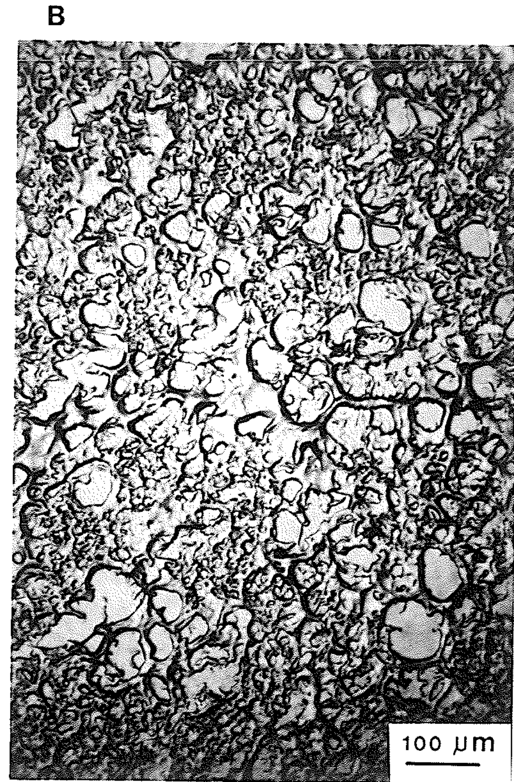
A. 10% ovalbumin in 0.15 M NaCl, pH 8.5.

B. 10% vicilin in 0.15 M NaCl, pH 8.5.

A

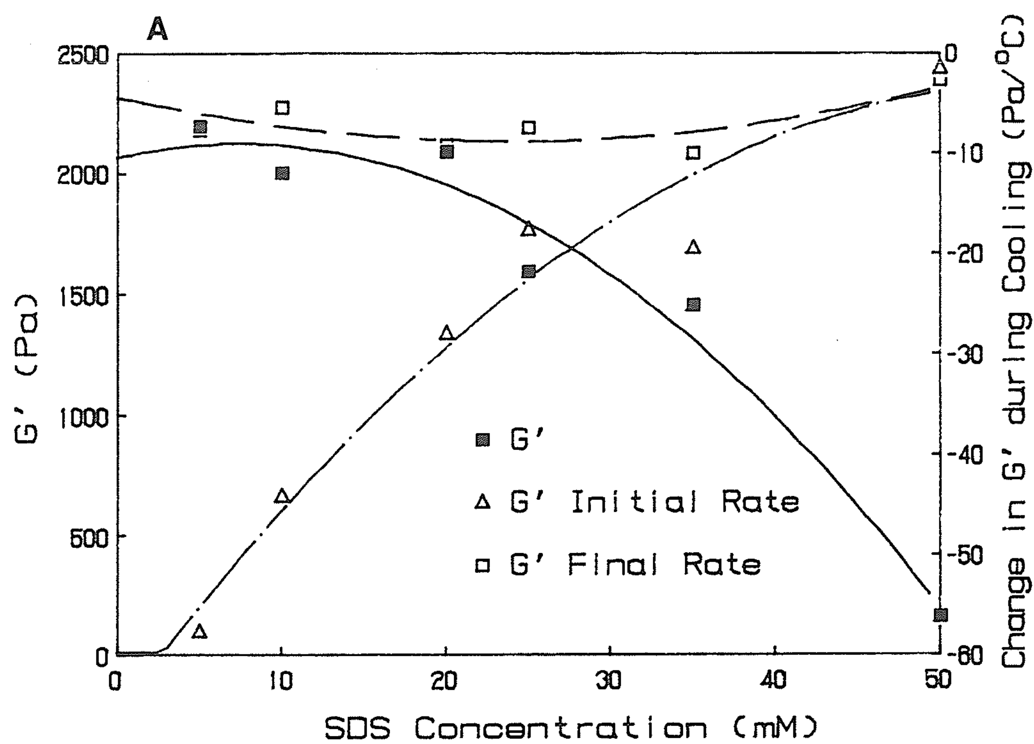


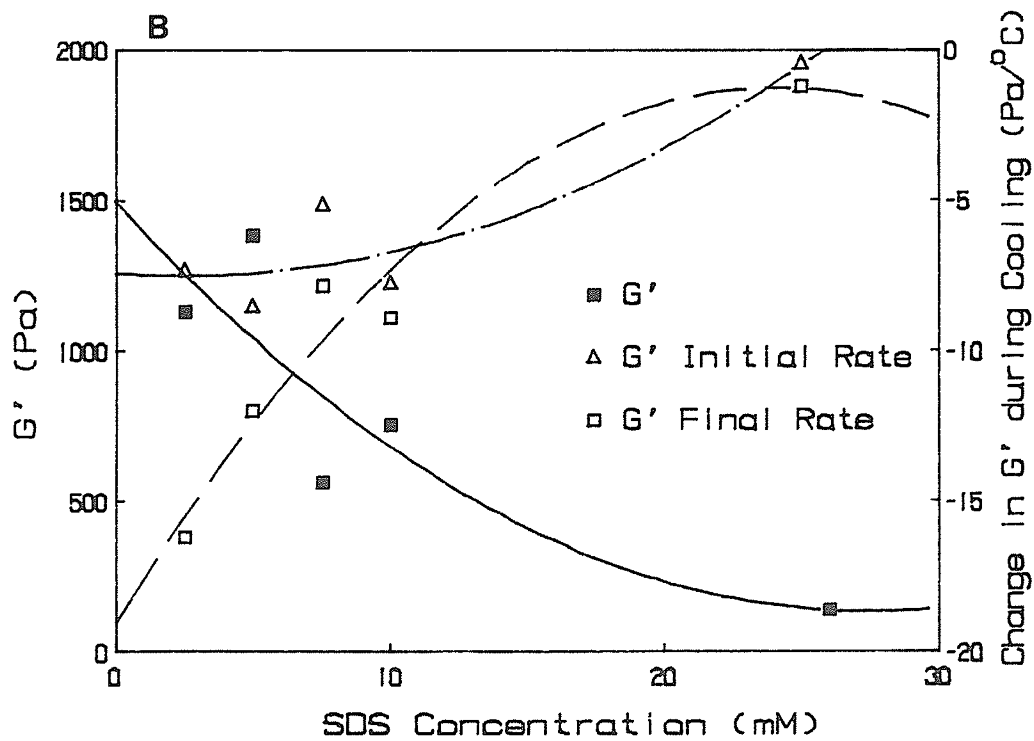
B



C

D


A
G' (Pa)
Change in G' during Cooling (Pa/°C)
SDS Concentration (mM)
G'
G' Initial Rate
G' Final Rate


B
G' (Pa)
Change in G' during Cooling (Pa/°C)
SDS Concentration (mM)
G'
G' Initial Rate
G' Final Rate

the use of pH 8.5 for this study, the optimum pH for a balance between attractive and repulsive electrostatic interactions (pH 7) had been past and there was an excess of net negative charge prior to the inclusion of SDS. Further increases in the net negative charge through SDS incorporation decreased the degree of interaction in a situation analogous to a further pH increase. It should by noted, however, that the addition of SDS also caused significant protein denaturation as evidenced by decreases in both Td and ΔH (Appendix 17). Unlike the situation at acidic pH values, the existence of a partially unfolded protein supported the formation of a highly elastic network (lower tan δ values) despite the overall decrease in the degree of interaction. It has also been suggested that stabilization at low SDS concentrations can be attributed to the formation of a bridge between a positively charged group and a hydrophobic region (Hegg and Lofqvist, 1974). It is conceivable that a similar bridge serves as a crosslink during network formation. This could account for the improved network structure despite the reduction in the G moduli.

The G′ moduli at different SDS concentrations were associated with changes in the rate of structure development during the initial cooling phase; the rate of change during the final cooling phase was independent of SDS concentration (Fig. 16A; Appendix 5A). As a result, the influence of SDS on the extent of interaction involved in producing an elastic network was determined primarily during network establishment. Electrostatic interactions as well as the bridges between positively charged groups and hydrophobic regions were established during initial network development. Factors responsible for

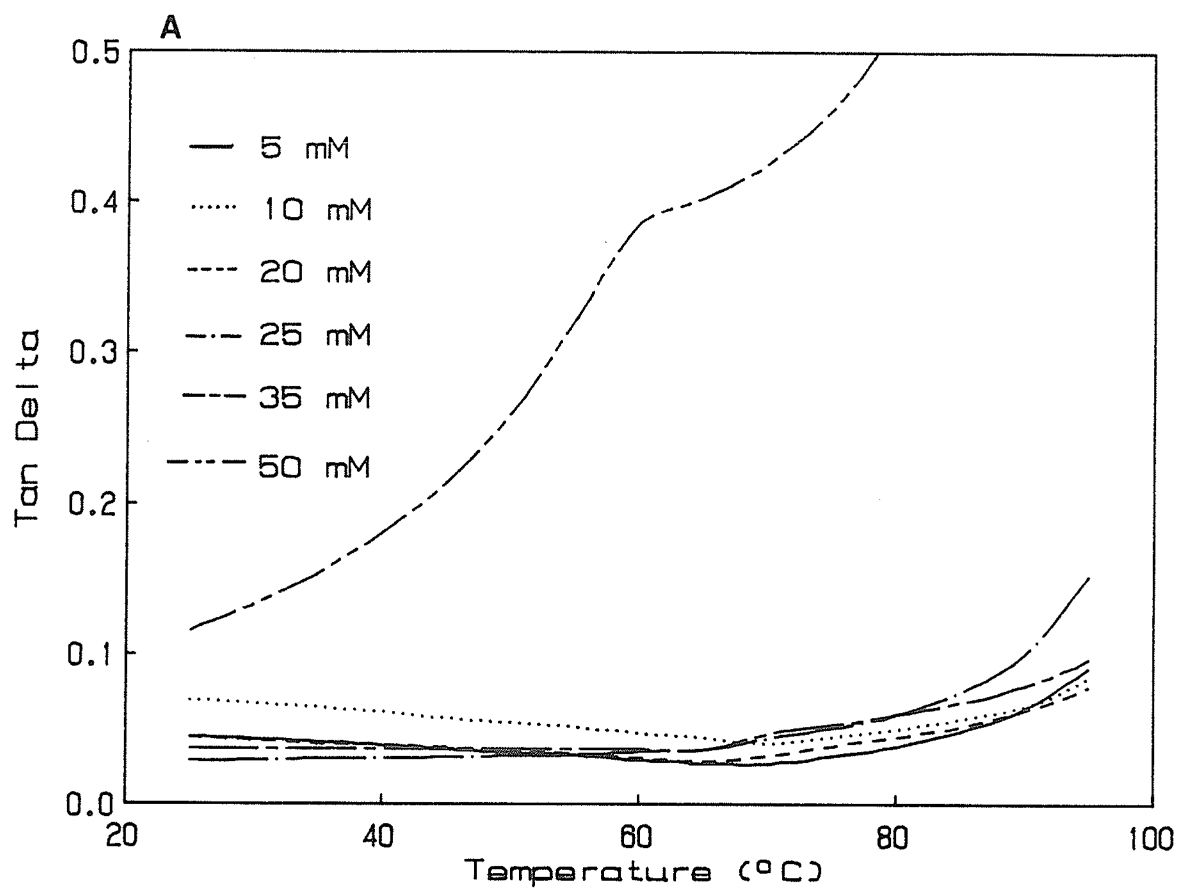
network stabilization did not respond to these variations in the SDS level. Changes in tan δ with temperature demonstrated the lack of variation in the network during cooling, up to a concentration of 35 mM SDS (Fig. 17A). At 50 mM SDS, the tan δ value was initially high and despite improvements during cooling, it did not reach values as low as those obtained at other concentrations. This behavior reflected the level of hydration of the protein seen in the microstructure and indicated that hydration may have occurred early in network formation process. Under these circumstances, both G' and G" were significantly reduced (Appendix 4A); however, the increase in tan δ values indicated that reduction was greater for the G' than the G" modulus.

Vicilin: The changes in heat induced networks for vicilin due to variations in SDS concentration were different from those observed for ovalbumin (Fig. 14; Appendix 4B). Tan δ values at low SDS concentrations ($\leq$ 10 mM) were not significantly different. Concentrations of 25 mM and 50 mM SDS resulted in increased tan δ values as was the case with ovalbumin in 50 mM SDS. Even at the lower concentrations, the tan δ values were all greater than 0.15 compared to values less than 0.1 for ovalbumin. Unlike ovalbumin, no evidence of protein denaturation was detected until 35 mM SDS (Appendix 17). The lack of well defined structure indicated by these high tan δ values were apparent in the microstructure (Fig. 18). At low SDS concentrations (2.5 and 5 mM), strands of protein were evident but they were not ordered into a good network. The excess of repulsive forces in these circumstances was not conducive to crosslinking. It is possible that conditions which promote protein unfolding may be necessary for

Figure 17. Influence of sodium dodecylsulfate on structure development during cooling for heat induced protein networks. Tan delta curves were calculated using a biphasic linear model to describe changes in G' and G".

A. 10% ovalbumin in 0.15 M NaCl, pH 8.5.

B. 10% vicilin in 0.15 M NaCl, pH 8.5.


A
0.5
0.4
0.3
0.2
0.1
0.0
Tan Delta
5 mM
10 mM
20 mM
25 mM
35 mM
50 mM
20
40
60
80
100
Temperature (°C)

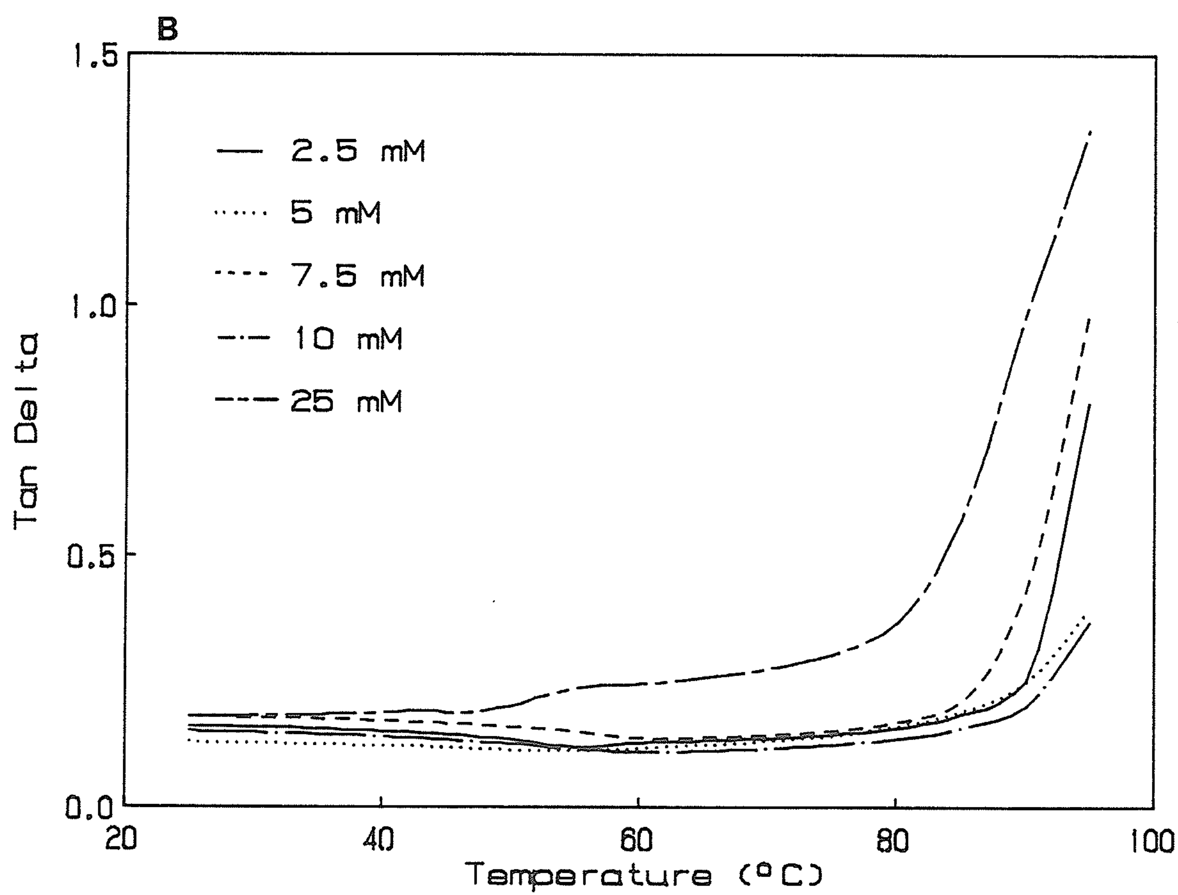

B
1.5
1.0
0.5
0.0
Tan Delta
2.5 mM
5 mM
7.5 mM
10 mM
25 mM
20
40
60
80
100
Temperature (°C)

Figure 18. Photomicrographs showing the effect of sodium dodecylsulfate on heat induced networks for 10% vicilin in 0.15 M NaCl, pH 8.5.

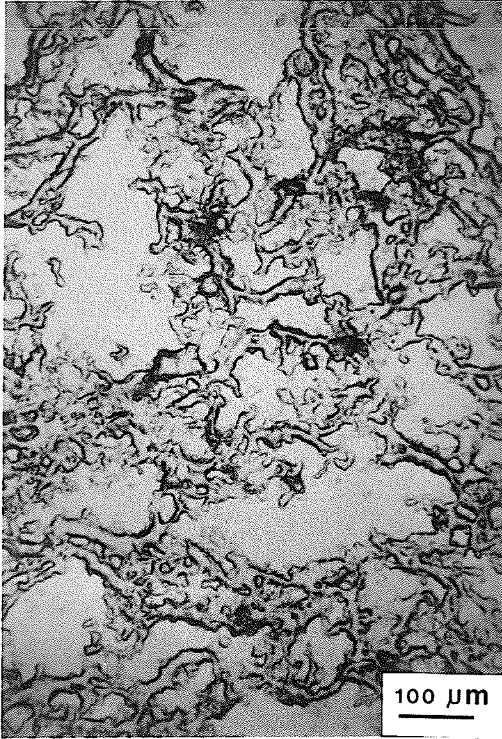
A. 2.5 mM

B. 5 mM

C. 10 mM

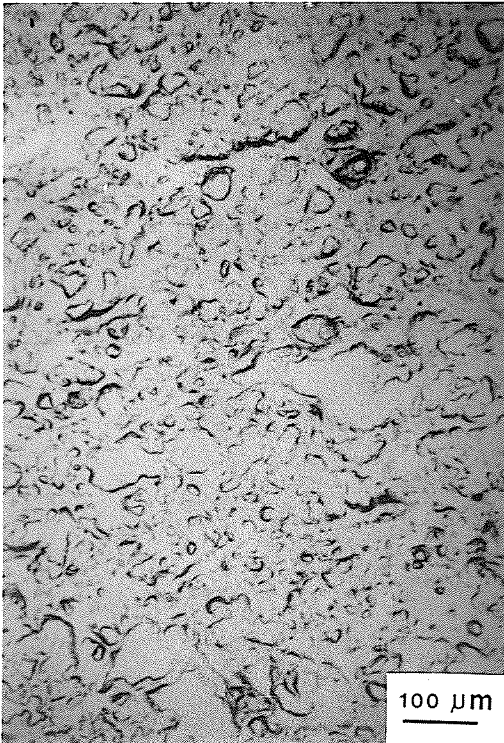
D. 25 mM

A

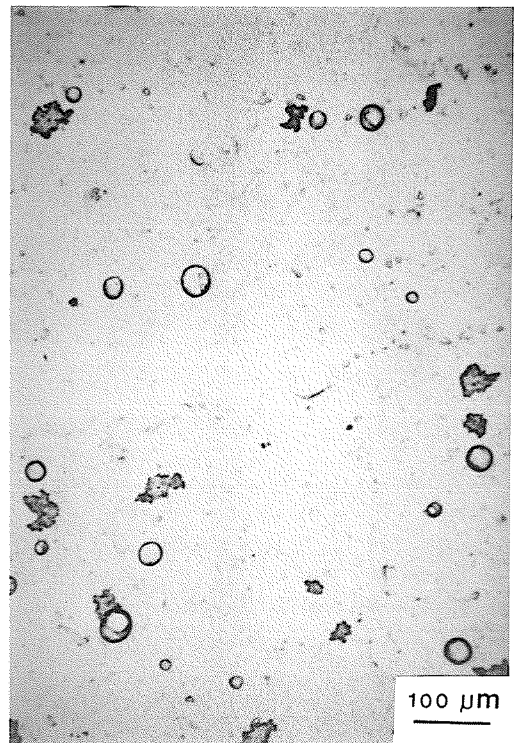


B

C



D



the low level SDS crosslinking that was observed with ovalbumin. In this respect, the conformation of the corpuscular structure involved in strand formation and the exposure of hydrophobic areas would be critical. With 10 mM SDS, the microstructure was similar to that seen for ovalbumin in 50 mM SDS in that the protein strands seemed to be highly hydrated. This hydration problem was even more noticeable with 25 mM SDS where a massive sheet containing a few air bubbles was observed. The liquid product resulting from the inclusion of 50 mM SDS could not be sectioned for microstructural analysis.

As with ovalbumin, G moduli decreased with increasing SDS concentration (Fig. 16B); the slight increases observed in 5 and 10 mM SDS were not significant (Appendix 4B). These changes, however, were related to the rate of change during the final rather than the initial cooling phase. Only at 25 mM SDS were the rates of change in G' and G" during the initial cooling phase significantly different (Appendix 5B), possibly reflecting the low level of structure development under these conditions. Overall, the contribution of electrostatic interactions to these rather poorly structured vicilin networks appeared to be in a stabilizing role during the latter stages of cooling. With the exception of the curve at 25 mM SDS, the changes in tan δ as a function of cooling temperature were the same (Fig. 17B). This supported the concept that the contribution of electrostatic interactions to network formation at low SDS concentration was through a stabilizing rather than a structure determining role. At higher SDS concentrations, the effect of electrostatic repulsion on the type of structure formed was more evident. The initially high tan δ value at 25 mM SDS was probably

due to the same hydration phenomenon observed with ovalbumin at high SDS concentrations. Although improvement during cooling reduced this value to be equivalent to those at lower SDS concentrations, it did not give a well structured network.

Calcium Chloride ($CaCl_2$)

Ovalbumin: Varying the $CaCl_2$ concentration between 0.1 and 0.5 M had no impact on the rheological characteristics of heat induced ovalbumin networks (Table 3A). The rate of change in G' and G" during cooling was also unresponsive to these $CaCl_2$ concentrations; the small variations showed no trend in terms of calcium effects (Table 3B). Of note in these data were the high tan δ values at all $CaCl_2$ concentrations, particularly when compared to the low values obtained in the pH and SDS studies. Furthermore, the rates of change in the G moduli were greater during the final cooling phase rather than the initial phase as was the case for previous networks. Despite these observations, the G' modulus was comparable to that for ovalbumin networks in the alkaline pH range. As expected from these high tan δ values, the microstructures for these networks were characterized by aggregated clumps of protein (Fig. 19). Alignment of these into strands at higher concentrations (0.3 - 0.5 M) was not sufficient to give a crosslinked network or improve rheological characteristics.

From a theoretical perspective, the inclusion of $CaCl_2$ should increase the number of positive charges on the protein through the binding of the calcium ion. At a pH of 8.5, the initial increase in positive charge would counteract the net negative charge so that a situation would result in which the effective charge would be

TABLE 3A. Effect of $CaCl_2$ on the rheological properties of heat set ovalbumin (10%, pH 8.5).

Concentration (M)	G' (Pascals)	G" (Pascals)	Tan δ
0.1	1684±454[a]	261±79[a]	0.153±0.005[ab]
0.2	1130±130[a]	190±20[a]	0.168±0.002[a]
0.3	1764±516[a]	280±76[a]	0.159±0.001[ab]
0.4	1430±308[a]	219±46[a]	0.153±0.002[ab]
0.5	1832±211[a]	270±18[a]	0.148±0.007[b]

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

TABLE 3B. Effect of $CaCl_2$ on the rate of structure development during cooling from 95°C to 25°C at 2°C/min for ovalbumin (10%, pH 8.5)

Concentration	Initial Cooling Phase		Final Cooling Phase	
(M)	G' (Pa/°C)	G" (Pa/°C)	G' (Pa/°C)	G" (Pa/°C)
0.1	-8.4±0.7[a]	-0.85±0.15[ab]	-25.1±5.2[a]	-4.82±1.54[a]
0.2	-3.9±0.5	-0.51±0.09[a]	-19.5±9.6[a]	-3.53±2.21[a]
0.3	-7.7±0.1[a]	-0.81±0.02[ab]	-31.2±7.6[a]	-5.65±1.53[a]
0.4	-7.4±0.3[a]	-0.86±0.03[ab]	-19.0±2.9[a]	-3.30±0.52[a]
0.5	-11.6±0.5	-1.47±0.34[b]	-24.8±4.2[a]	-4.01±1.09[a]

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

Figure 19. Photomicrographs showing the effect of $CaCl_2$ on heat induced networks for 10% ovalbumin, pH 8.5.

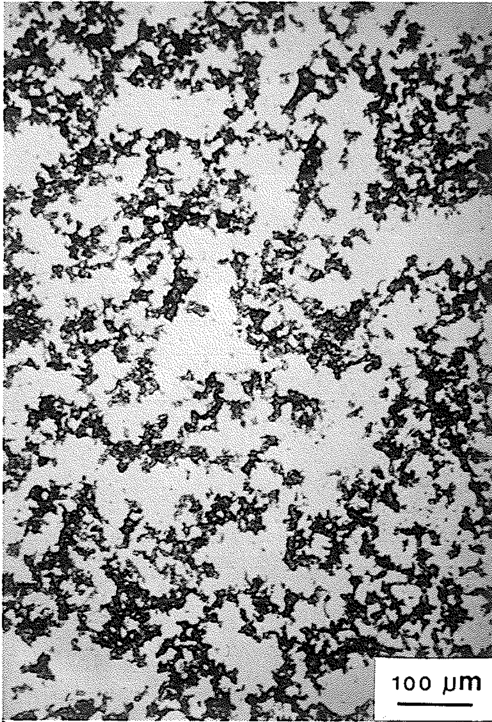
A. 0.1 M

B. 0.2 M

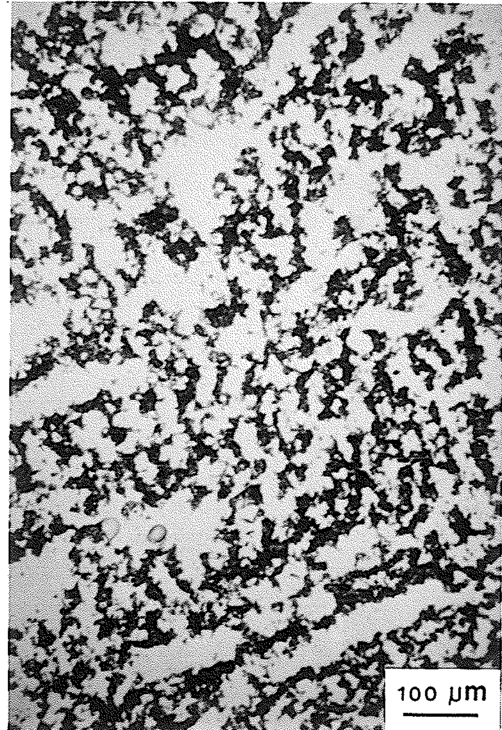
C. 0.3 M

D. 0.5 M

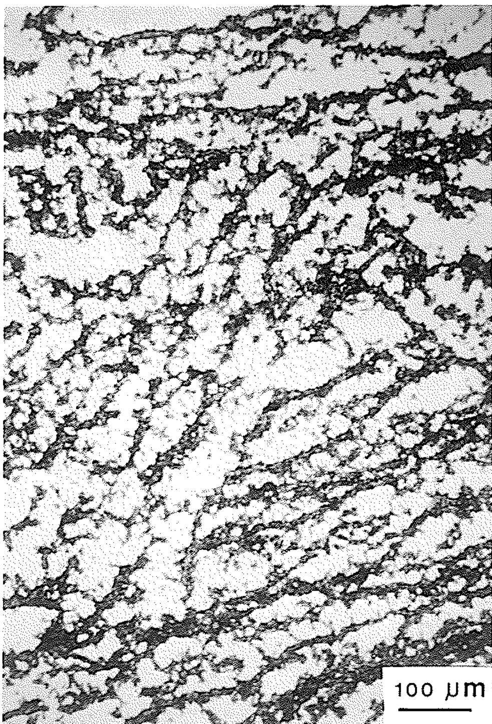
A



B



C



D

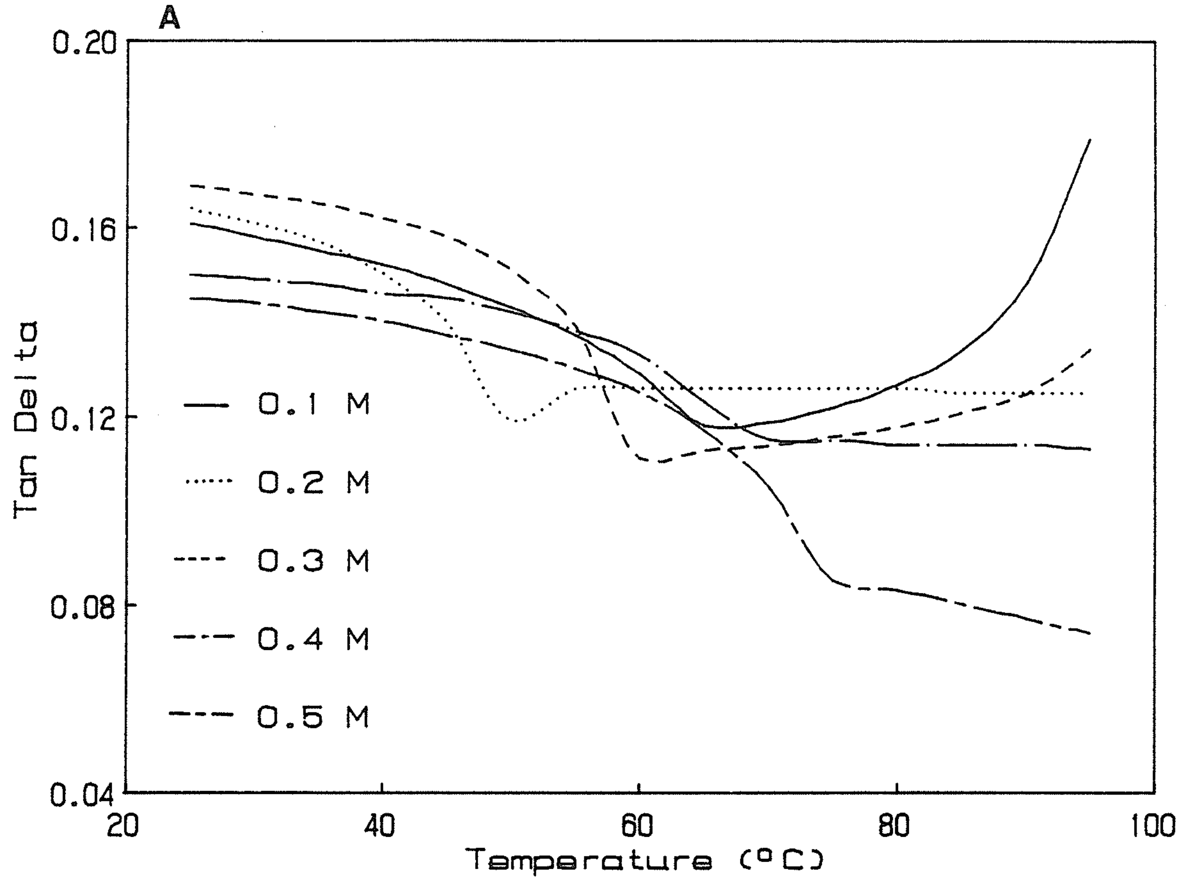
neutralized and aggregation such as that seen around the isoelectric point would be expected. Further addition of $CaCl_2$ would increase the net positive charge leading to conditions favoring network formation and eventually solubilization. The high tan δ values and poor microstructure at low $CaCl_2$ levels, could be interpreted as the point at which the net charge was essentially neutralized. Alignment of the protein into strands as was seen in the microstructure could signify some network formation due to the increase in net positive charge providing a balance between attractive and repulsive forces. Evidence of this network, however, was not supported by the rheological data, where there was no improvement in these characteristics. The high tan δ values, in conjunction with the high G moduli demonstrated that attractive forces were high in this system but the interactions which resulted did not contribute to good network characteristics. In this respect, the preferential binding of calcium to the protein may have been sufficient to alter this balance, and thus render $CaCl_2$ a poor choice of solute for investigating charge effects with ovalbumin.

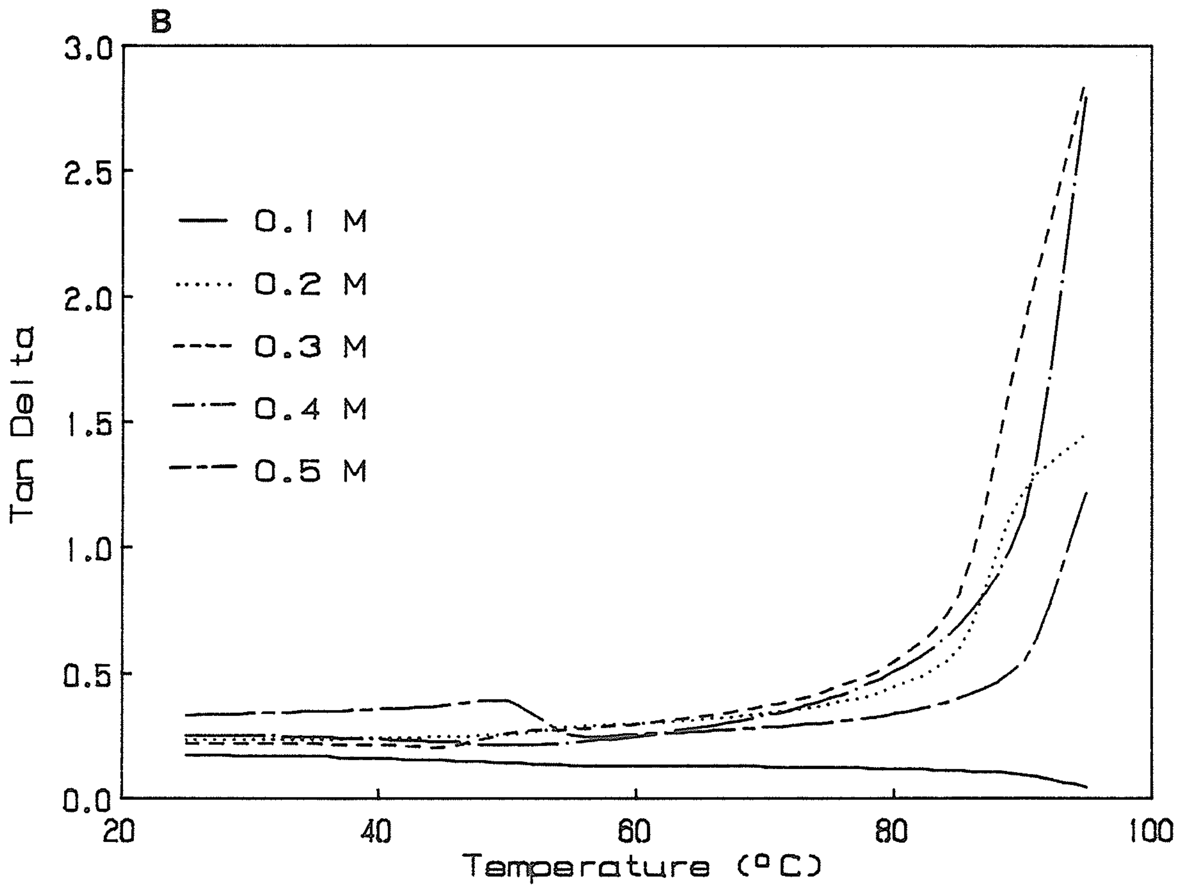
The changes in tan δ with cooling temperature followed a similar pattern of structure development for all $CaCl_2$ concentrations (Fig. 20A). Any evidence of structure formation (decreased tan δ values) occurred during the initial cooling phase. At all concentrations, however, this structure was not maintained and interactions responsible for the changes in the G moduli were not beneficial to producing a crosslinked network. This may also be related to the binding of the calcium ion, which seemed to promote aggregation. There was no evidence of ovalbumin denaturation at these $CaCl_2$

Figure 20. Influence of $CaCl_2$ on structure development during cooling for heat induced protein networks. Tan delta curves were calculated using a biphasic linear model to describe changes in G' and G".

A. 10% ovalbumin, pH 8.5.

B. 10% vicilin, pH 8.5.


A
0.20
0.16
0.12
0.08
0.04
Tan Delta
20
40
60
80
100
Temperature (°C)
0.1 M
0.2 M
0.3 M
0.4 M
0.5 M

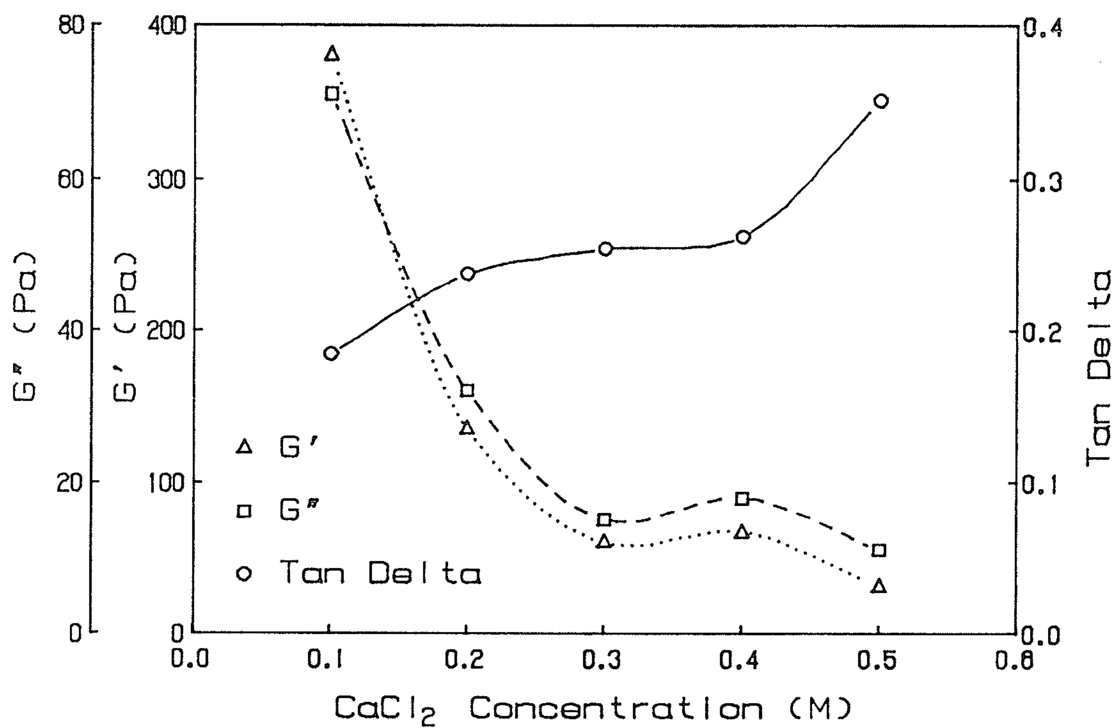

B
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Tan Delta
20
40
60
80
100
Temperature (°C)
0.1 M
0.2 M
0.3 M
0.4 M
0.5 M

concentrations, although the stability (Td value) was reduced at $CaCl_2$ concentrations of 0.3 and above (Appendix 18).

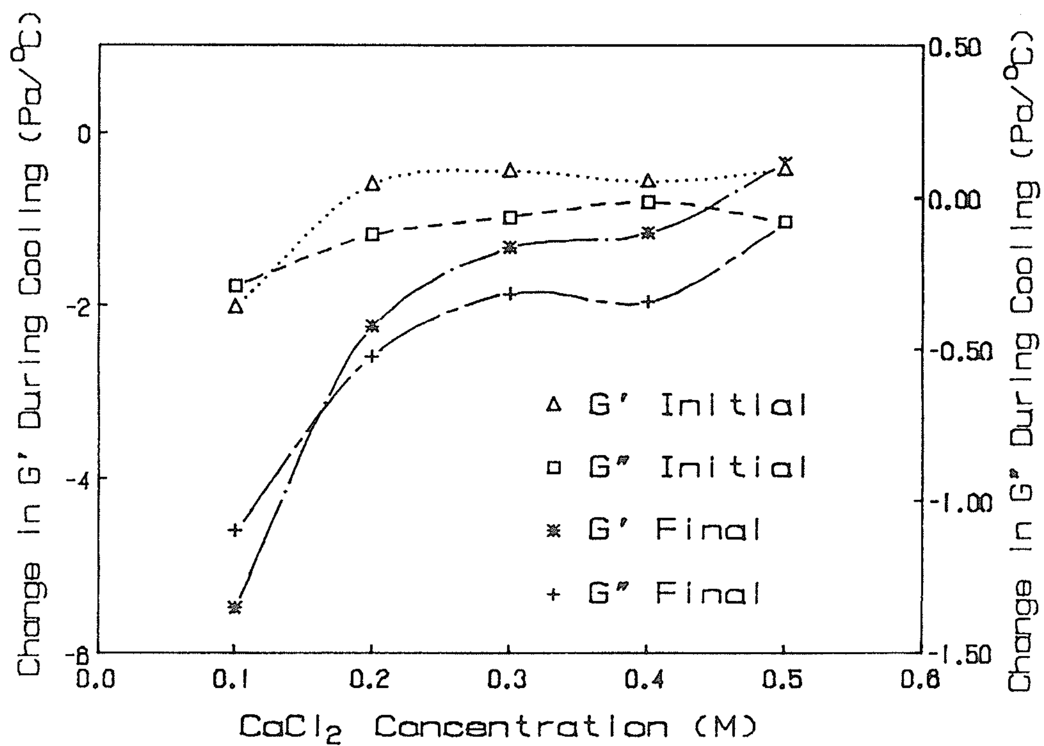
Vicilin: Unlike ovalbumin, the inclusion of $CaCl_2$ had a significant effect on the rheological and microstructural properties of heat induced vicilin networks (Fig. 21A; Appendix 6A). An increase in $CaCl_2$ caused a gradual decrease in the degree of interaction (decreased G moduli) and general deterioration of the network structure (increased tan δ values). The microstructure at 0.1 M $CaCl_2$ contained small aggregates similar to those for ovalbumin under similar conditions. At higher concentrations, the strands either became hydrated (0.2 and 0.3 M) or the network was too liquid to section and determine the microstructure (0.4 and 0.5 M). These results demonstrated the influence of excess positive charge. The progressive solubilization of the network with increased $CaCl_2$ concentration, due to this charge, was responsible for poor network characteristics. The calcium related aggregation seen for ovalbumin did not seem to be a factor at these concentrations for vicilin. As with ovalbumin, these was no significant vicilin denaturation at these $CaCl_2$ levels (Appendix 18).

As was seen for both the pH and SDS studies, the rates of change in G moduli during the final cooling phase were more sensitive to the variation in the $CaCl_2$ level (Fig. 21B; Appendix 6B), though a significant increase in the rate of structure development during the initial cooling phase at 0.1 M $CaCl_2$ was also observed. It would appear that the progressive solubilization with $CaCl_2$ was associated with changes in the network during the final cooling phase rather than during structure formation. Differences in the curves monitoring tan δ as a

Figure 21. Effect of $CaCl_2$ on the rheological properties (A) and rate of structure development (change in G') during initial and final cooling phases (B) for 10% vicilin pH 8.5.



G″ (Pa)
G′ (Pa)
Tan Delta
G′
G″
Tan Delta
CaCl2 Concentration (M)



Change in G′ During Cooling (Pa/°C)
Change in G″ During Cooling (Pa/°C)
G′ Initial
G″ Initial
G′ Final
G″ Final
CaCl2 Concentration (M)

function of cooling temperature were not informative. With the exception of the curve at 0.1 M $CaCl_2$, all other salt environments resulted in very high initial tan δ values (> 1), reflecting the trend toward solubilization. Following the initial establishment of structure at temperatures greater that 80°C, there were no major difference in the tan δ values. The relatively low tan δ values with the 0.1 M $CaCl_2$ M network indicated that structure development had occurred during the heating phase.

Charge in Relation to Network Characteristics

Despite a fair amount of confusion in the literature over the rheological changes associated with pH manipulation, the microscopic techniques have been well suited to assessing these structural variations (Gill and Tung, 1978; Woodward and Cotterill, 1986; van Kleef, 1986). The structures observed in this study with light microscopy were also effective in characterizing the response to environmental manipulation and enabled aggregated products (at IEP or in $CaCl_2$) to be distinguished from the well crosslinked networks (alkaline pH and ovalbumin in SDS) and those networks where protein solubility was a significant factor (vicilin in high concentrations of SDS or $CaCl_2$). These microstructural data were crucial for the identification of conditions suited to good network formation. In terms of rheological data, the response of the tan δ values were most closely related to the microstructures obtained; the discrepancy at pH 3 and 4 with vicilin may have resulted from sample preparation. As previous investigations on the fundamental rheological values as a function of pH have focused on G moduli (Gill and Tung, 1978, van Kleef, 1986),

this relationship between tan δ and microstructure had not been noted.

The G moduli, therefore, represented an additional parameter for network characterization. Unlike the situation with variation in protein concentration, the G moduli could not be directly related to microscopically detectable changes in the extent of interaction and were dependent on the nature of the proteinaceous structure formed. For example, the G′ modulus at pH 7 was higher than at the IEP, though on the basis of charge alone, the number of protein - protein interactions at the IEP would be expected to be higher. In fact, this rationale was used by van Kleef (1986) to explain a high G′ modulus at pH 5 for ovalbumin networks. In this study, however, the three dimensional, highly elastic structure produced at pH 7 and above, translated into a higher G′ modulus. The use of the G′ modulus as a indication of the number of protein - protein interactions is, therefore, questionable particularly when there are fundamental changes in network structure. At alkaline pH values and for ovalbumin in the presence of higher concentrations of SDS, decreases in G moduli were accompanied by lower tan δ values yet the microstructure remained essentially the same. This improvement in the network structure (decrease in tan δ) resulted despite an overall weakening of the system. In this situation a decrease in the number of protein - protein interactions reduced the overall network strength yet maintained the elastic crosslinked structure. In situations where the type of network remains the same, the G moduli can be used as an indicator of network strength.

Based on both rheological and microstructural data, it can be seen that protein charge was a major factor in establishing the attractive -

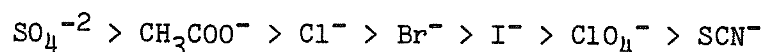
repulsive balance required for the production of good networks. In this respect, the generation of a specific negative or positive charge was essential for providing the repulsive component in this balance, so necessary for crosslinked network formation with both ovalbumin and vicilin. This was particularly apparent for the pH data, though data from SDS and $CaCl_2$ studies were also supportive. An increase in repulsive forces by increasing the net negative charge (alkaline conditions and the inclusion of SDS) promoted interactions to a greater extent than an increase in the net positive charge (acid pH and inclusion of $CaCl_2$) as evidenced by the higher G moduli. For ovalbumin, lower tan δ values were also associated with manipulation of the negative charge; however, for vicilin the lowest tan δ values were obtained at low pH values (high net positive charge). An excess in the repulsive force due to either charge lead to network solubility as was seen in the microstructure and poor rheological properties.

In addition to the contribution of charge to network formation through its role as an electrostatic repulsive force, the role of electrostatic attractive forces must be considered. This function of electrostatic interactions in the network was dependent on the type of structure obtained. For the highly aggregated structures produced around the IEP or in the presence of $CaCl_2$ the rate of structure development (change in G') was greater during the final cooling phase. Furthermore, variations in the G' modulus for networks which were not well crosslinked (including most vicilin networks) were attributed to changes during the final rather than the initial cooling phase. In effect, electrostatic forces contributing to the increased G' modulus

appeared to be of a strengthening or stabilizing nature. For reasonable to well crosslinked networks with low tan δ values, this did not appear to be the case. The rate of structure development in the initial cooling phase was always greater than the final cooling phase. In addition, variations in the G moduli due to solvent manipulation were associated with differences in the rate of structure development during this initial cooling phase. In this situation, the importance of attractive electrostatic forces was related to their contribution to the balance of attractive and repulsive forces and hence their ability to establish the appropriate structure. There was no evidence to indicate any further contribution to the strength of the network.

Salts

Neutral salts can have a profound effect on the conformational stability of proteins (von Hippel and Schleich, 1969). This influence has been shown to be concentration dependent. At low concentrations, there is a "salting - in" effect resulting from a direct interaction of the ions with specific charged groups on the protein. At higher concentrations, neutral salts can exert an indirect influence through solvent modification. In this situation, stabilization is related to strengthened intramolecular hydrophobic interactions (Melander and Horvath, 1977). The effectiveness with which salts alter protein stability at this concentration has been the basis for the Hofmeister or lyotropic series. For anions, a typical series is as follows (von Hippel and Wong, 1964):



$$SO_4^{-2} > CH_3COO^- > Cl^- > Br^- > I^- > ClO_4^- > SCN^-$$

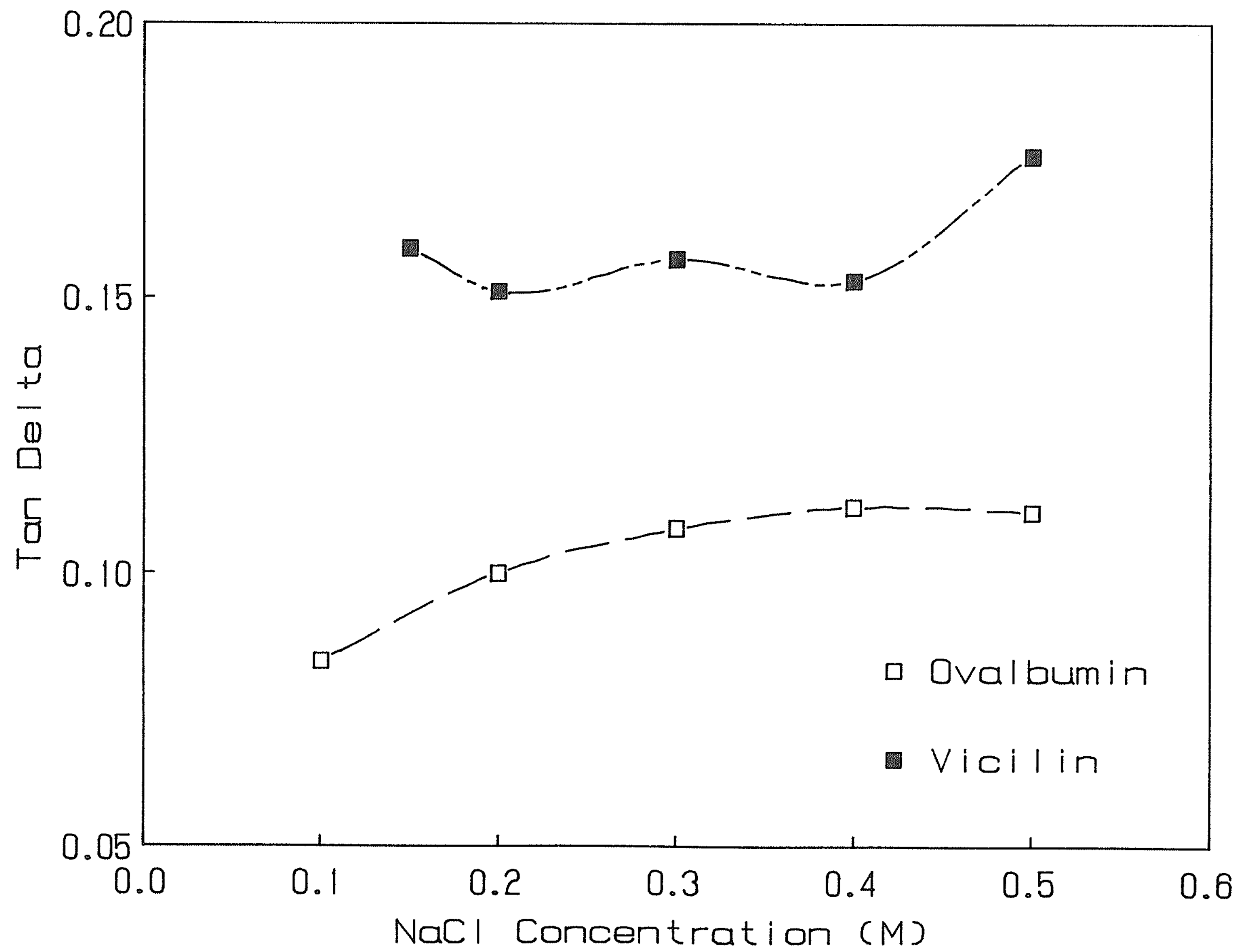
where anions on the left are more stabilizing. A similar response has been demonstrated for the vicilin storage protein in both a protein isolate (Arntfield _et al_., 1986) and a purified form (Ismond _et al_., 1986). The impact of anions in this series on network characteristics is not as well documented although increased turbidity for egg protein (Gossett _et al_., 1984) and decreased viscosity for soy protein (Babajimopoulos _et al_., 1983) have been attributed to the stabilizing salts.

A similar approach has been used to investigate the importance of hydrophobic interactions to the microstructural and rheological properties of ovalbumin and vicilin networks. Initially, networks were examined at various NaCl concentrations to see if the increase in stability was reflected in network characteristics. Subsequently, a series of anions of sodium salts were examined at concentrations where electrostatic (0.1 M) and lyotropic (0.5 M) influences should predominate.

NaCl Concentration

Ovalbumin: Increasing the NaCl concentration resulted in a gradual increase in the tan δ values for ovalbumin such that values at 0.1 and 0.2 M NaCl were significantly lower that those at the higher concentrations of 0.3 to 0.5 M (Fig. 22; Appendix 7A). Despite this difference, the range for these values was quite narrow (0.084 - 0.112) and no differences were seen in the microstructure. For all concentrations, a good crosslinked network, similar to that for ovalbumin at pH 9 in 0.15 M NaCl (Fig. 11) were obtained. For the G moduli, however, there were significant decreases between 0.3 and 0.4 M

Figure 22. Effect of NaCl on the tan delta values of heat induced protein networks from 10% ovalbumin, pH 8.5 and 10% vicilin, pH 8.5.



0.20
0.15
0.10
0.05
Tan Delta
0.0
0.1
0.2
0.3
0.4
0.5
0.6
NaCl Concentration (M)
Ovalbumin
Vicilin

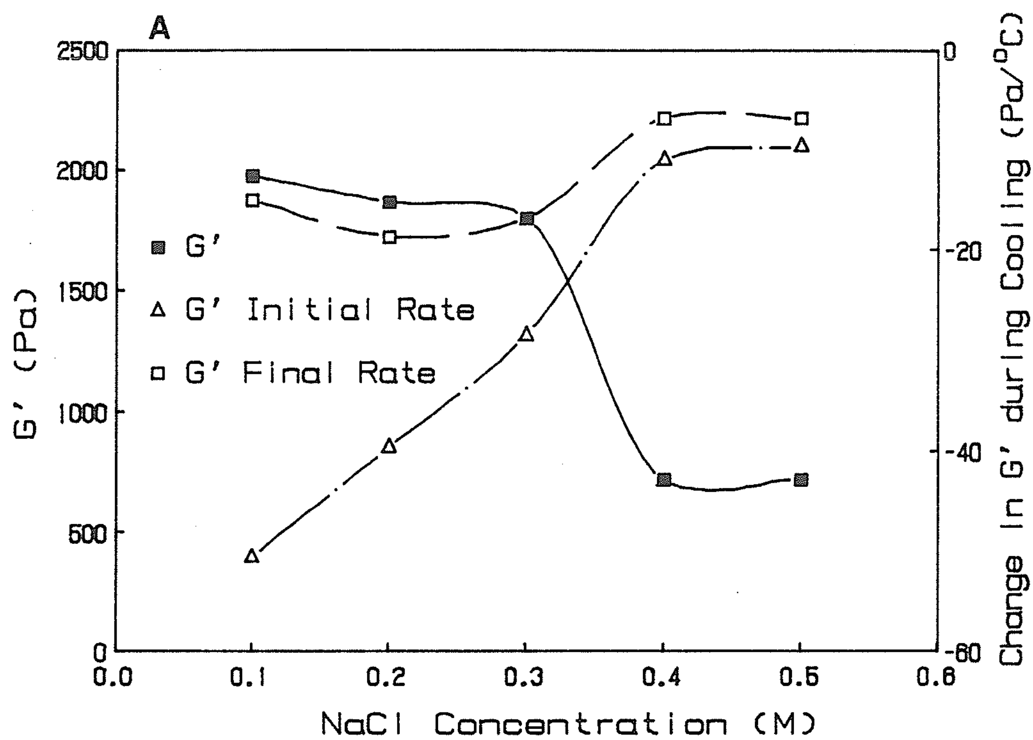
NaCl (Fig. 23). It would appear that at the higher salt concentrations where the influence of salt is related to the promotion of intramolecular hydrophobic interactions, the degree of interaction between proteins was greatly reduced. Although this change had little influence on the type of structure obtained, 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 in the network was the same. It is interesting to note that the thermal properties of ovalbumin did not indicate any stabilization at these salt concentrations. The Td values obtained, ranging from 85.4 to 86.8^{o}C, were not significantly different (Appendix 19). 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.

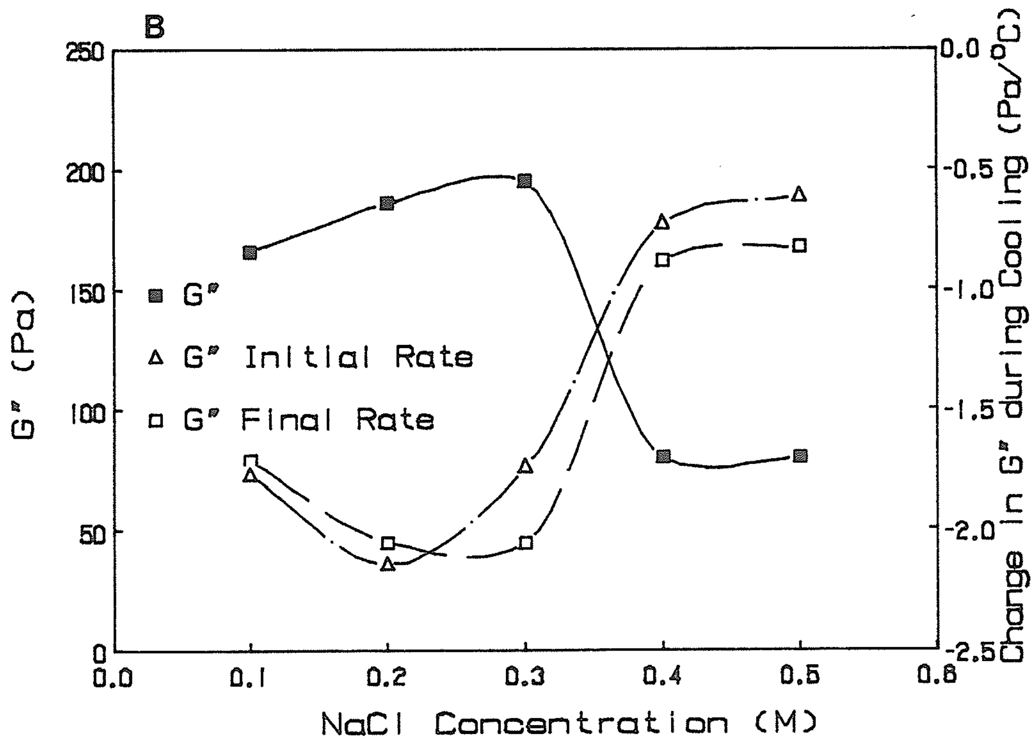
The influence of NaCl concentration was seen during both the initial and final cooling phases for G' and G" (Fig. 23A). The decrease in the rates of change in the G moduli for both phases was such that values at 0.3 M and below were significantly different from those above 0.3 M (Appendix 8A). In addition, there was a 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.

Figure 23. Effect of NaCl on the rate of structure development during the initial and final cooling phases in relation to the G moduli for the resulting networks for 10% ovalbumin, pH 8.5.

A. G' values

B. G" values


A
2500
2000
1500
1000
500
0
G' (Pa)
0
-20
-40
-60
Change in G' during Cooling (Pa/°C)
G'
G' Initial Rate
G' Final Rate
0.0
0.1
0.2
0.3
0.4
0.5
0.6
NaCl Concentration (M)


B
250
200
150
100
50
0
G" (Pa)
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
Change in G" during Cooling (Pa/°C)
G"
G" Initial Rate
G" Final Rate
0.0
0.1
0.2
0.3
0.4
0.5
0.6
NaCl Concentration (M)

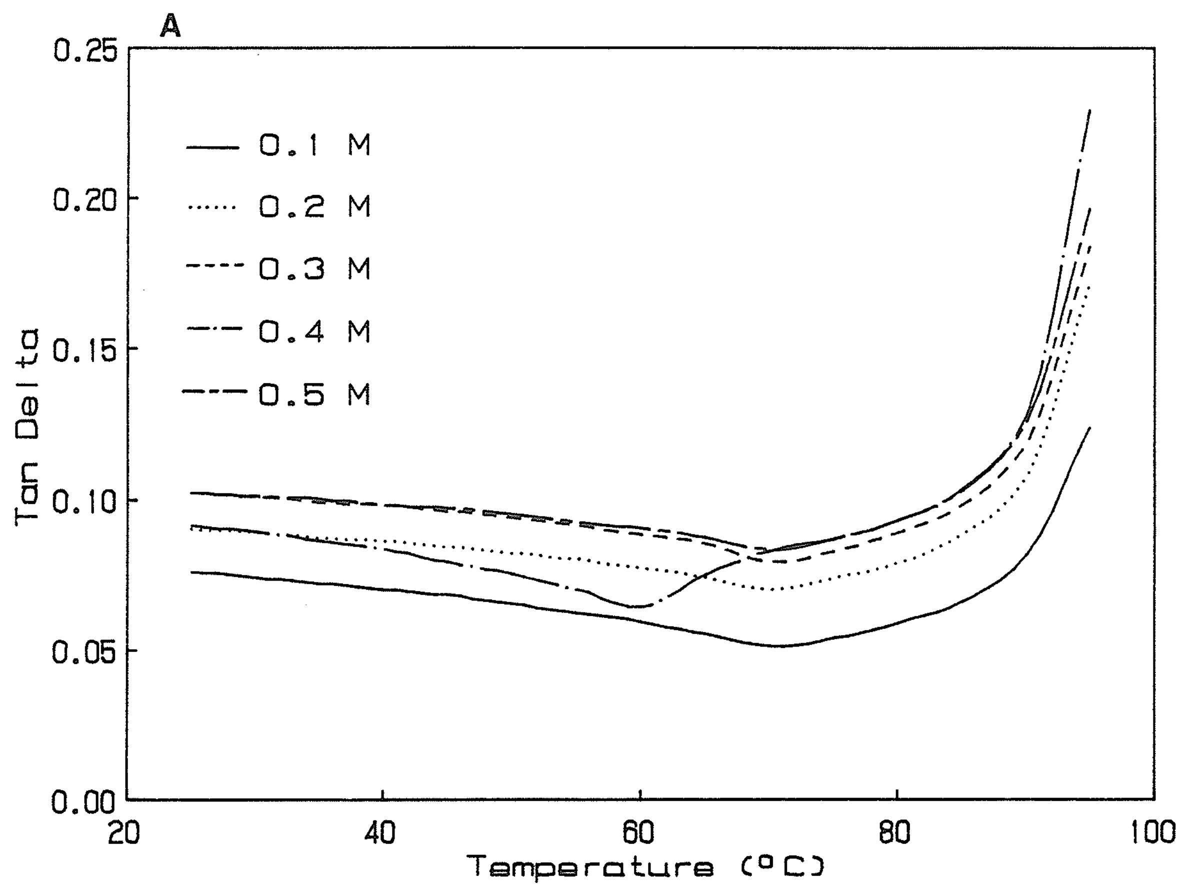
The similarities in terms of the type of network structure (microstructure and tan δ values) were also apparent in the changes in tan δ as a function of temperature (Fig. 24A). The shapes of the curves were essentially the same with only minor variations in the absolute tan δ values. Overall, the influence of hydrophobic interactions on both the viscous and elastic network components appeared to be a factor during both the establishment and strengthening of the network.

Vicilin: The influence of increasing NaCl concentration on vicilin network characteristics was comparable to that for ovalbumin. Tan δ values were essentially unaltered (Fig. 22) with only a slight, but significant, increase in 0.5 M NaCl (Appendix 7B). Microstructure was also unaffected over this salt concentration range; structures at all NaCl concentrations were similar to that for vicilin in 0.15 M NaCl at pH 9 (Fig. 13). 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 (Fig. 25; Appendix 7B). 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. Furthermore, the thermal properties for vicilin indicated a gradual increase in protein stability with increasing salt concentration, with Td values ranging from 78.1^{o}C at 0.1 M to 84.2^{o}C at 0.5 M (Appendix 19). This increased stability in conjunction with the decreased G' modulus added support to the theory that the interactions which promoted protein stability were also factors in determining the strength of the network that resulted from this heat treatment. The

Figure 24. Influence of NaCl on structure development during cooling for heat induced protein networks. Tan delta curves were calculated using a biphasic linear model to describe changes in G' and G".

A. 10% ovalbumin, pH 8.5.

B. 10% vicilin, pH 8.5.


A
0.25
0.20
0.15
0.10
0.05
0.00
Tan Delta
0.1 M
0.2 M
0.3 M
0.4 M
0.5 M
20
40
60
80
100
Temperature (°C)

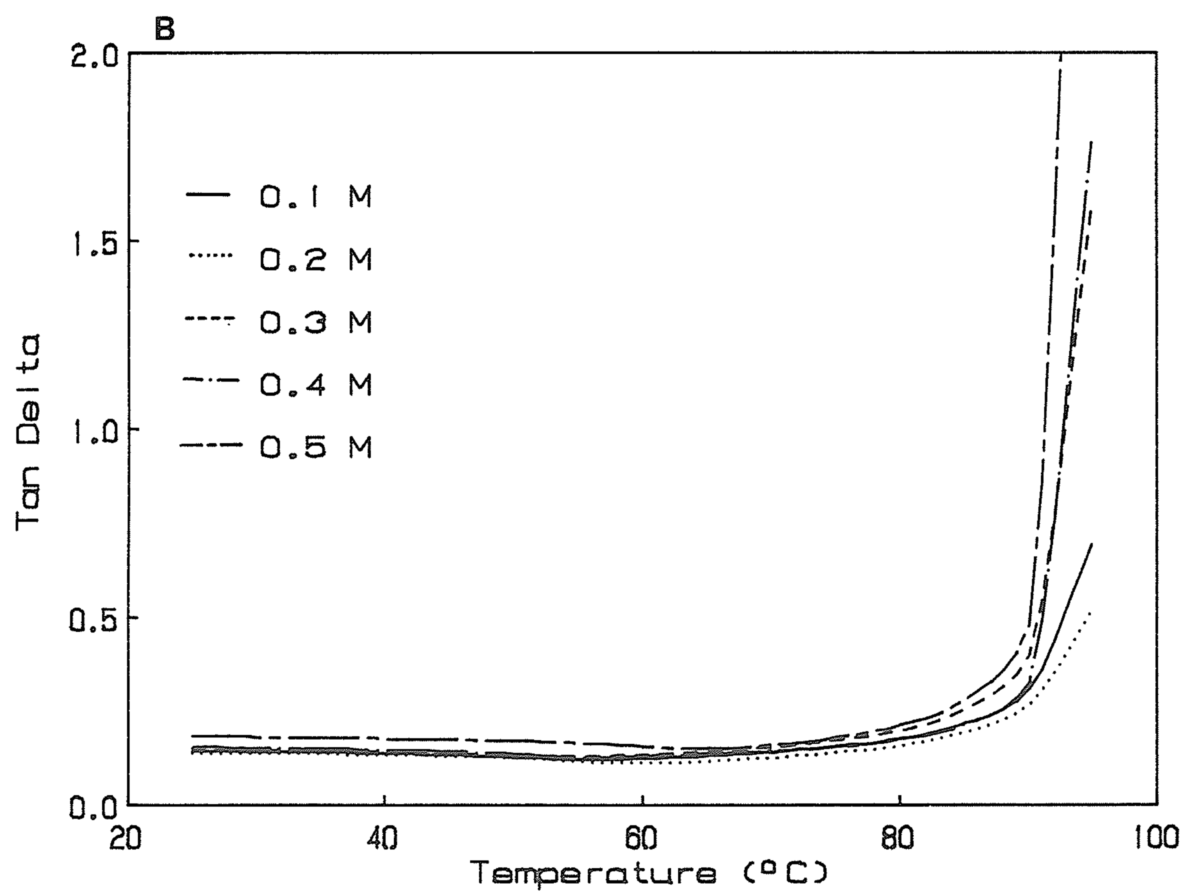
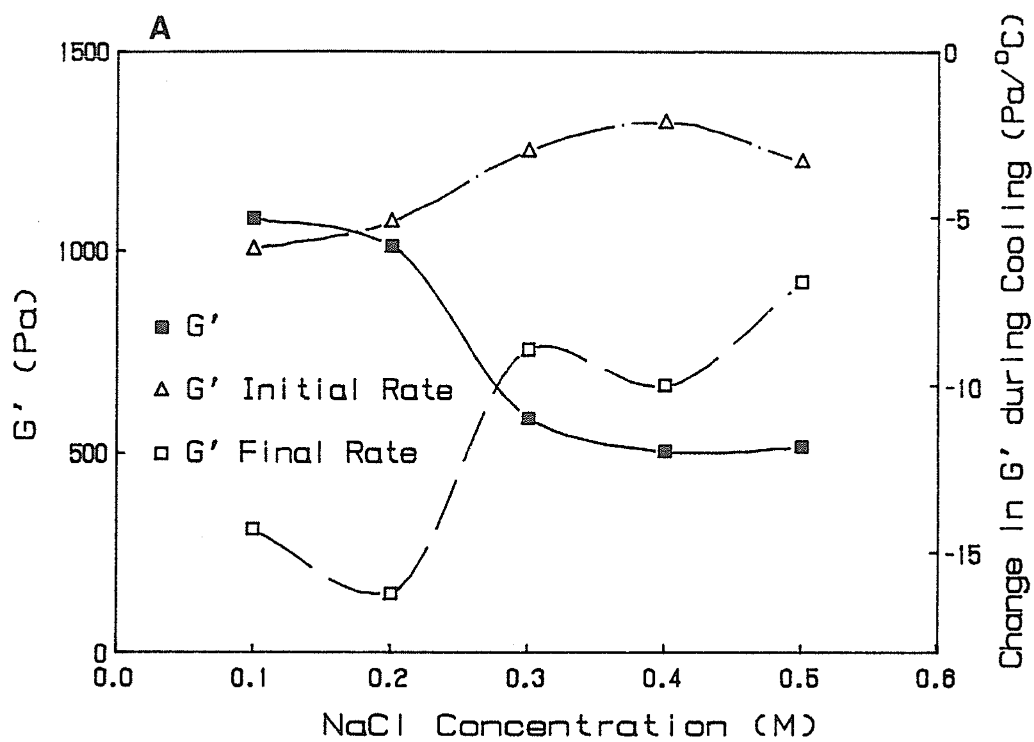

B
2.0
1.5
1.0
0.5
0.0
Tan Delta
0.1 M
0.2 M
0.3 M
0.4 M
0.5 M
20
40
60
80
100
Temperature (°C)

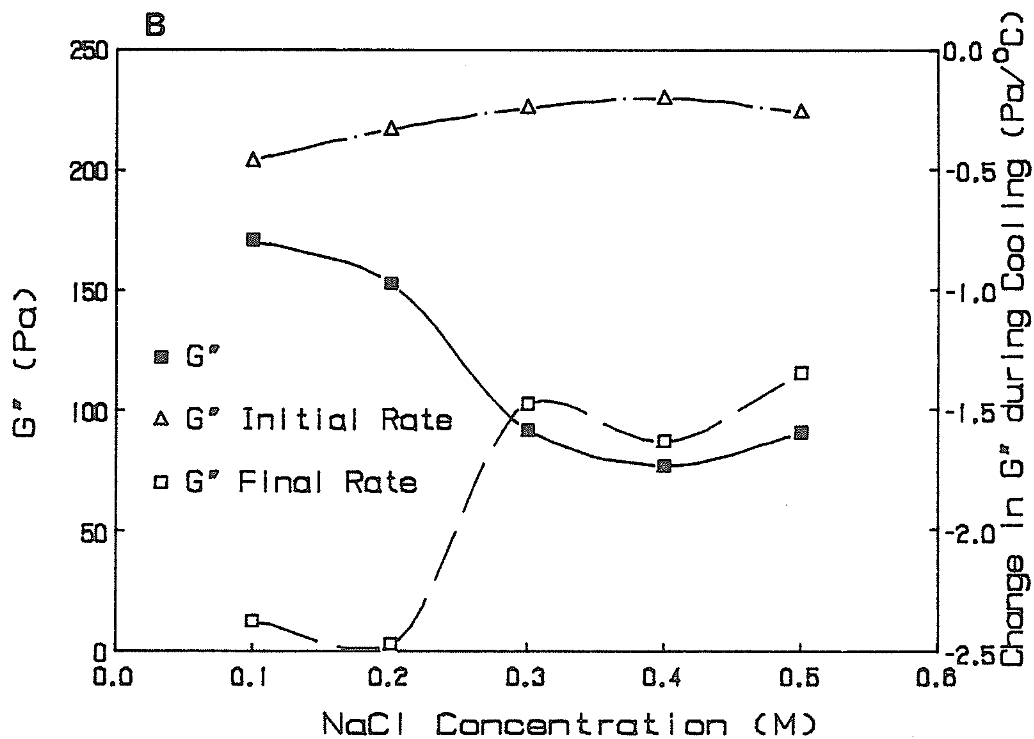
Figure 25. Effect of NaCl on the rate of structure development during the initial and final cooling phases in relation to the G moduli for the resulting networks for 10% vicilin, pH 8.5.

A. G' values

B. G" values



A
1500
1000
500
0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
-5
-10
-15
G' (Pa)
Change in G' during Cooling (Pa/°C)
G'
G' Initial Rate
G' Final Rate
NaCl Concentration (M)



B
250
200
150
100
50
0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
G" (Pa)
Change in G" during Cooling (Pa/°C)
G"
G" Initial Rate
G" Final Rate
NaCl Concentration (M)

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 (Fig. 25); however, the rates of change in the G moduli during the initial cooling phase at concentration of 0.1 and 0.2 M NaCl were also significantly different from those at higher salt concentrations (Appendix 8B). 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. The fact that both elastic and viscous components were affected by the manipulation of salt concentration was seen in the similarity of tan δ values during the cooling process (Fig. 24B); only initial values (at 95°C) varied slightly reflecting different degrees of structure development during the heating phase.

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 which may result from the inclusion of these anions. This is particularly true for anions which 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

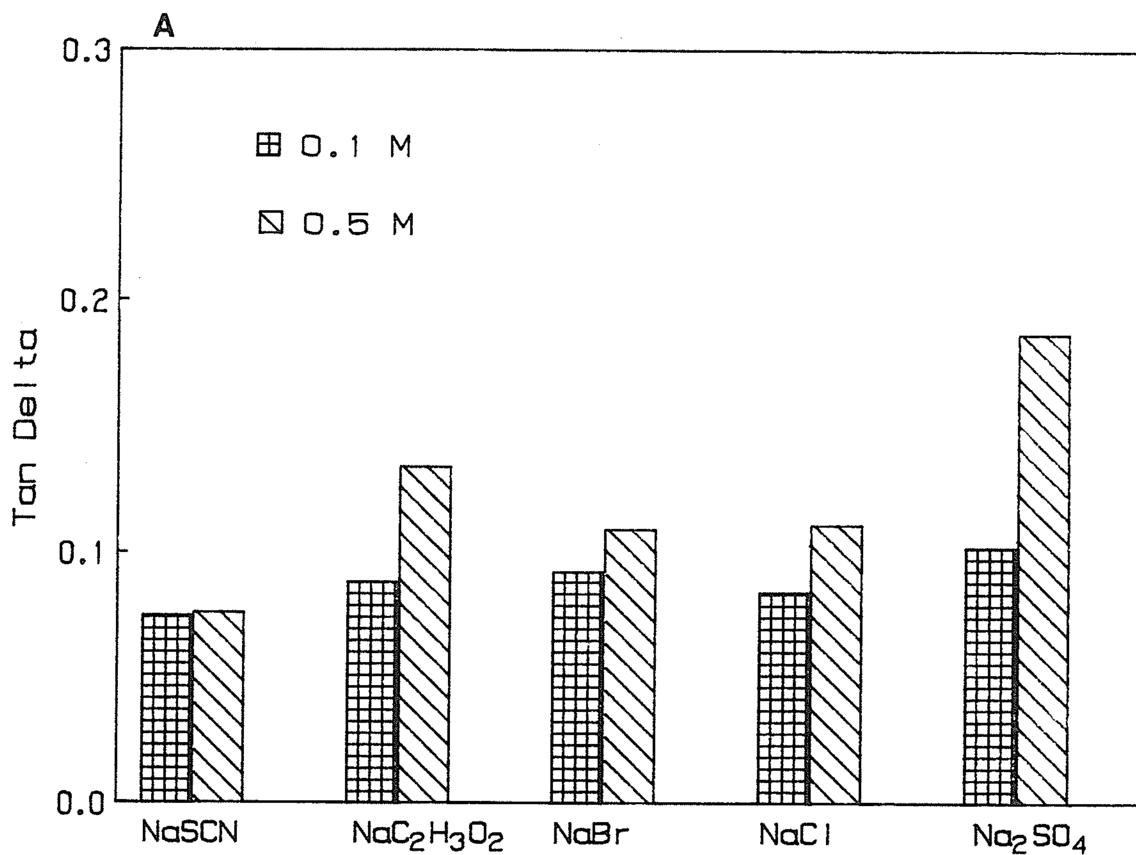
isolate a response that can be attributed to hydrophobic interactions. In an attempt to account for possible charge effects, the anions have been examined at concentrations of 0.1 and 0.5 M. Differences seen at 0.5 M but not 0.1 M should provide a better evaluation of the importance of hydrophobic interactions, as the lyotropic influence should not be a significant factor at the lower salt concentration.

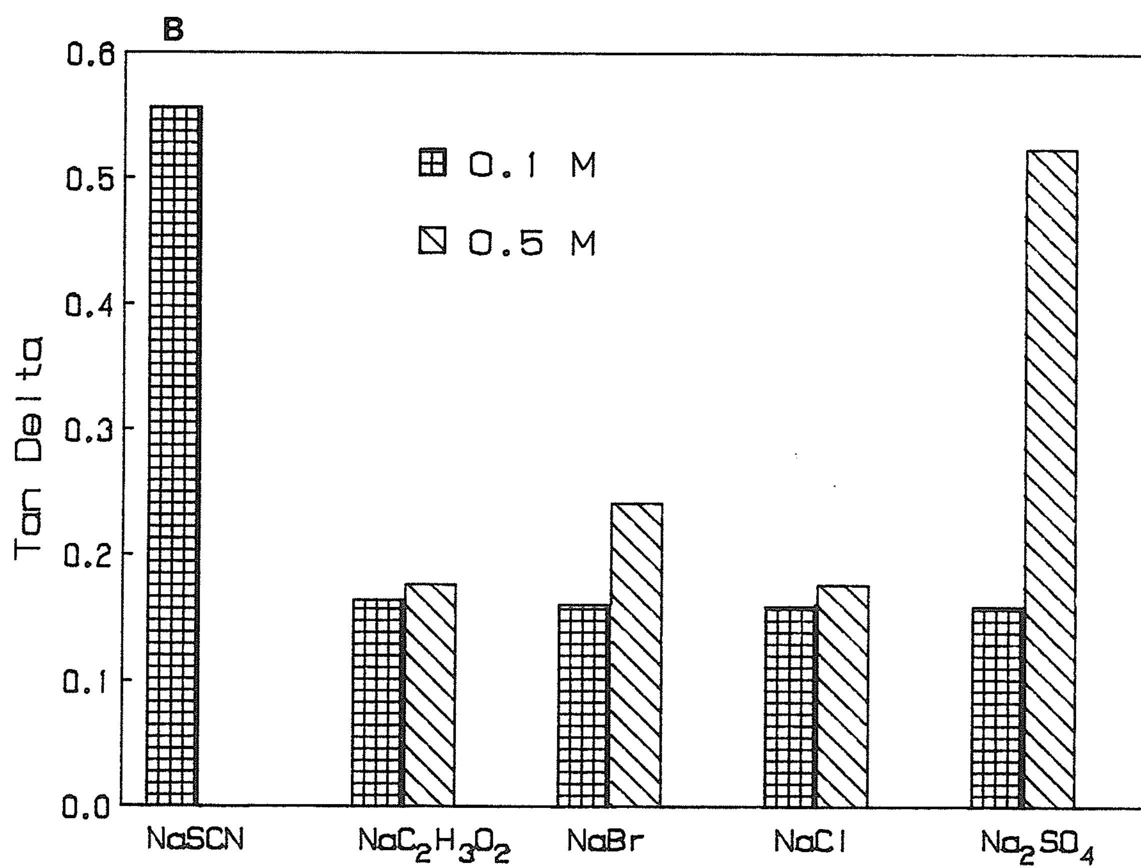
Ovalbumin: Variations in tan δ values for various sodium salts are given in Figure 26A. The salts have been presented in terms of their position in the lyotropic series with the more stabilizing salts (based on the molal surface tension increments as suggested by Melander and Horvath, 1977) on the right hand side of the graph. At a concentration of 0.1 M, tan δ values were all approximately 0.1 or less, values which in the previous experiments represented well crosslinked networks. Within this range, 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 (Appendix 9A). According to DSC data, both the NaSCN (Td = 83.9°C) and NaBr (Td = 84.3°C) environments resulted in destabilization of ovalbumin (Appendix 20A). Destabilization by the thiocyanate anion has been attributed to preferentially binding to the protein (Arakawa and Timasheff, 1982). This would increase the net negative charge on the protein and result in a response similar to that seen with an increase in pH or the addition of SDS. The interaction between NaSCN and the protein would thus account for the low tan δ values observed in this environment. The fact that a similar response was not observed for NaBr, despite a similar destabilizing effect, may be attributed to a lower degree of binding for this anion. The increase

Figure 26. Effect of anions of sodium salts (0.1 and 0.5 M) on the tan delta values for heat induced protein networks.

A. 10% ovalbumin, pH 8.5.

B. 10% vicilin, pH 8.5.


A
0.3
0.2
0.1
0.0
Tan Delta
0.1 M
0.5 M
NaSCN
NaC2H3O2
NaBr
NaCl
Na2SO4


B
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Tan Delta
0.1 M
0.5 M
NaSCN
NaC2H3O2
NaBr
NaCl
Na2SO4

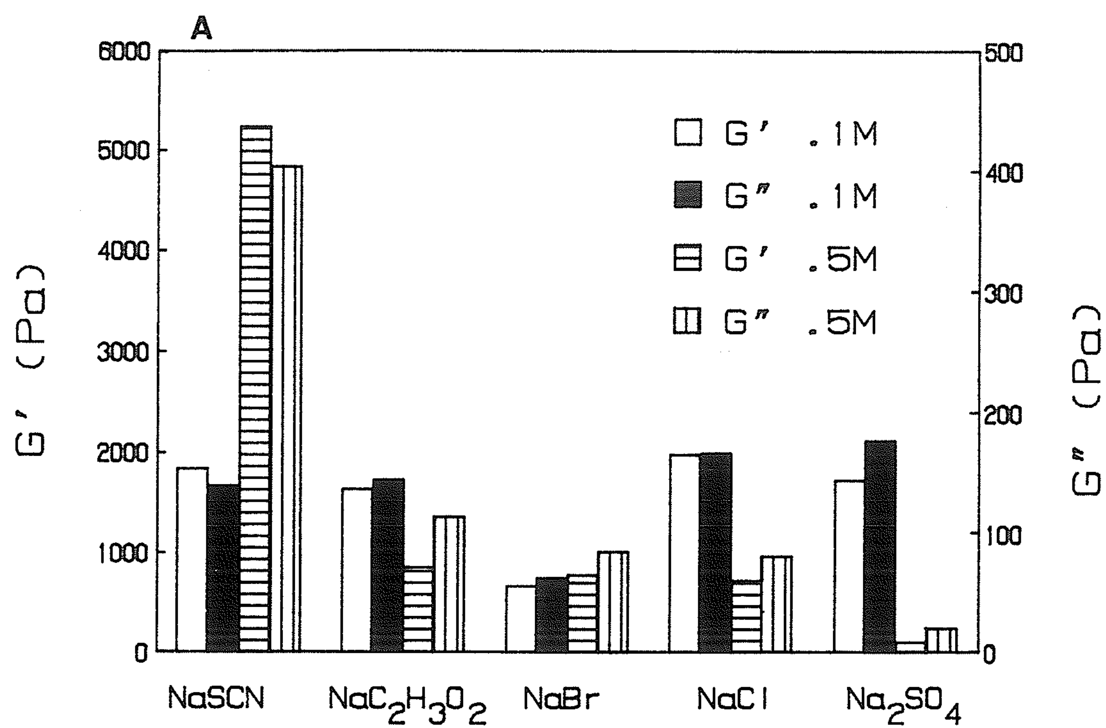
in tan δ in the presence of 0.1 M Na_2SO_4 may result from the higher ionic strength ($\mu = 0.3$) associated with this salt. Except for NaBr, the G moduli were not affected by anion type at this low salt concentration (Fig. 27A). The destabilization of NaBr may be responsible for the significantly lower G moduli in this environment (Appendix 9A). The differences in the tan δ values were also reflected in the microstructure (Fig. 28). As expected, the NaSCN environment produced an intense well crosslinked network. With $NaC_2H_3O_2$ and NaCl, the networks were again characterized as well crosslinked strands, but the degree of crosslinking appeared to be slightly lower as evidenced by the number of strands which stopped abruptly and were not connected to an adjacent strand. With NaBr, the crosslinked strands contained large areas of unstructured material, suggesting a certain level of aggregation. It was difficult to understand why this was not reflected in the rheological data. The poor network in the sulfate environment-many strands but little evidence of crosslinking - was also worse than expected considering the tan δ value was only slightly higher than those for the other salts.

In terms of tan δ values, the trend with the inclusion of salts at a concentration of 0.5 M was similar to that at the lower salt concentration in that the tan δ value was significantly lower in the thiocyanate environment and significantly higher in the sulfate environment (Fig. 26A; Appendix 9A). The tan δ value in the acetate environment was also higher than in bromide and chloride but lower than sulfate, though the reason for this was not clear. In all cases, the values were higher than at the lower salt concentration supporting the

Figure 27. 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.


A
G' (Pa)
G" (Pa)
G' .1M
G" .1M
G' .5M
G" .5M
6000
5000
4000
3000
2000
1000
0
500
400
300
200
100
0
NaSCN
$NaC_2H_3O_2$
NaBr
NaCl
Na_2SO_4

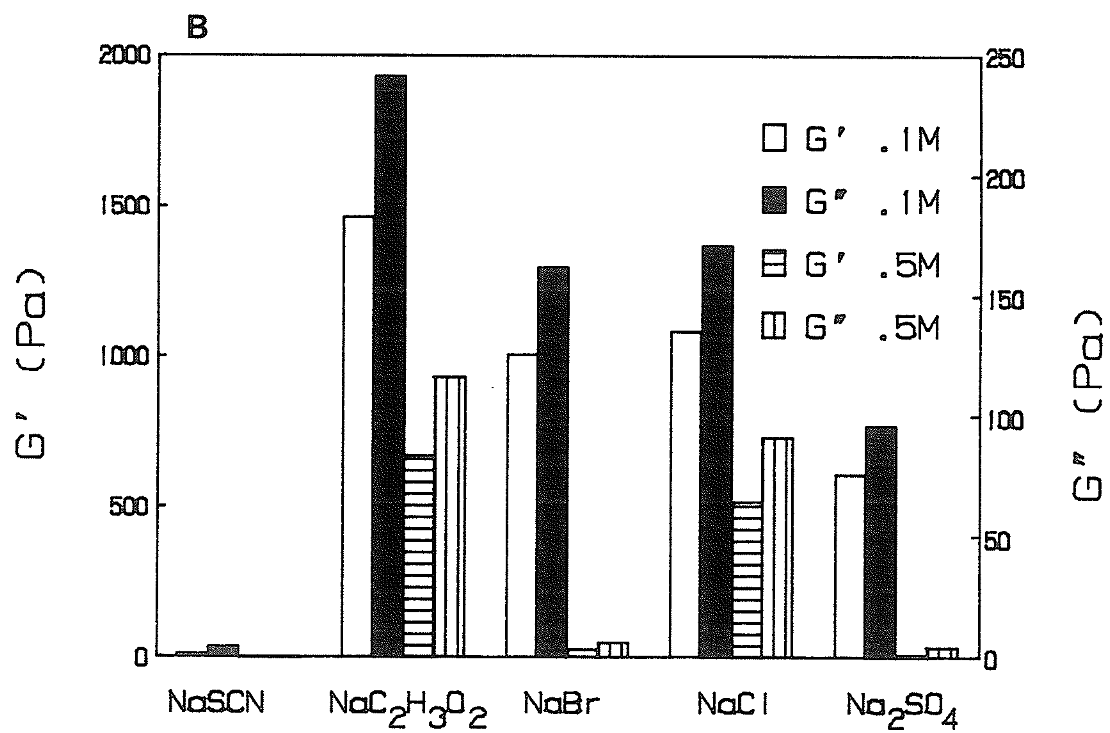

B
G' (Pa)
G" (Pa)
G' .1M
G" .1M
G' .5M
G" .5M
2000
1500
1000
500
0
250
200
150
100
50
0
NaSCN
$NaC_2H_3O_2$
NaBr
NaCl
Na_2SO_4

Figure 28. Photomicrographs showing the effect of anions of sodium salts on heat induced networks for 10% ovalbumin, pH 8.5.

A. 0.1 M NaSCN

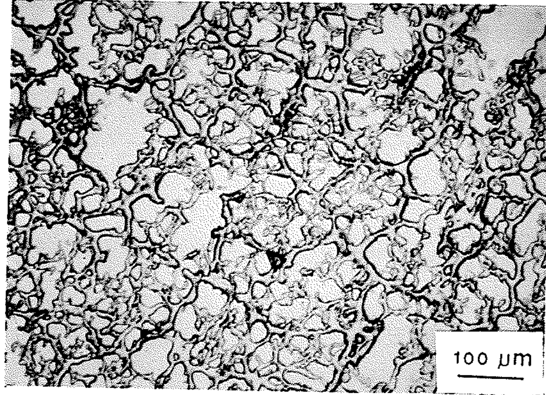
B. 0.1 M $NaC_2H_3O_2$

C. 0.1 M NaBr

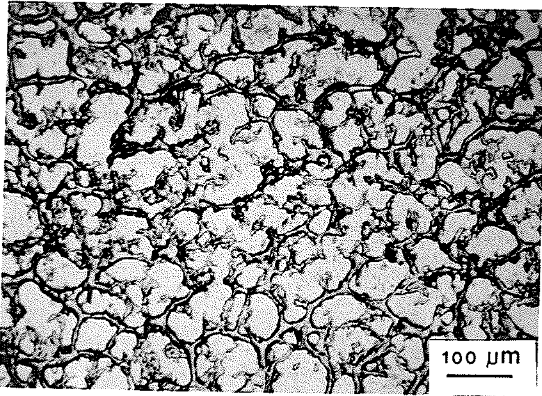
D. 0.1 M NaCl

E. 0.1 M Na_2SO_4

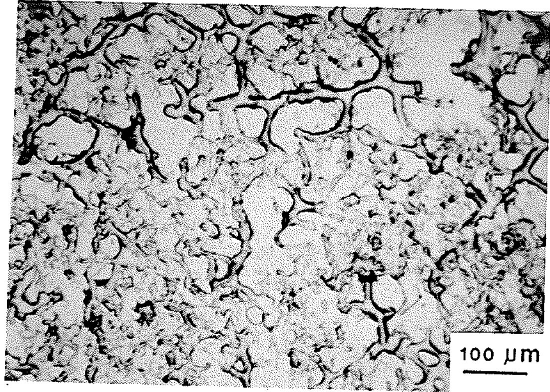
A



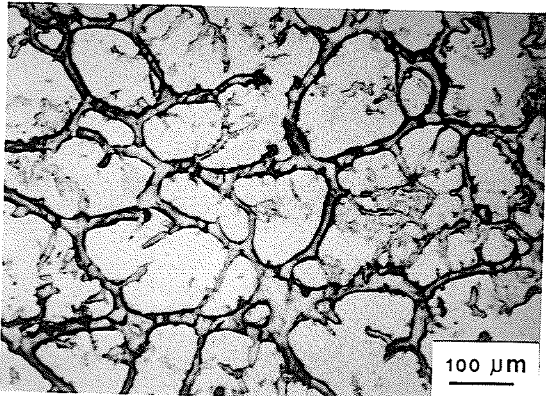
B



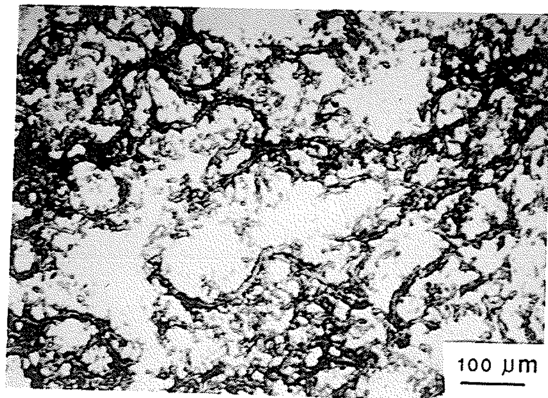
C



D



E



observation made with NaCl that increased salt concentrations results 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 and some improvement in crosslinking for the NaBr environment. From these results, it appeared that hydrophobic interactions have little influence on the type of structure that is formed in heated induced ovalbumin networks.

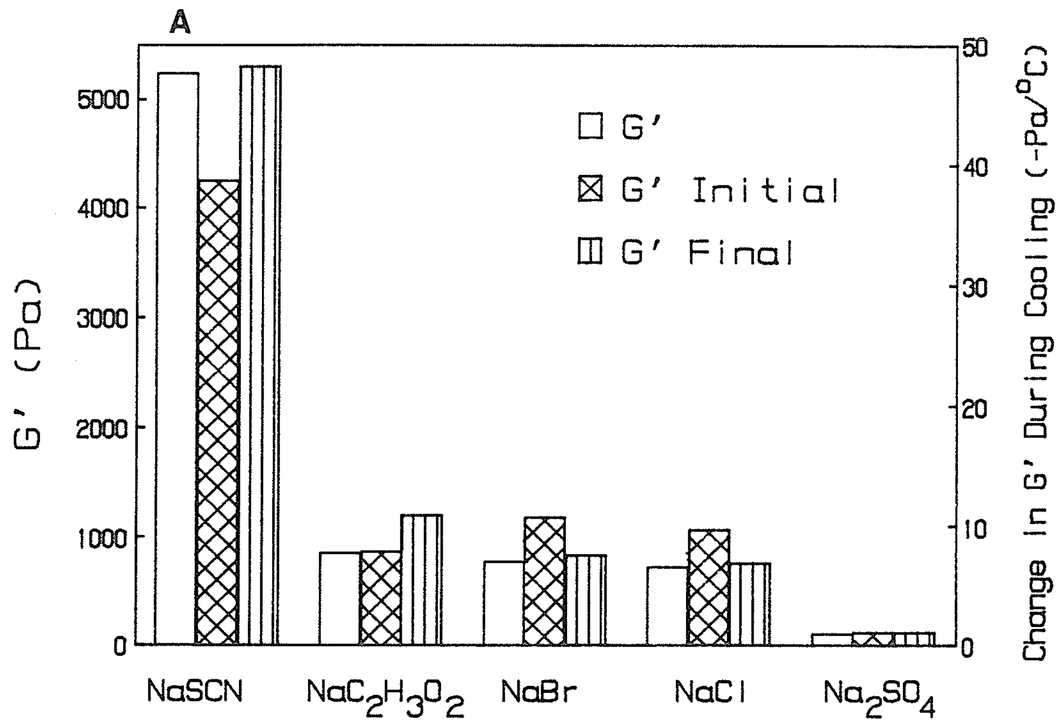
For the G moduli, however, the increase in salt concentration had a profound effect. Those salts at the destabilizing end of the series, especially NaSCN, produced high G' and G" moduli (Fig. 27A). These moduli decreased with the inclusion of the more stabilizing salts so that with Na_2SO_4, the G moduli were extremely low (Appendix 9A). 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.

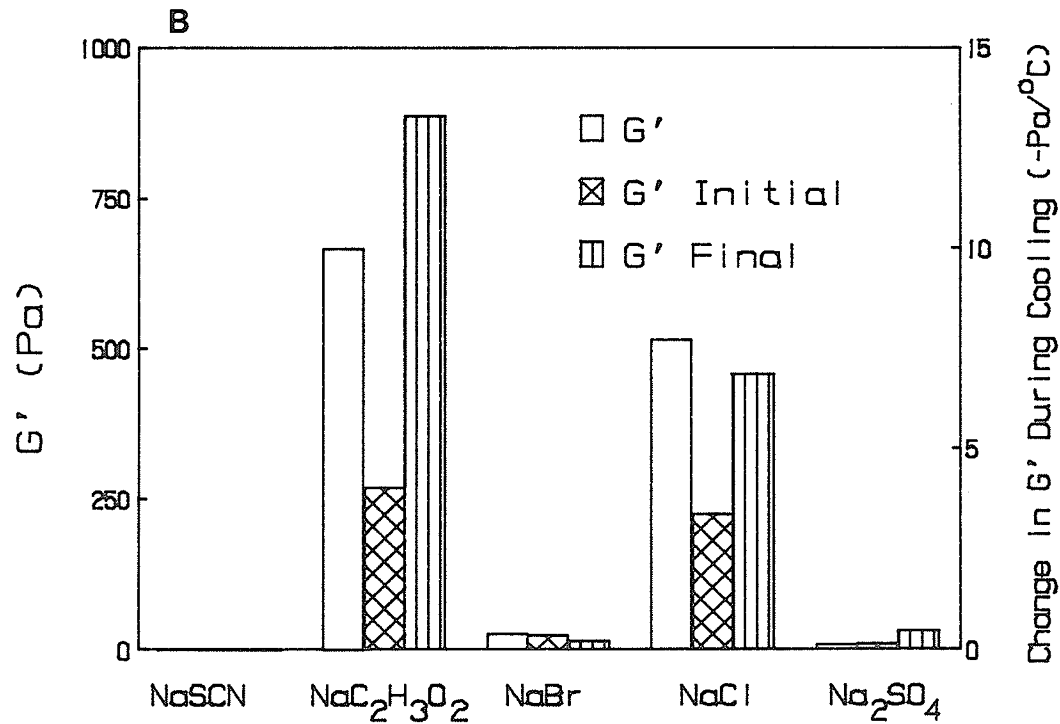
Examination of the cooling curve data for 0.5 M salts showed that changes in G' during both the initial and final cooling phases followed the same trend as the G' modulus for the final product (Fig. 29A; Appendix 10A). The more stabilizing salts were associated with a lower rate of structure development thus accounting for the lower G' modulus in the final product. A similar trend was noted for the G" modulus. It

Figure 29. Effect of anions of sodium salts (0.5 M) on the rate of structure development (change in G') during the initial and final cooling phases in relation to the G' moduli for the resulting networks.

A. 10% ovalbumin, pH 8.5.

B. 10% vicilin, pH 8.5.


A
G' (Pa)
5000
4000
3000
2000
1000
0
50
40
30
20
10
0
Change In G' During Cooling (-Pa/°C)
G'
G' Initial
G' Final
NaSCN
NaC2H3O2
NaBr
NaCl
Na2SO4


B
G' (Pa)
1000
750
500
250
0
15
10
5
0
Change In G' During Cooling (-Pa/°C)
G'
G' Initial
G' Final
NaSCN
NaC2H3O2
NaBr
NaCl
Na2SO4

should be noted that unlike previous cooling curve data, the rates of change in G′ for these bar graphs have been plotted as positive values (despite the negative correlation) in order to make comparisons easier.

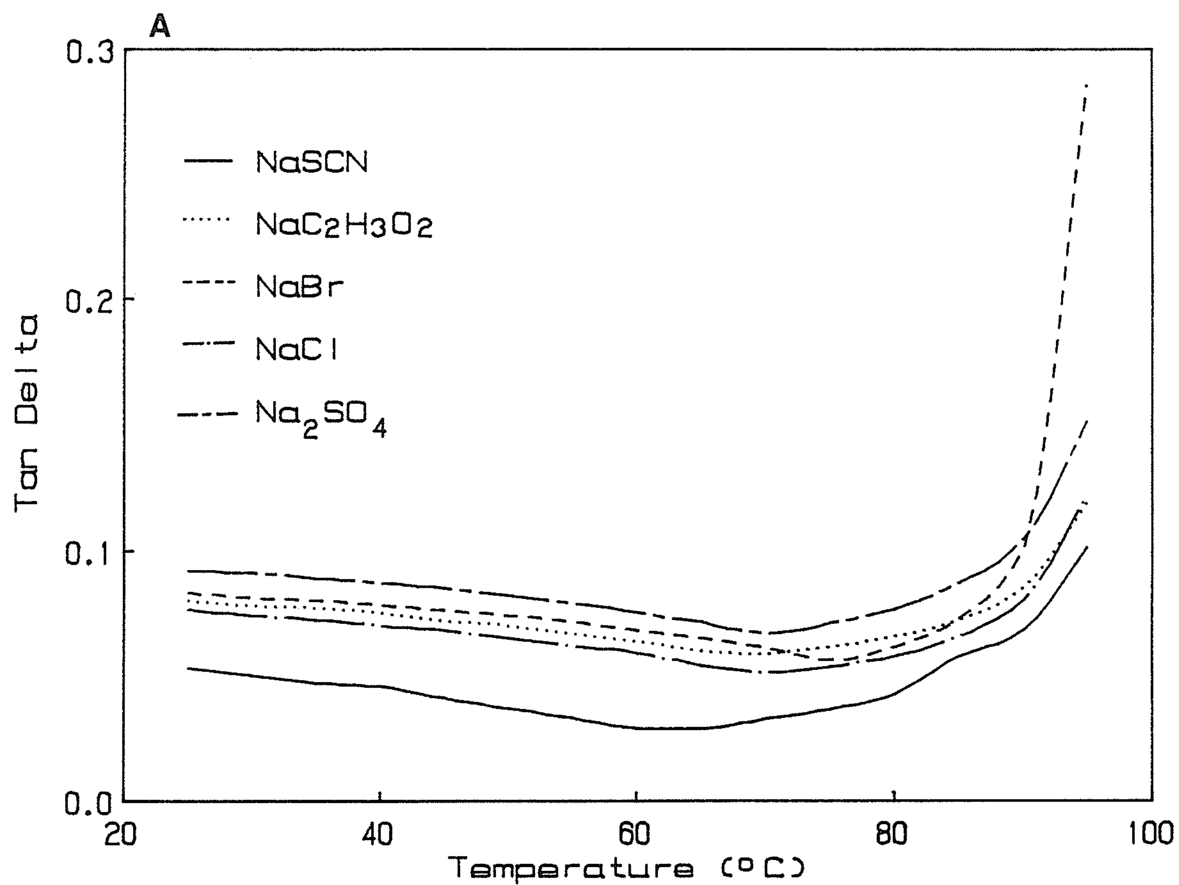
Changes in tan δ as a function of cooling temperature were essentially the same for all salts at a concentration of 0.1 M (Fig. 30A). The low tan δ values for NaSCN throughout reflected the improved network structure due to the charge effect even at the onset of the cooling regime. At this higher salt concentration, the poor structure for the Na_2SO_4 environment and good structure for the NaSCN environment were even greater at the onset of cooling than in the final product (Fig. 30B). From these results, it is 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.

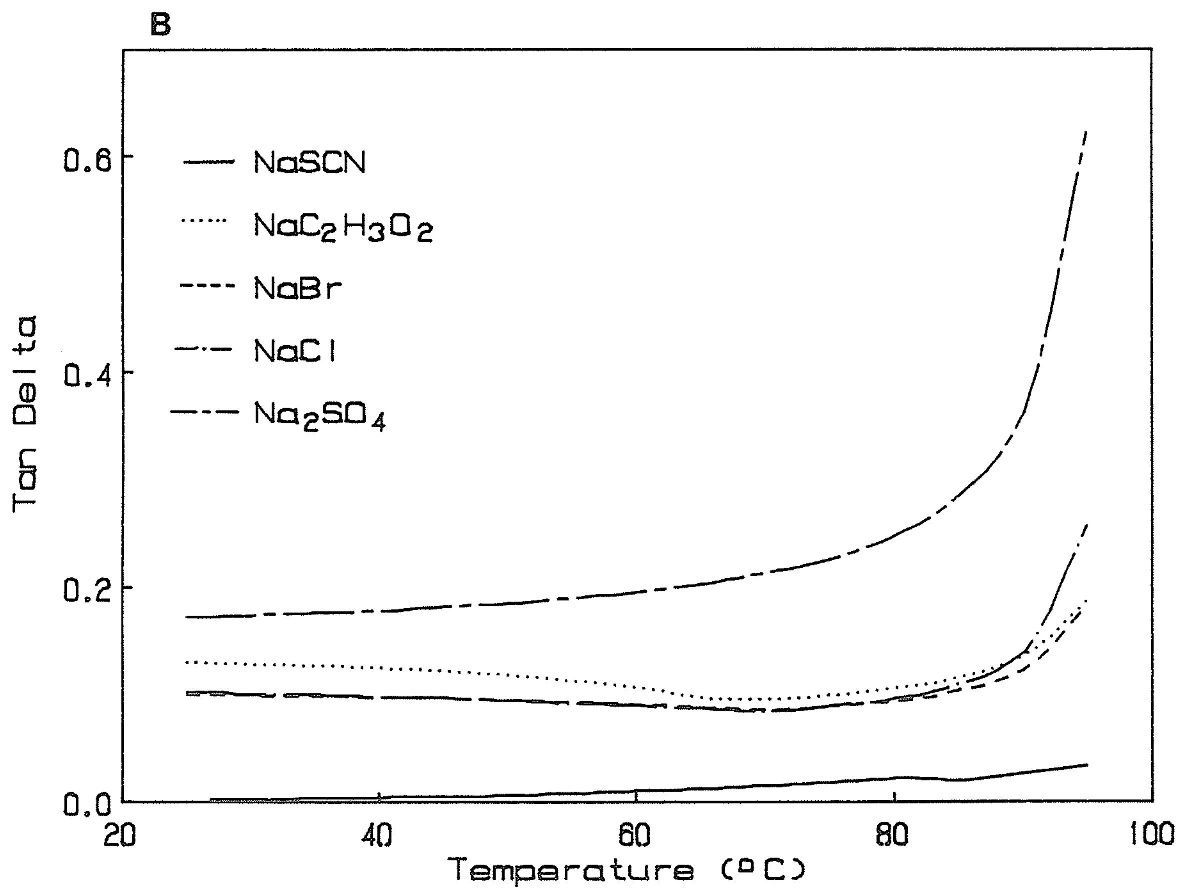
Vicilin: With the exception of NaSCN, the inclusion of various anions of sodium salts at the 0.1 M level had no impact on the tan δ values for heat induced vicilin networks (Fig. 26B; Appendix 9B). As with ovalbumin, the thiocyanate environment resulted in protein destabilization (significantly lower Td value - Appendix 20A) 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 associated with 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, similar to those seen at higher concentrations of SDS

Figure 30. Influence of anions of sodium salts on structure development during cooling for heat induced protein networks from 10% ovalbumin, pH 8.5. Tan delta curves were calculated using a biphasic linear model to describe changes in G' and G".

A. salts at a concentration of 0.1 M.

B. salts at a concentration of 0.5 M.


A
0.3
0.2
0.1
0.0
NaSCN
NaC2H3O2
NaBr
NaCl
Na2SO4
Tan Delta
20
40
60
80
100
Temperature (°C)


B
0.6
0.4
0.2
0.0
NaSCN
NaC2H3O2
NaBr
NaCl
Na2SO4
Tan Delta
20
40
60
80
100
Temperature (°C)

(Fig. 18). The consistency in terms of tan δ values for the other salts was apparent throughout the entire cooling process, indicating similarities in network structure during the initial network establishment (Fig. 31A).

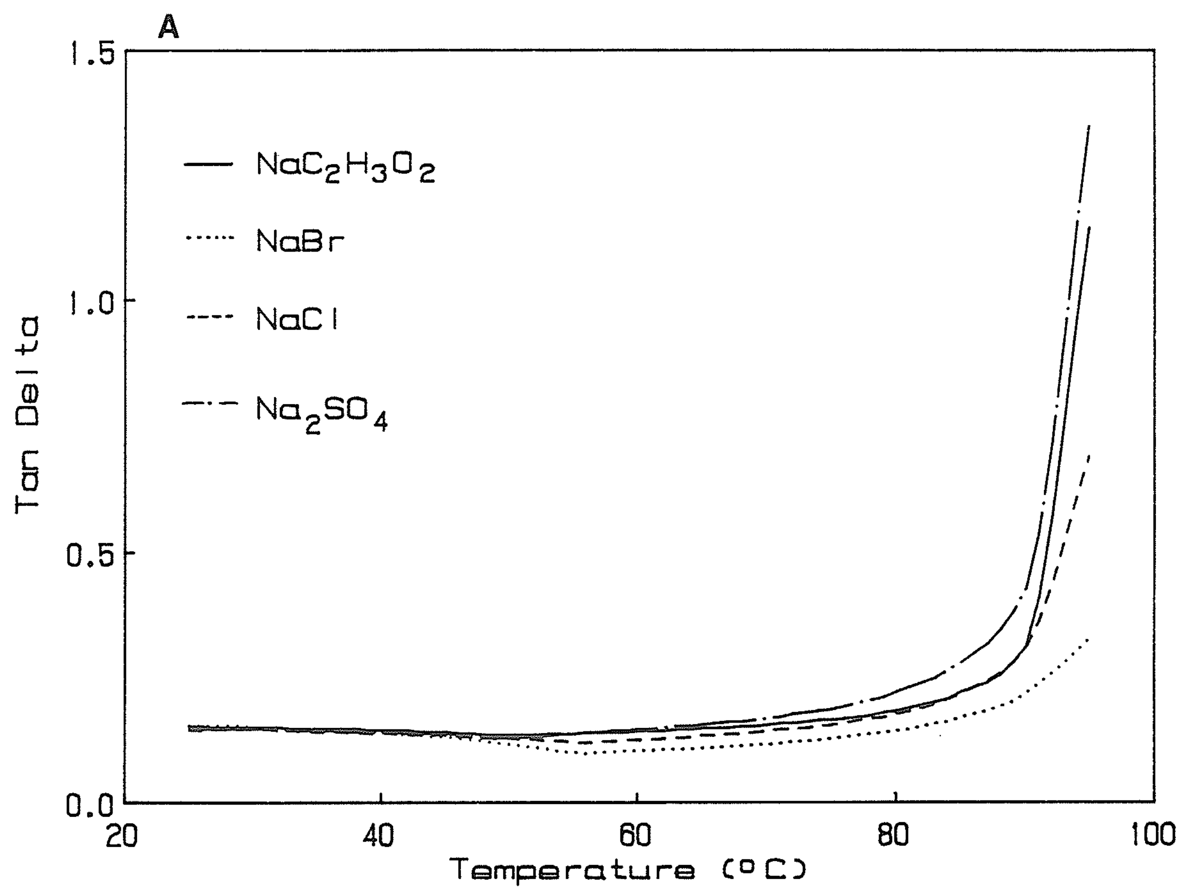
The very low G moduli in the 0.1 M NaSCN environment were consistent with the trend towards protein solubilization (Fig. 27B; Appendix 9B). For the other anions, there was a gradual decrease in the G moduli with the inclusion of the more stabilizing salts, with no significant difference between the moduli for NaBr and NaCl. Although vicilin stability was not affected at this low salt concentration, the interactions in the heat induced networks were. It is possible that under certain conditions, even this low salt concentration may impact hydrophobic interactions.

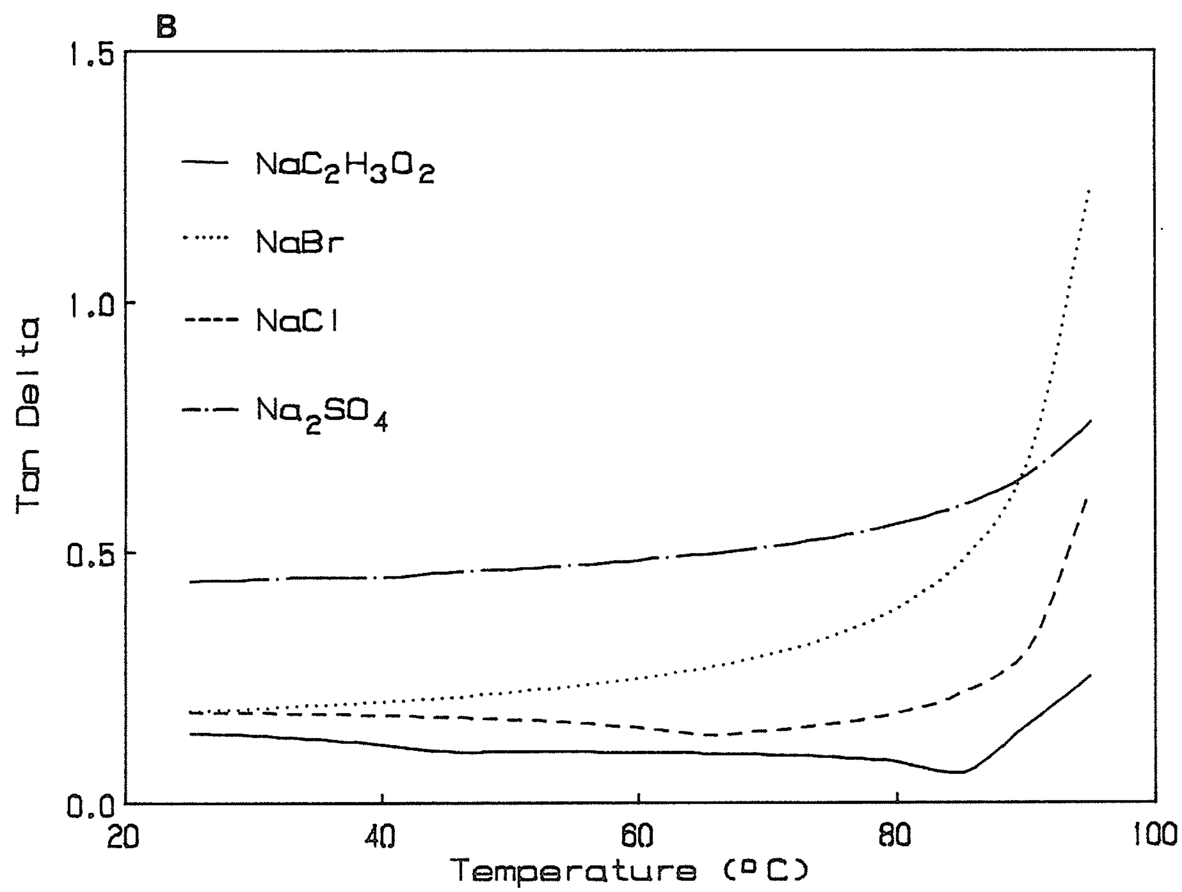
Results with 0.5 M salts were somewhat complex. With NaSCN, vicilin remained soluble during the heat treatment so that no rheological or microstructural data could be obtained. The influence of the other anions on protein stability was not completely as expected (Appendix 20B). The Td values for vicilin in $NaC_2H_3O_2$ (85.1^oC) and NaCl (84.2^oC) were not significantly different, but significantly lower than that for Na_2SO_4 (Td = 92.2^oC), as has been reported previously (Ismond <u>et al</u>., 1986). The NaBr environment, however, resulted in a significant reduction in the Td value (81.5^oC). This was uncharacteristic of the position of bromide in the lyotropic series and in conflict with previously reported data (Ismond <u>et al</u>., 1986). This problem with the NaBr environment was also reflected in the rheological and microstructural data for the heat induced networks. These networks were

Figure 31. Influence of anions of sodium salts on structure development during cooling for heat induced protein networks from 10% vicilin, pH 8.5. Tan delta curves were calculated using a biphasic linear model to describe changes in G' and G".

A. salts at a concentration of 0.1 M.

B. salts at a concentration of 0.5 M.


A
Tan Delta
Temperature (°C)
$NaC_2H_3O_2$
NaBr
NaCl
Na_2SO_4
0.0
0.5
1.0
1.5
20
40
60
80
100


B
Tan Delta
Temperature (°C)
$NaC_2H_3O_2$
NaBr
NaCl
Na_2SO_4
0.0
0.5
1.0
1.5
20
40
60
80
100

characterized by high tan δ values (Fig. 26B), low G moduli (Fig. 27B) and a microstructure which contained highly hydrated protein strands tending towards a soluble mass (Fig. 32). The binding of bromide to vicilin could account for both the calorimetric and rheological results; however, the lack of literature to support this type of interaction makes this interpretation questionable.

As a result, the importance of hydrophobic interactions was examined using only the three remaining salts. Tan δ values in $NaC_2H_3O_2$ and NaCl were the same while values in the Na_2SO_4 environment were significantly higher (Fig. 26B; Appendix 9B). In terms of microstructure, none of these environments produced particularly good networks (Fig. 32). With NaCl, there were clear protein strands while the degree of crosslinking 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 crosslinking was again poor. With Na_2SO_4, only protein aggregates were present as expected from the tan δ values. The G moduli, however, did decrease in the presence of more stabilizing anions (Fig. 27B; Appendix 9B) as was observed with ovalbumin. This influence was evident during both the initial and final cooling phases (Fig. 29B; Appendix 10B). In this respect, the response was similar to that for ovalbumin and indicated the importance of hydrophobic interactions during both the establishment and stabilization of heat induced vicilin networks.

Evidence for the role of hydrophobic interactions in network establishment could also be found in the changes in tan δ during the cooling regime (Fig. 31B). Unlike the situation with the 0.1 M salts

Figure 32. Photomicrographs showing the effect of anions of sodium salts on heat induced networks for 10% vicilin, pH 8.5.

A. 0.5 M $NaC_2H_3O_2$

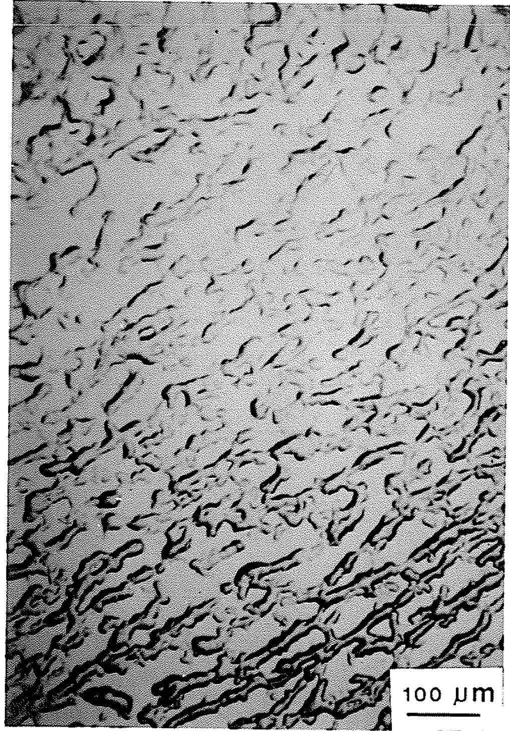
B. 0.5 M NaBr

C. 0.5 M NaCl

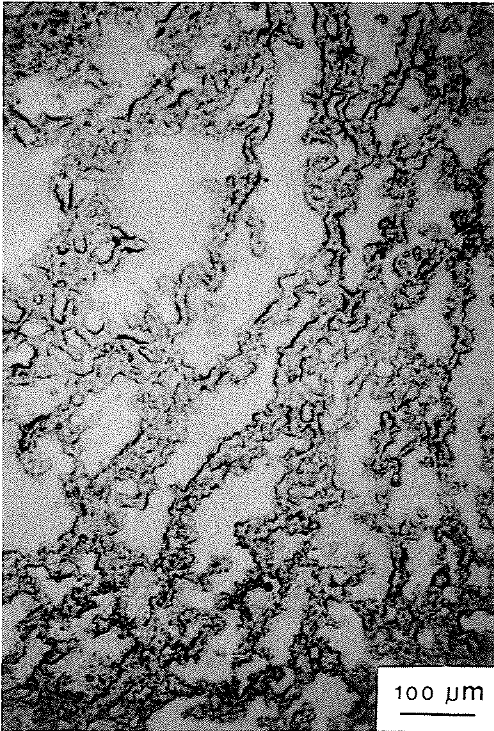
D. 0.5 M Na_2SO_4

A

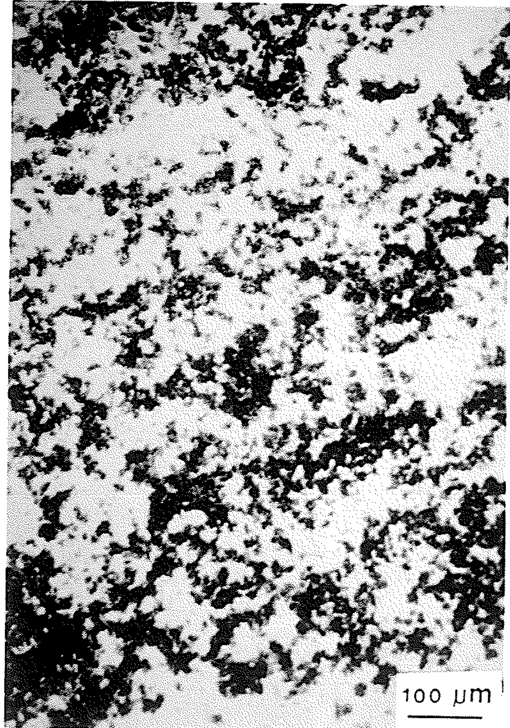
B



C



D



(Fig. 31A), the difference due to the various anions were evident at the onset of cooling and persisted throughout. It appeared that, with vicilin, hydrophobic interactions may have had an influence on both network type and strength. Unfortunately, the high salt concentration required to give this lyotropic effect was not conducive to good network formation so that differences in microstructure and tan δ values for the final products were not too informative. It is possible that the altered electrostatic profile at these concentrations was responsible for the poor network formation.

Hydrophobic Interactions in Relation to Network Characteristics

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 these interactions in heat induced network formation. The impact of hydrophobic 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 few salts which gave usable data and the poor networks at the high salt concentrations 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, 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 influences. In this respect, the data agreed with that obtained by Paulson and Tung (1989) where they found that 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 suppresion of surface hydrophobicity within the corpuscular structure with other salt environments, merely reduced network strength rather than alter the type of network formed.

Urea

Although urea is well known as a protein denaturant, there is no

generally accepted mechanism to explain its effectiveness. Traditional explanations focused on its potential for hydrogen bonding and it was felt that urea could disrupt protein hydrogen bonds. It is questionable, however, whether urea is more potent in this respect than water (Creighton, 1984). As a result, it has also been proposed that in protein denaturation, urea acts indirectly by decreasing hydrophobic interactions; although direct interactions with both the folded and unfolded state can also occur following denaturation (Creighton, 1984). In support of this approach are the high concentrations of denaturant required. Studies with model systems have indicated that urea-water interactions, resulting in displacement of several water molecules by each urea molecule introduced into the solvation shell around the protein, may be the dominant factor in the solubilization of nonpolar residues (Kuharski and Rossky, 1984). As a result, the consensus at this time favors the theory that the influence of urea on hydrophobic interactions has a significant impact on its ability to unfold proteins (Kamoun, 1988).

Although the indirect influence on hydrophobic interactions may be the driving force in denaturation by urea, the binding of urea to proteins has also received considerable attention. The extent of binding has been related to a combination of the number of peptide bonds and the number of aromatic residues in a protein (Prakash et al., 1981; Nandi and Robinson, 1984; Suresh Chandra et al., 1986).

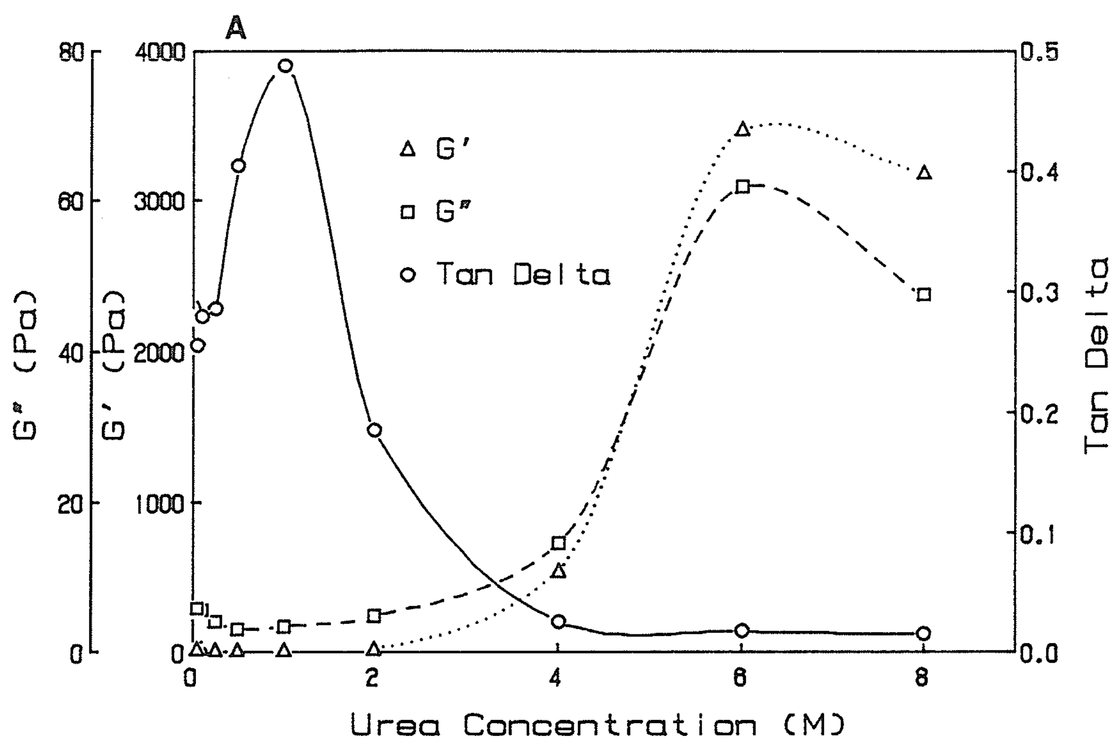
The implication of these mechanisms in terms of protein aggregation and network development is not well established. Aggregation of sesame α-globulin in the presence of low concentrations

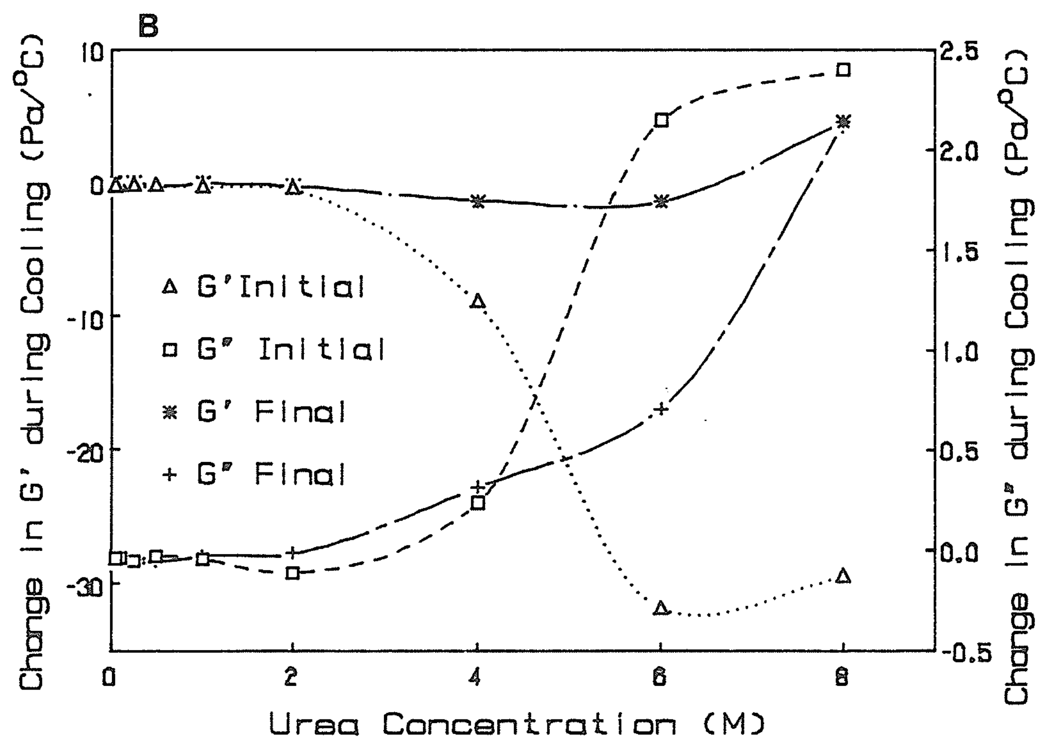
of urea has been attributed to hydrophobic interactions between aliphatic side chains of specific subunits presumably because these groups do not bind to urea (Prakash and Nandi, 1977). For the heat induced networks from the 12S rapeseed glycoprotein, on the other hand, the inclusion of 1 M urea lowered the tan δ value slightly (Gill and Tung, 1978), indicating the formation of a gel-like rather than an aggregated product. It is therefore clear that when examining the influence of urea on the formation of heat induced protein networks, all possible mechanisms must be considered. Hydrophobic interactions, hydrogen bonds, and increased solvation of peptide bonds and aromatic amino acids may all impact the results in some way.

Ovalbumin: At concentrations below 1.0 M, urea had little effect on ovalbumin stability with Td values all about 84.0^{o}C (Appendix 21). At concentrations of 2.0 M and above, however, there was a gradual decrease in stability (from 79.9 to 67.8^{o}C) and at 6.0 and 8.0 M urea, this was accompanied by significant decreases in the ΔH values. The average ΔH value up to 4.0 M urea was 14.5 J/g protein, while at 6.0 M urea it was 10.2 J/g protein and at 8.0 M urea, 4.8 J/g protein. In other words, low concentrations of urea had little effect on ovalbumin conformation, but destabilization and denaturation resulted at higher concentrations.

The low urea concentrations, however, did have a significant impact on the rheological properties of heat induced networks (Fig. 33A; Appendix 11A). Even at the minimum urea concentration examined (0.05 M), the network was characterized by a high tan δ value (0.255) and extremely low G moduli (<25 Pa), particularly when compared

Figure 33. Effect of urea on the rheological properties (A) and rate of structure development (change in G') during initial and final cooling phases (B) for 10% ovalbumin, pH 8.5.


A
G'
G"
Tan Delta
G" (Pa)
G' (Pa)
Tan Delta
Urea Concentration (M)
0
20
40
60
80
1000
2000
3000
4000
2
4
6
8
0.0
0.1
0.2
0.3
0.4
0.5


B
G'Initial
G" Initial
G' Final
G" Final
Change in G' during Cooling (Pa/°C)
Change in G" during Cooling (Pa/°C)
Urea Concentration (M)
10
0
-10
-20
-30
2
4
6
8
2.5
2.0
1.5
1.0
0.5
0.0
-0.5

to previous ovalbumin networks. Increasing the urea concentration up to 1.0 M had no significant impact on the G moduli and tan δ values actually increased indicating further deterioration in network structure. In terms of microstructure, very low urea concentrations (e.g. 0.1 M) produced distinct protein strands which tended to be hydrated and not well crosslinked (Fig. 34). At increased urea concentrations (e.g. 0.5 M) the network strands tended to be thicker and clear. This lead to the interpretation that level of moisture associated with the network had increased. The poor rheological characteristics for these networks seemed to result from increased interactions with the solvent rather than the promotion of protein aggregation.

It was not until conditions which promoted ovalbumin destabilization (2.0 M urea) that the trend towards poor network characteristics was reversed. Higher urea concentrations resulted in remarkable network improvement, such that at 6.0 and 8.0 M urea, the G' modulus was over 3000 Pa and tan δ values were less than 0.02 (Appendix 11A). To give some perspective as to how low this tan δ value was, the lowest tan δ values reported to this point in the study were 0.064 for vicilin at pH 3 and 0.075 for ovalbumin in 0.1 or 0.5 M NaSCN. The improvement in network characteristics was also evident in the microstructure (Fig. 34). With 2.0 M urea, protein strands were more evident than at 0.5 M yet the strand thickness was again indicative of a high level of hydration. At the higher urea concentrations (e.g. 6.0 M), there appeared to be a well crosslinked network, though it was somewhat obscured by the urea crystals in the system. The conditions at

Figure 34. Photomicrographs showing the effect of urea on heat induced networks for 10% ovalbumin, pH 8.5.

A. 0.1 M

B. 0.5 M

C. 2.0 M

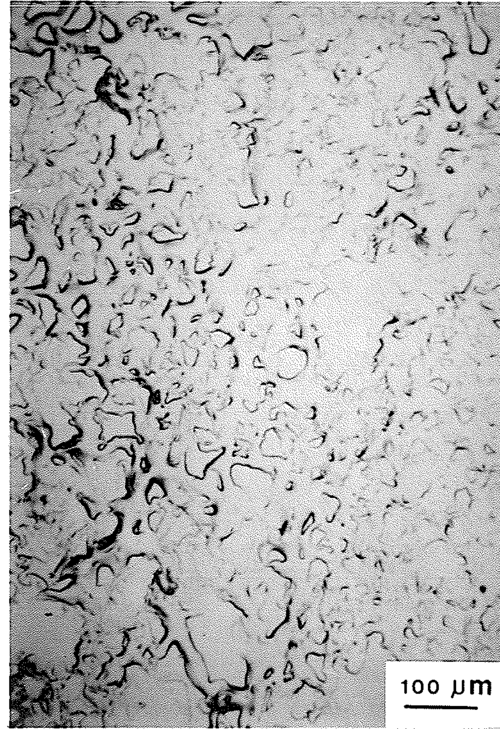
D. 6.0 M

high urea concentrations are such that noncovalent intermolecular protein interactions are inhibited and network formation under these circumstances is thought to be through covalent disulfide linkages (van Kleef et al., 1978). In fact, the characteristics (especially the low tan δ value) of this ovalbumin/urea network are such that in the work of van Kleef et al. (1978), they felt is was appropriate to describe this material using the classical theory of elasticity. In terms of food proteins, this represents an atypical system and not one that can be described as a corpuscular protein gel normally associated with the gelation of globular proteins (Clark and Lee-Tuffnell, 1986). As a result, this type of network provided little information as to the type of interactions normally associated with heat induced network formation. It does, however, demonstrate the potential for disulfide bond formation in the gelation of ovalbumin. The lack of structure at the lower urea levels indicated that disulfide interactions were probably not significant at these concentrations, a conclusion similar to that reached by Prakash and Nandi (1977) when investigating the aggregation of sesame α-globulin.

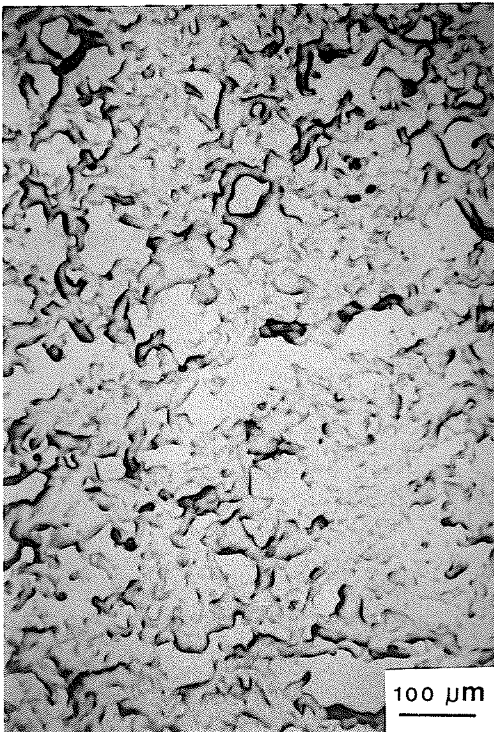
Examination of the data during cooling indicated that increased G′ moduli at high urea concentrations were associated with increased rates of change in G′ during the initial cooling phase only (Fig. 33B; Appendix 12A). Changes in G′ during the final cooling phase were not significantly different up to a concentration of 6.0 M urea. In 8.0 M urea, however, a positive value for the final rate of change in G′ was obtained, indicating structural breakdown. For the G" modulus, changes in rate during both the initial and final cooling phases were observed

A

B



C



D

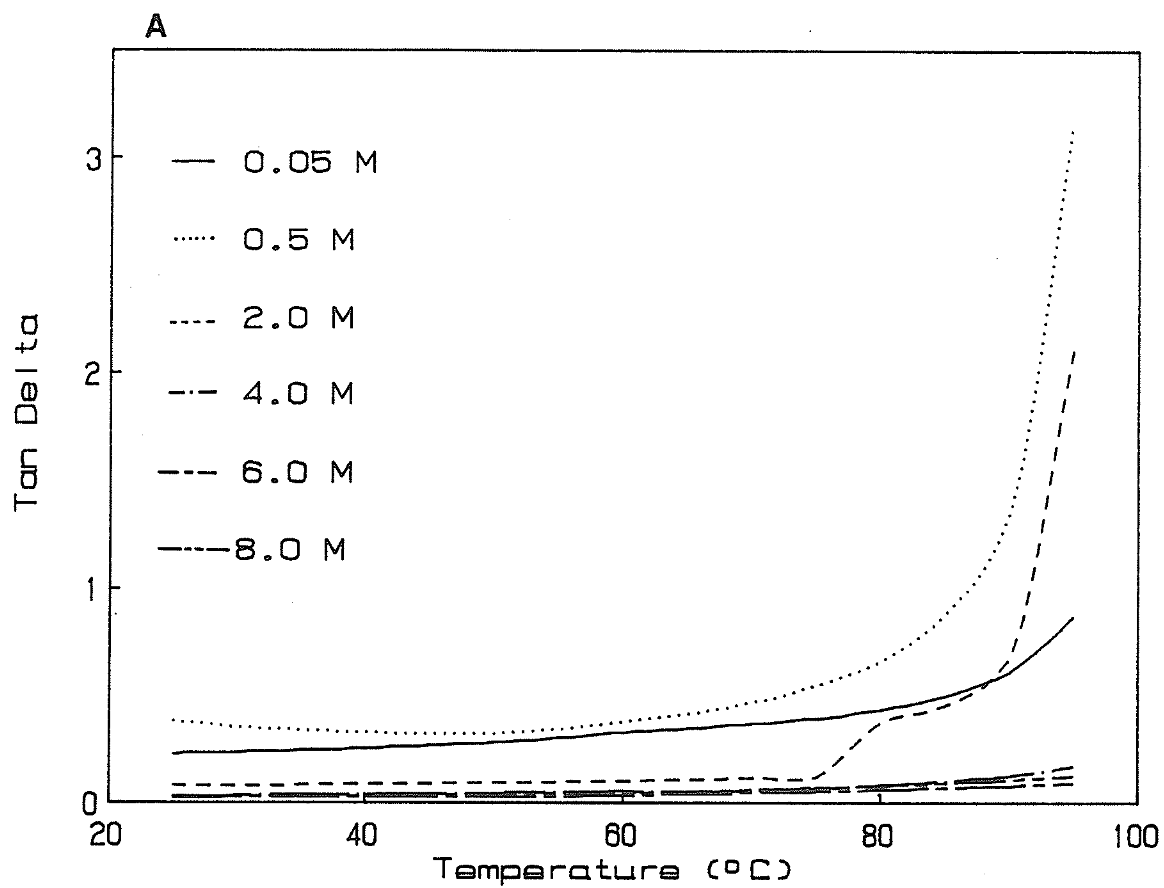
at urea concentrations greater than 2.0 M. In fact, positive values for the rate of change modulus were observed at these concentrations. It would appear that at high urea concentrations, changes during cooling promoted the breakdown of these viscous components in order to strengthen the three dimensional network. Despite evidence of breakdown in the components contributing to G" at high urea concentrations, the G" moduli in the resulting network were significantly higher than at lower urea concentrations (Fig. 33A; Appendix 11A). A possible explanation for this can be seen by examining the changes in tan δ as a function of cooling temperature (Fig. 35A). At low urea concentrations, tan δ values were high, particularly at the beginning of the cooling regime. Therefore, the limited structure which developed did so during the cooling regime. At higher urea concentrations, tan δ values were low at the onset of cooling due to structure development during heating. The changes during cooling merely strengthened the network and incorporated structures contributing to the viscous component into the elastic three dimensional network. It was the overall high degree of interaction in these conditions that made it possible to have relatively high G" moduli (compared to low urea concentrations) despite the very low tan δ values.

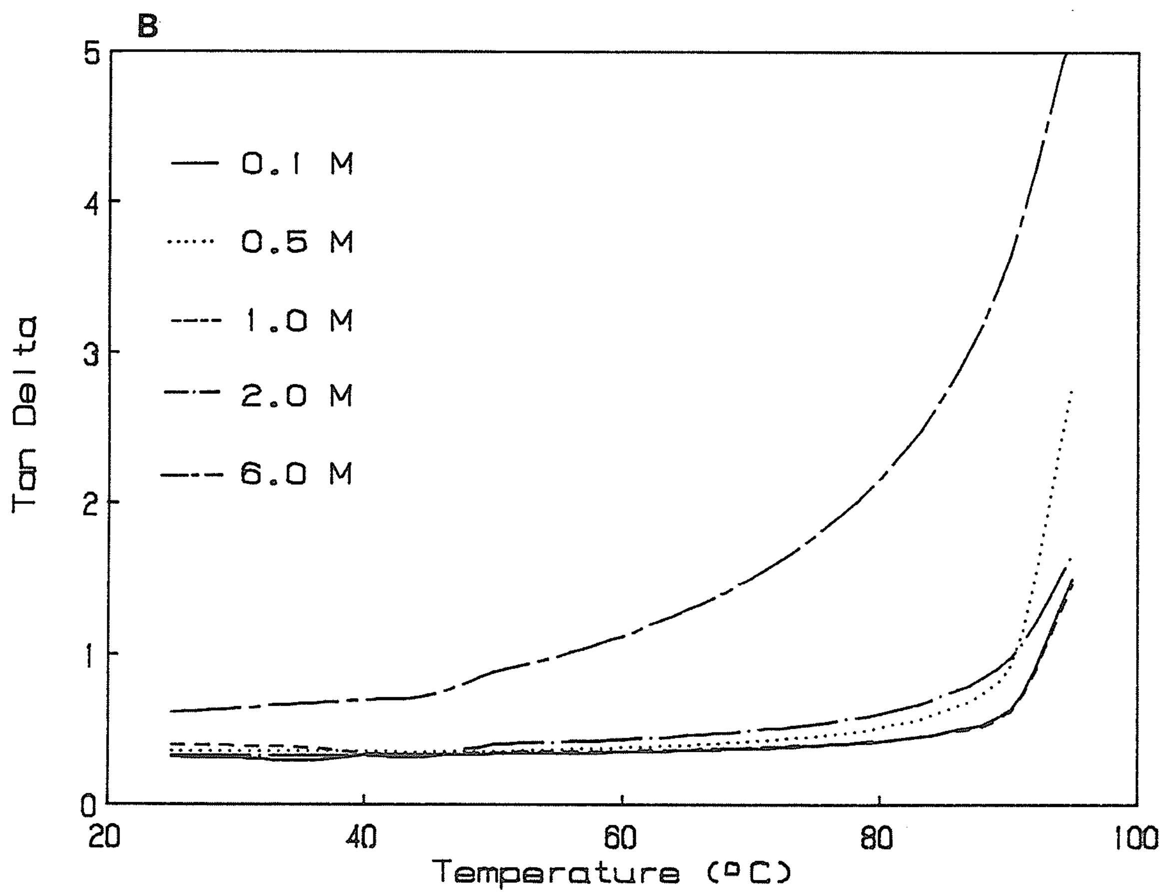
Vicilin: Addition of urea to vicilin resulted in progressive destabilization (decrease in Td values) and denaturation (decrease in ΔH values) of vicilin; however, only at concentration of 1.0 M and higher were these changes significant (Appendix 21). Heat induced networks from vicilin, on the other hand, were affected by minimum urea addition and were characterized by high tan δ (0.296 and up) and low G

Figure 35. Influence of urea on structure development during cooling for heat induced protein networks. Tan delta curves were calculated using a biphasic linear model to describe changes in G' and G".

A. 10% ovalbumin, pH 8.5.

B. 10% vicilin, pH 8.5.

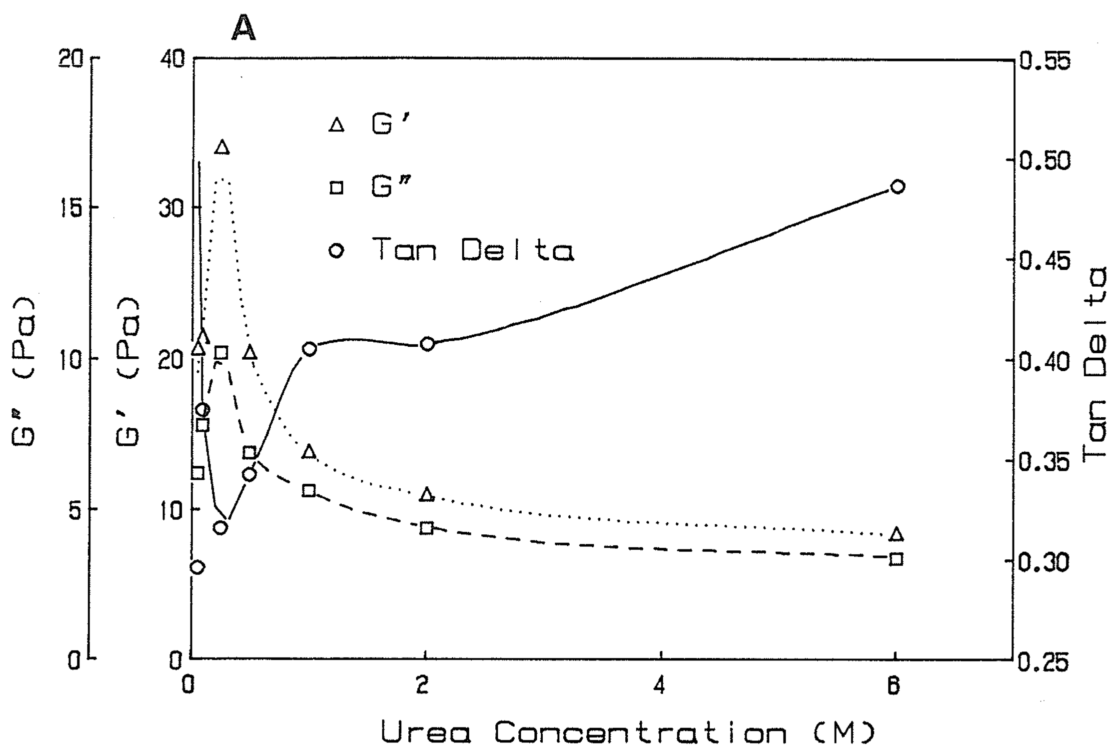

A
Tan Delta
0.05 M
0.5 M
2.0 M
4.0 M
6.0 M
8.0 M
Temperature (°C)
0
1
2
3
20
40
60
80
100


B
Tan Delta
0.1 M
0.5 M
1.0 M
2.0 M
6.0 M
Temperature (°C)
0
1
2
3
4
5
20
40
60
80
100

moduli (Fig. 36A; Appendix 11B). Despite a significant improvement in network characteristics at 0.25 M urea, the general trend indicated that increased urea concentrations resulted in increased deterioration of the network. The changes within this trend were minor; the only significant difference in tan δ values was between the network in 6.0 M urea and those in 0.05 or 0.25 M urea (Appendix 11B). The very poor network characteristics were also evident in the microstructure (Fig. 37). Although a few protein strands were visible in 0.1 M urea, at a urea concentration of 2.0 M only a highly hydrated mass was obtained. Microstructure could not be determined at higher urea concentration due to the fluidity of the products. Like ovalbumin, the inhibition of network formation in the presence of urea, appeared to be related to increased interactions with the solvent rather than the promotion of aggregation.

Unlike ovalbumin, high concentration of urea did not promote network formation. In view of the low levels of cysteine in vicilin (three residues per molecule - Ismond, 1984), it is not surprising that a network based solely on disulfide interactions could not be obtained. This inability to form networks in urea is in contrast to the low tan δ values reported previously for networks formed from the 12S rapeseed glycoprotein in 1.0 M urea (Gill and Tung, 1978). In that study, disulfide bonds were implicated as a possible crosslinking mechanism in matrix formation; it is conceivable that, unlike the situation with vicilin or ovalbumin, in the presence of even this relatively low concentration of urea 1.0 M, disulfide bonds are important to network formation with the rapeseed protein.

Figure 36. Effect of urea on the rheological properties (**A**) and rate of structure development (change in G') during initial and final cooling phases (**B**) for 10% vicilin, pH 8.5.


A
G'
G"
Tan Delta
G" (Pa)
G' (Pa)
Tan Delta
Urea Concentration (M)
0
2
4
6
0
5
10
15
20
0
10
20
30
40
0.25
0.30
0.35
0.40
0.45
0.50
0.55

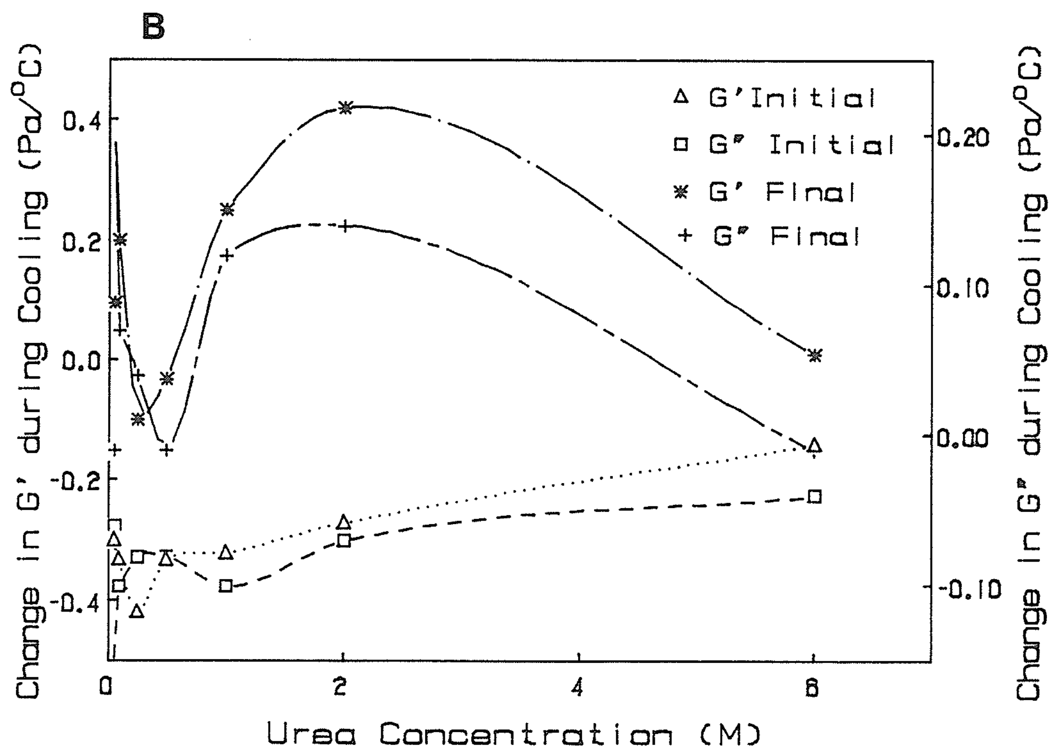
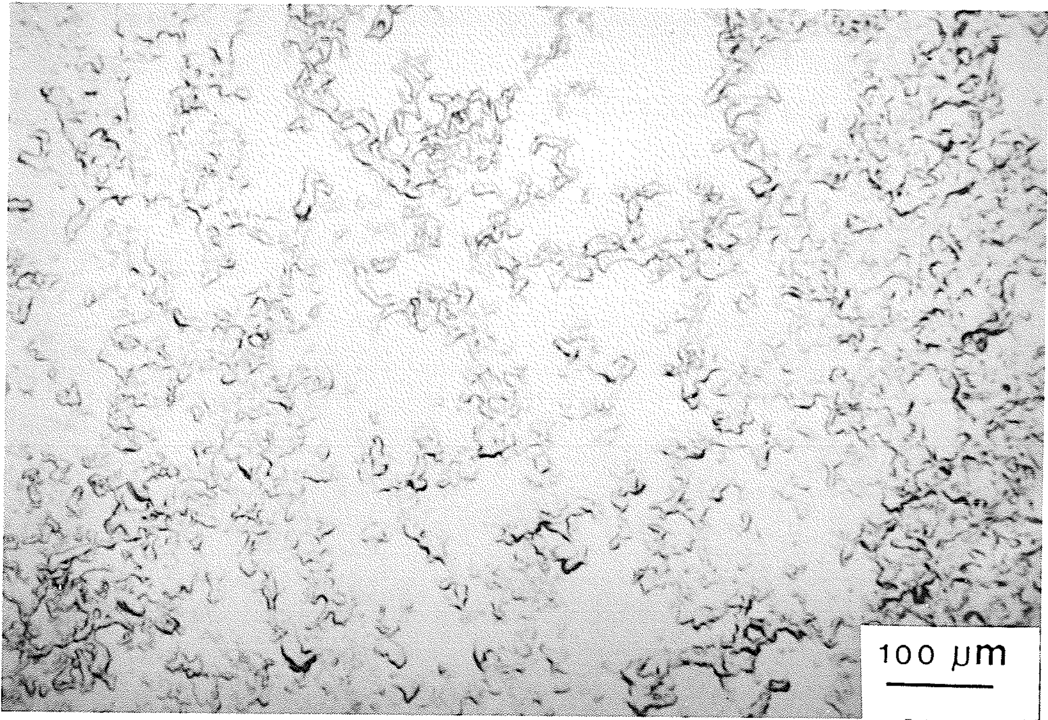

B
G'Initial
G" Initial
G' Final
G" Final
Change in G' during Cooling (Pa/°C)
Change in G" during Cooling (Pa/°C)
Urea Concentration (M)
0
2
4
6
-0.4
-0.2
0.0
0.2
0.4
-0.10
0.00
0.10
0.20

Figure 37. Photomicrographs showing the effect of urea on heat induced networks for 10% vicilin, pH 8.5.

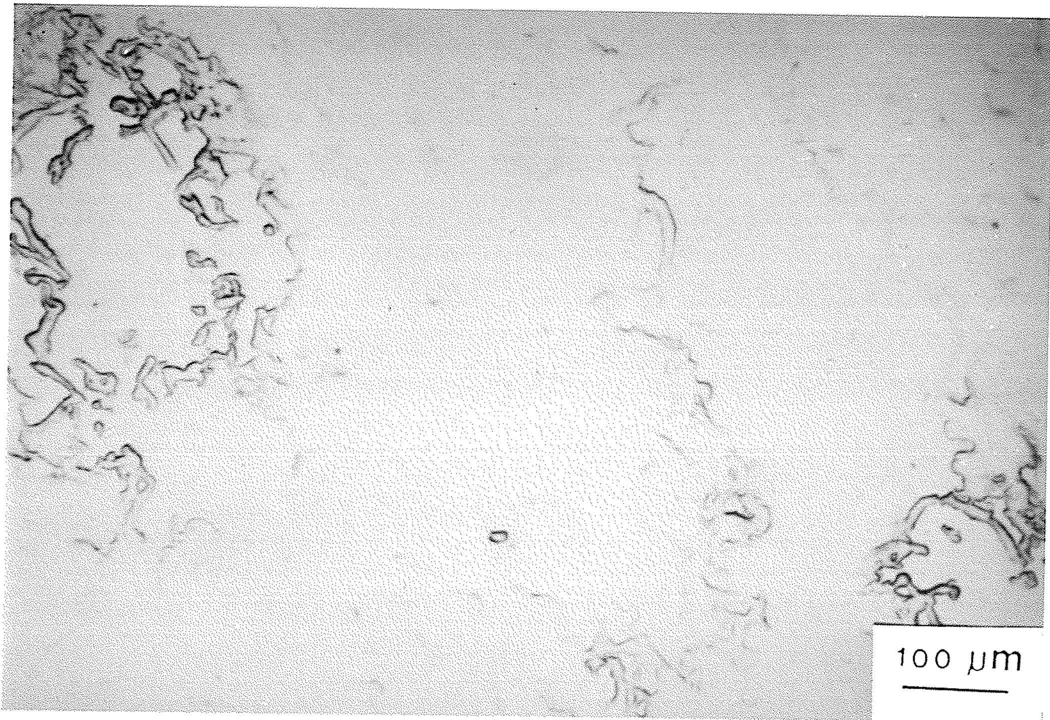
A. 0.1 M

B. 0.5 M

A



B



As with previous vicilin networks, the slight variations in the G moduli that were obtained were primarily associated with changes in the rate of structure development during the final rather than the initial cooling phase (Fig. 36B; Appendix 12B). It should be noted, however, that variation in these rates of change during both initial and final cooling phases were not significant, so that such a comparison is of little value. Similarities were also evident in the changes in tan δ as a function of cooling temperature (Fig. 35B). The only exception was for the network in 6.0 M urea, where the high level of solubility was particularly noticeable at the onset of cooling and despite structural improvements during cooling the tan δ value remained high. As a result, the poor network in 6.0 M urea appeared to be associated with problems during the establishment of the network structure.

Urea in Relation to Network Characteristics

From this study, it is clear that the presence of urea has major influence on the formation of heat induced protein networks. In fact, ever at low concentrations, which did not result in significant protein denaturation for either ovalbumin or vicilin, networks formed upon heat treatment tended to be highly hydrated with poor rheological characteristics.

When considering the influence of urea on the intermolecular protein interactions responsible for network formation, the situation is different from that when examining protein denaturation. A major factor in assessing the mechanism of protein denaturation by urea is an appreciation of the driving force responsible for protein unfolding. It is in establishing this driving force that the influence of urea on

hydrophobic interactions plays such as important role.

With the protein networks in this study, the driving force for protein unfolding was the thermal treatment, so the influence of urea on the intermolecular interactions involved in network formation was probably through the binding of urea to the protein. In this respect, urea has been shown to bind to both the peptide bonds and the aromatic amino acids and thus aid in the solvation of these groups (Prakash et al., 1981; Nandi and Robinson, 1984; Suresh Chandra et al., 1986). Thus, even low concentrations of urea could limit intermolecular protein interactions as was the case in this study.

Due to the potential for urea binding to both peptide bonds and aromatic amino acids, it appears that both hydrogen bonding and hydrophobic interactions could be impaired in the presence of urea. In view of the minor changes in network characteristics (particularly tan δ values) with the various stabilizing salts, it is reasonable to conclude that the influence of urea on the potential formation of intermolecular hydrogen bonds may be the major factor contributing to the poor network characteristics at low urea concentrations. As a result, it appears that hydrogen bonds are important interactions contributing to the attractive component in the balance required for network formation. If the potential for hydrogen bond formation is limited through the addition of urea, then the repulsive electrostatic forces dominate and there is a tendency toward network solvation. The increased solubility of the aromatic residues would also contribute to this increased network solvation. The importance of hydrogen bonds to network strength has been reported previously (Paulson and Tung, 1989),

where increasing gel firmness during cooling implicated the involvement of hydrogen bonds.

At higher urea concentrations, there is an additional influence on protein structure. Specifically, the formation of the corpuscular structure involved in the gelation of globular proteins would be inhibited and the protein would exist as an unfolded polypeptide chain. Furthermore, network formation via noncovalent interactions such as hydrogen bonds and hydrophobic interactions would be inhibited by the binding of urea. This appeared to be the situation with vicilin where no network was formed as these noncovalent interactions were necessary for network formation. With ovalbumin, the presence of the unfolded polypeptide chain increased the availability of sulfhydryl groups and thus promoted network formation via covalent disulfide bonds. This was not possible for vicilin as the number of sulfhydryl groups (cysteine residues) was limited. It should be kept in mind that the formation of the disulfide linked three dimensional networks at high urea concentrations does not implicate disulfide bonds as factors in network formation under other conditions.

Disulfide Bond Modifying Agents

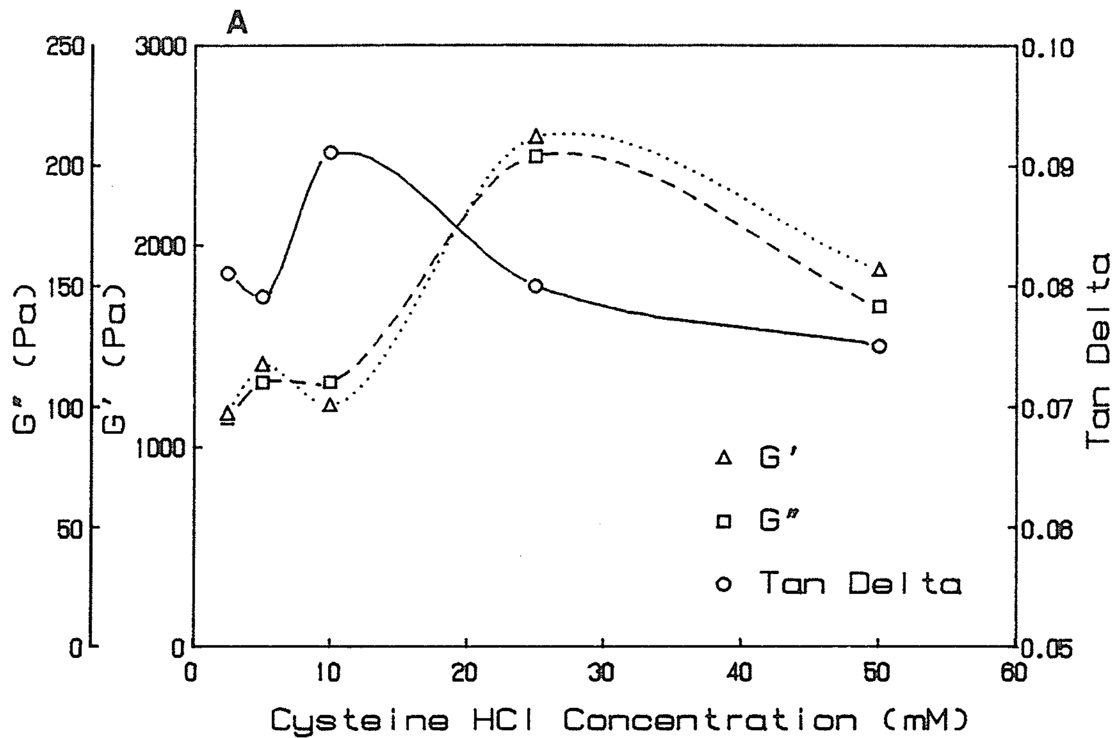
Unlike the other intermolecular protein interactions examined in this study, disulfide bonds are covalent and thus their interactions energies are high. Due to their strength, the presence of disulfide bonds could have a significant impact on network characteristics. The highly elastic gels resulting from the ovalbumin/urea system in which only disulfide interactions could exist demonstrated the potential for this type of interaction. The possibility of disulfide bond formation

varies with the protein and even for protein networks in which disulfide bond formation has been implicated, the exact role of these bonds has not been well established. In order to examine the role of disulfide bonds in network formation for ovalbumin and vicilin, under conditions where noncovalent interactions can also exist, three disulfide modifying agents were included with the protein dispersion prior to the heat treatment. These reagents, including cysteine hydrochloride (CysHCl), 2-mercaptoethanol (ME) and N'-ethylmaleimide (NEM), have all been shown to disrupt disulfide bonds or block their formation (Lakshmi and Nandi, 1979; Schmidt, 1981; Utsumi and Kinsella, 1985; Hayakawa and Nakamura, 1986).

Cysteine Hydrochloride (CysHCl)

Ovalbumin: The inclusion of CysHCl had little influence on the rheological properties of the heat induced ovalbumin networks produced (Fig. 38A). Although the tan δ value with 10 mM CysHCl was significantly higher than at other CysHCl concentrations (Appendix 13A), the tan δ values were all less than 0.1, values normally associated with good network formation. The G moduli increased with the inclusion of 25 mM CysHCl but decreased again at a higher concentration. Examination of the microstructure of some of these networks, showed that at concentrations up to 25 mM CysHCl, good crosslinked networks were formed (Fig. 39). Unlike previous ovalbumin networks, these structures lacked homogeneity and there were distinct patches of amorphous material in the network. The fact that these structures were integrated into the stranded networks would account for the good rheological properties. At a concentration of 50 mM CysHCl,

Figure 38. Effect of cysteine hydrochloride on the rheological properties (**A**) and rate of structure development (change in G') during initial and final cooling phases (**B**) for 10% ovalbumin in 0.15 M NaCl, pH 8.5.


A
G″ (Pa)
G′ (Pa)
Tan Delta
G′
G″
Tan Delta
Cysteine HCl Concentration (mM)

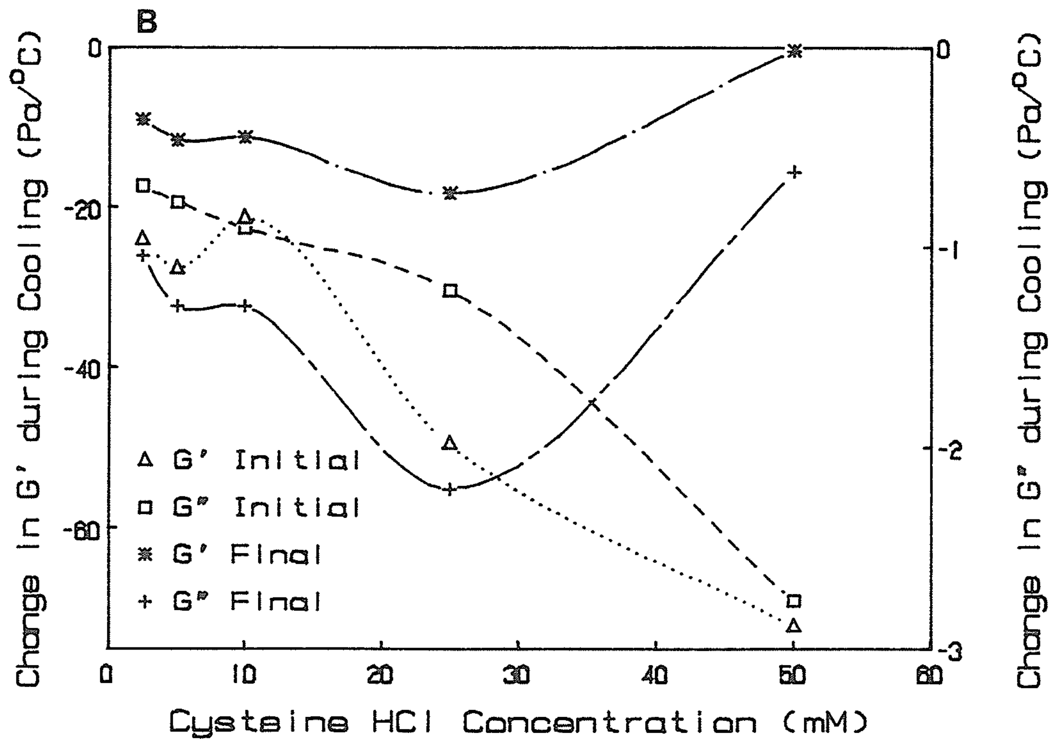

B
Change in G′ during Cooling (Pa/°C)
Change in G″ during Cooling (Pa/°C)
G′ Initial
G″ Initial
G′ Final
G″ Final
Cysteine HCl Concentration (mM)

Figure 39. Photomicrographs showing the effect of cysteine hydrochloride on heat induced networks for 10% ovalbumin in 0.15 M NaCl, pH 8.5.

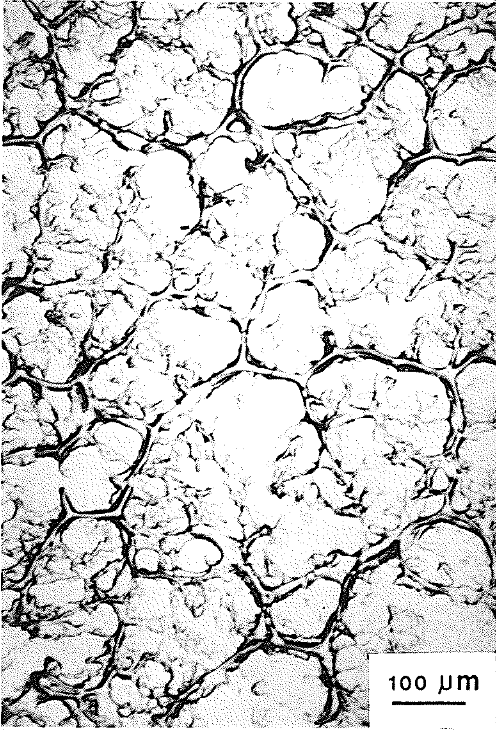
A. 2.5 mM

B. 5 mM

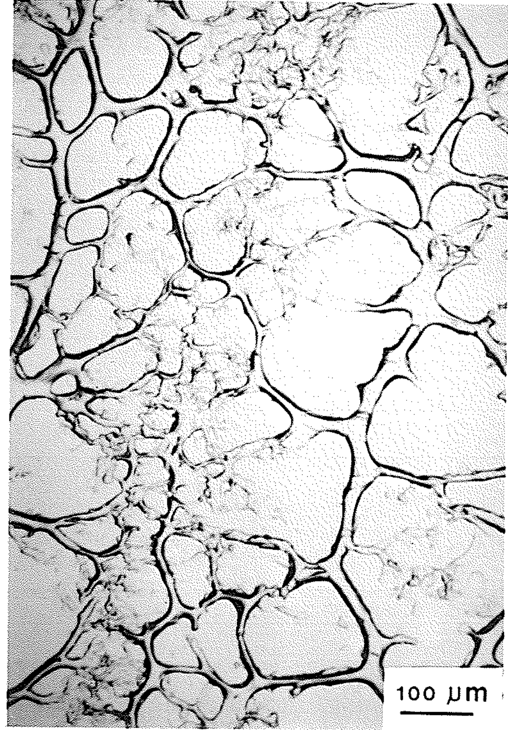
C. 25 mM

D. 50 mM

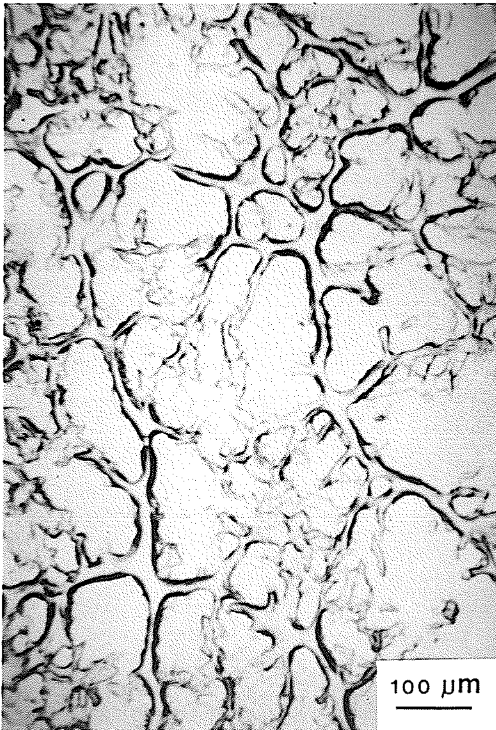
A



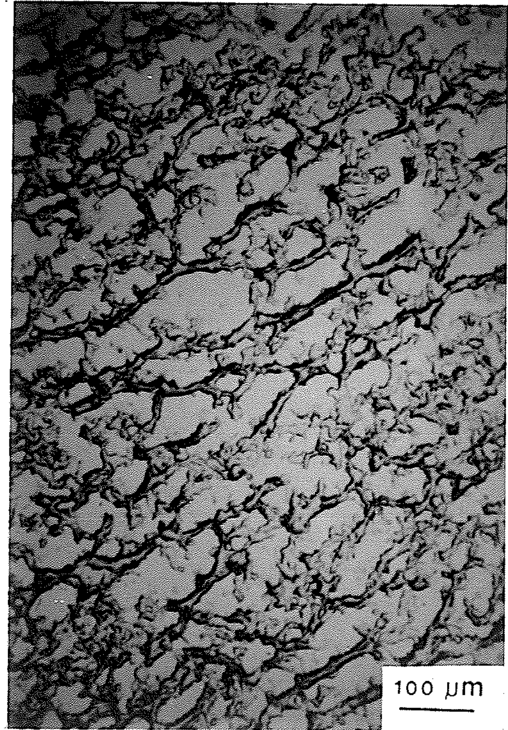
B



C



D



these amorphous structures disappeared and crosslinking within the structure was not as uniform as at lower concentrations. As there was no evidence of ovalbumin denaturation at these CysHCl concentrations based on similar Td (mean = 84.9°C) and ΔH (mean = 15.6 J/g protein) values (Appendix 22), these variations in microstructure probably reflected changes in disulfide bond formation.

Examination of the data obtained during cooling gave some indication of the reasons for the variations in G moduli in the final product (Fig. 38B). For both G′ and G", there was a gradual increase in the rate of structural development during the initial cooling phase, such that the moduli at 25 and 50 mM CysHCl were significantly different (Appendix 13B). A gradual increase in the rate of structure 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 effect of the increase in the rate of change in the G moduli during the initial cooling phase was counteracted by the drastic decrease during the final cooling phase, thus accounting for the slightly lower G moduli in the final product.

In attempting to explain these results, it must be kept in mind that CysHCl has the ability to disrupt both intramolecular and intermolecular disulfide bonds. Although there was no evidence of conformational changes in ovalbumin due to the effect of CysHCl on intramolecular disulfide bonds at these concentrations, the unfolding of the molecule during heat treatment may be sufficient to expose the disulfide bonds to this modifying agent. The disruption of

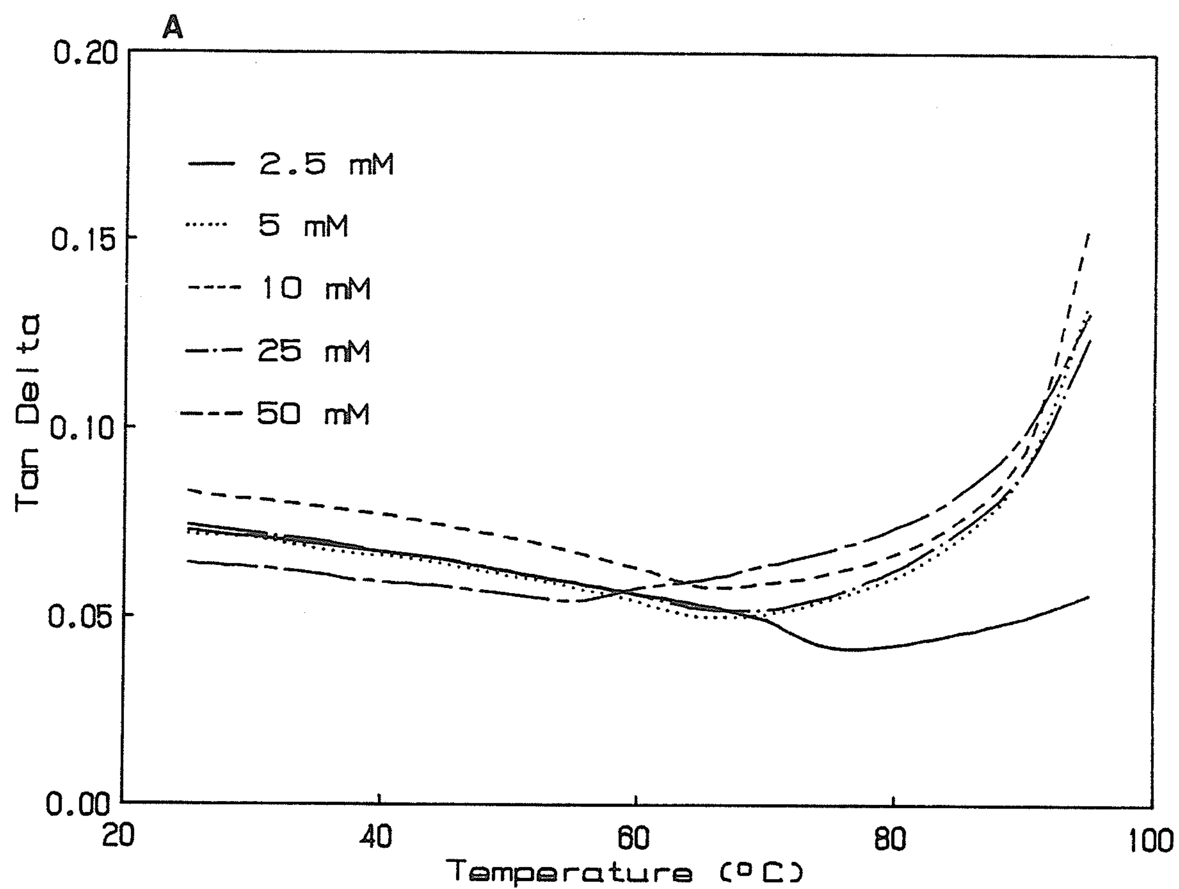
intramolecular disulfide bonds during this heat treatment could have promoted a rearrangement in the corpuscular structure involved in strand formation such that intermolecular protein interactions were encouraged. These interactions could include both covalent (disulfide bonds) and noncovalent associations. This would account for the higher G moduli in 25 mM CysHCl and the increase in the rate of change in the G moduli during the initial cooling phase at CysHCl concentrations of 25 and 50 mM. At a concentration of 50 mM CysHCl, however, there was a substantial reduction in the rate of change in the G moduli during the final cooling phase. At this concentration CysHCl appeared to be preventing the formation of intermolecular disulfide bonds. It was this combination of factors that was presumably responsible for the G moduli being maximized at 25 mM CysHCl. 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), though the concentrations of the modifying agent in these cases were lower (< 10 mM for each).

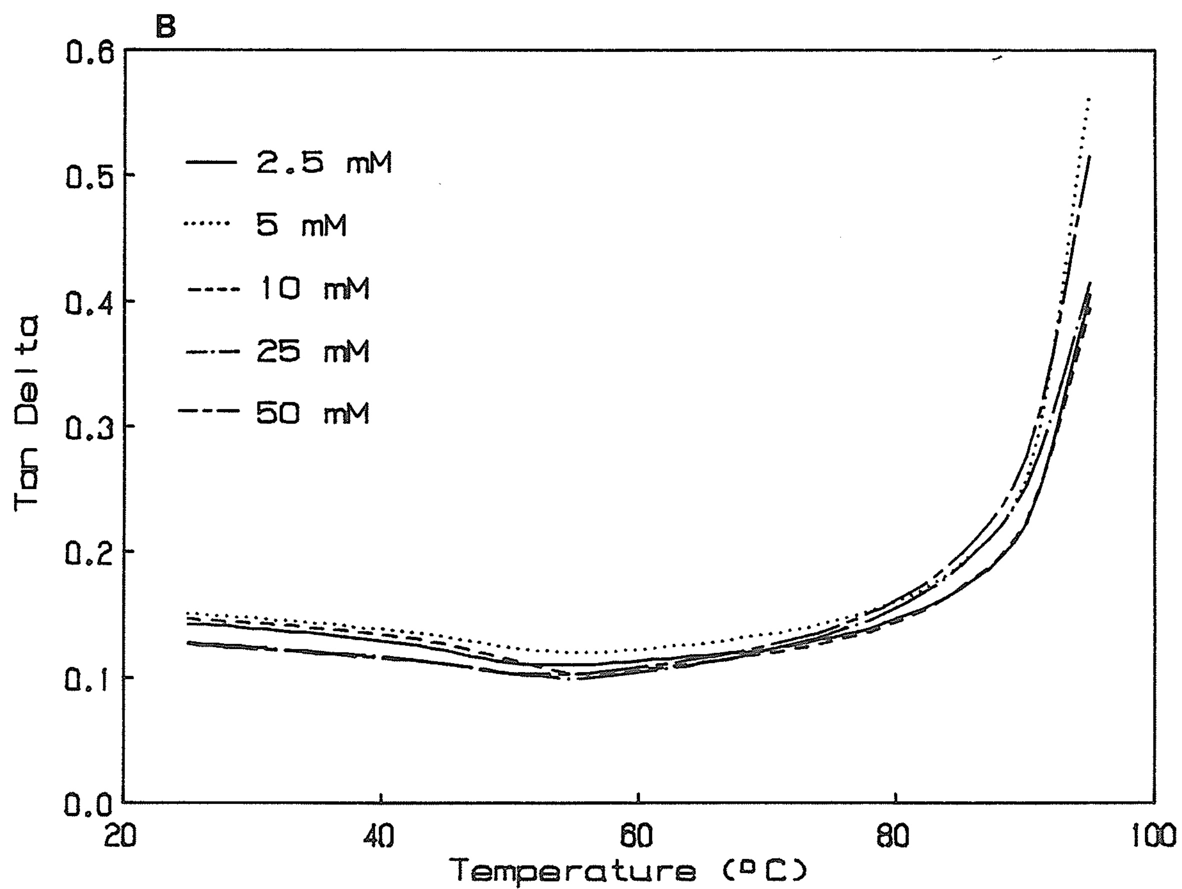
The similarity in tan δ values at different CysHCl concentrations was also reflected in the changes in tan δ as a function of cooling temperature (Fig. 40A). It would appear that the counteracting influences during the initial and final cooling phases involved both the viscous and elastic components. The impact of CysHCl on disulfide bonds, therefore, was related to changes in both the viscous and elastic components of the network. The shapes of all the tan δ cooling curves for ovalbumin in CysHCl were slightly different from those seen previously and obtained for vicilin under the same conditions

Figure 40. Influence of cysteine hydrochloride on structure development during cooling for heat induced protein networks. Tan delta curves were calculated using a biphasic linear model to describe changes in G' and G".

A. 10% ovalbumin in 0.15 M NaCl, pH 8.5.

B. 10% vicilin in 0.15 M NaCl, pH 8.5.


A
0.20
0.15
0.10
0.05
0.00
Tan Delta
2.5 mM
5 mM
10 mM
25 mM
50 mM
20
40
60
80
100
Temperature (°C)


B
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Tan Delta
2.5 mM
5 mM
10 mM
25 mM
50 mM
20
40
60
80
100
Temperature (°C)

(Fig. 40B). The ovalbumin curves were characterized by a gradual increase in the tan value at the lower end of the cooling range. This may represent some disruption of disulfide bonds at all CysHCl concentrations, resulting in a reduction in network elasticity. The impact of this disruption on the final network characteristics was minimal as seen by the similarities in tan δ values.

Vicilin: At the levels of CysHCl used in this investigation, there were no significant changes in the rheological characteristics of heat induced vicilin networks (Table 4A) despite significant denaturation at concentrations of 25 and 50 mM CysHCl (Appendix 22). The microstructure for these networks were also comparable, although there did appear to be an increase in the intensity of strands at the 50 mM CysHCl level (Fig. 41). Unlike the situation with ovalbumin, there was no conflicting trend with respect to the initial and final phases of the cooling regime as no significant differences were observed for these data (Table 4B). This was also reflected in the similarity of the curves monitoring the changes in tan δ as a function of cooling temperature (Fig. 40B). These curves were typical of those which resulted in good vicilin network formation. As a result, there was no evidence to implicate disulfide bond involvement in vicilin network formation at these CysHCl concentrations.

Mercaptoethanol (ME) and N'-ethylmaleimide (NEM)

Due to the similarity in the data obtained for these two disulfide modifying agents, they will be discussed together. As was the case with CysHCl, the levels of reagent used in these studies was not sufficient to alter the conformation of native ovalbumin or vicilin according to

TABLE 4A. Effect of cystine hydrochloride on the rheological properties of heat set vicilin (10% in 0.15 M NaCl, pH 8.5).

Concentration (mM)	G′ (Pascals)	G″ (Pascals)	Tan δ
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]

[1] Column values followed by the same letter are not significantly different (P≤0.05).

TABLE 4B. Effect of cysteine hydrochloride on the rate of structure development during cooling 95°C to 25°C at 2°C/min for vicilin (10% in 0.15 M NaCl, pH 8.5).

Concentration	Initial Cooling Phase		Final Cooling Phase	
(mM)	G′ (Pa/°C)	G″ (Pa/°C)	G′ (Pa/°C)	G″ (Pa/°C)
2.5	-6.32±2.02[a]	-0.52±0.18[a]	-12.5±3.7[ab]	-2.22±0.68[ab]
5.0	-5.61±0.92[a]	-0.50±0.05[a]	-10.0±2.2[b]	-1.78±0.41[ab]
10.0	-5.67±0.56[a]	-0.40±0.02[a]	-18.8±1.1[a]	-3.18±0.40[a]
25.0	-6.64±0.12[a]	-0.34±0.04[a]	-10.5±0.5[b]	-1.73±0.04[ab]
50.0	-5.46±0.29[a]	-0.31±0.05[a]	-8.1±0.5[b]	-1.31±0.06[b]

[1] Column values followed by the same letter are not significantly different (P≤0.05).

Figure 41. Photomicrographs showing the effect of cysteine hydrochloride on heat induced networks for 10% vicilin in 0.15 M NaCl, pH 8.5.

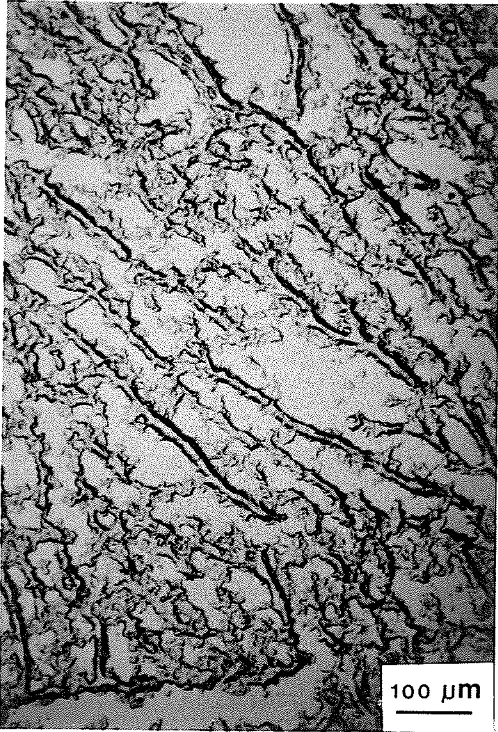
A. 5 mM

B. 10 mM

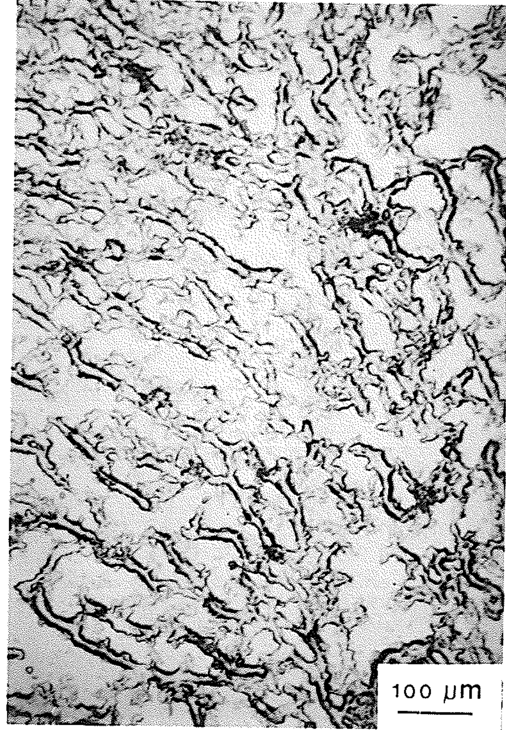
C. 25 mM

D. 50 mM

A

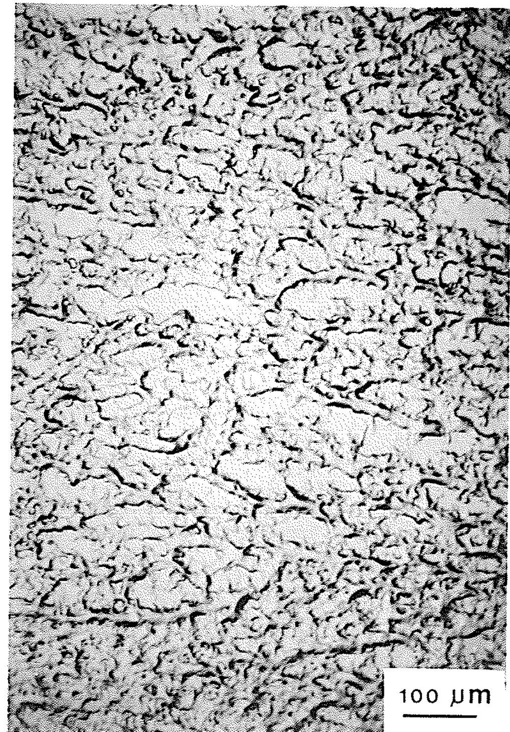


B



C

D

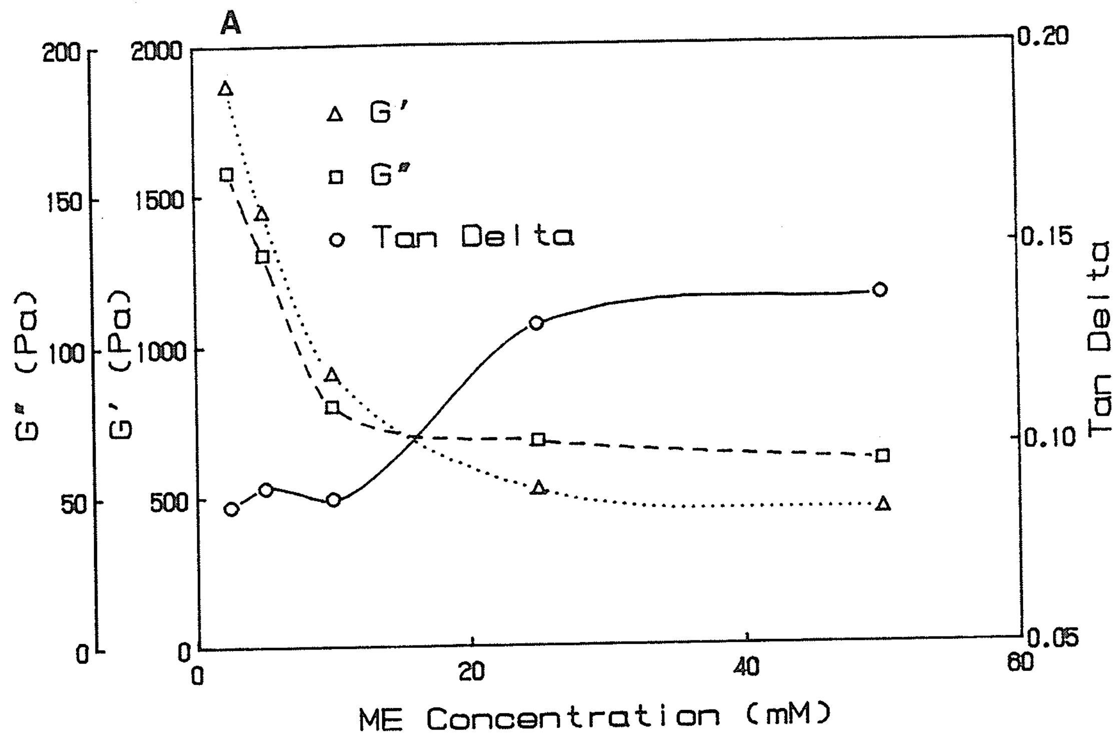


DSC data (Appendices 23A and 23B).

Ovalbumin: The inclusion of increasing concentrations of ME resulted in a gradual decrease in the G moduli up to a concentration of 10 mM; G moduli between 10 and 50 mM were not significantly different (Fig. 42; Appendix 14A). The type of structure was not affected by the initial decrease in the G moduli, but differences in the influence on 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) contained well crosslinked networks (Fig. 43). The appearance of amorphous areas within the network at 5 mM ME may account for the decrease in the 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 well crosslinked. To keep perspective, however, the microstructure in these networks was comparable to what has been rated as reasonably good networks for vicilin (e.g. vicilin in CysHCl - Table 4A; Fig. 41).

Based on the data obtained during cooling; the differences in the characteristics of the resulting network could be attributed to changes which occurred during the final phase of the cooling regime (Fig. 42B; Appendix 14B). With the exception of an unexplained lower rate of change in G' in 10 mM ME, there were no differences in the rate of change in G' or G" during the initial cooling phase as a consequence of ME inclusion. For the final cooling phase, however, there was a decrease in the rate of structure development with increasing ME concentration such that the values at 25 and 50 mM ME were

Figure 42. Effect of mercaptoethanol on the rheological properties (A) and rate of structure development (change in G') during initial and final cooling phases (B) for 10% ovalbumin in 0.15 M NaCl, pH 8.5.


A
G'
G"
Tan Delta
G" (Pa)
G' (Pa)
Tan Delta
ME Concentration (mM)
200
150
100
50
0
2000
1500
1000
500
0
0.20
0.15
0.10
0.05
0
20
40
60

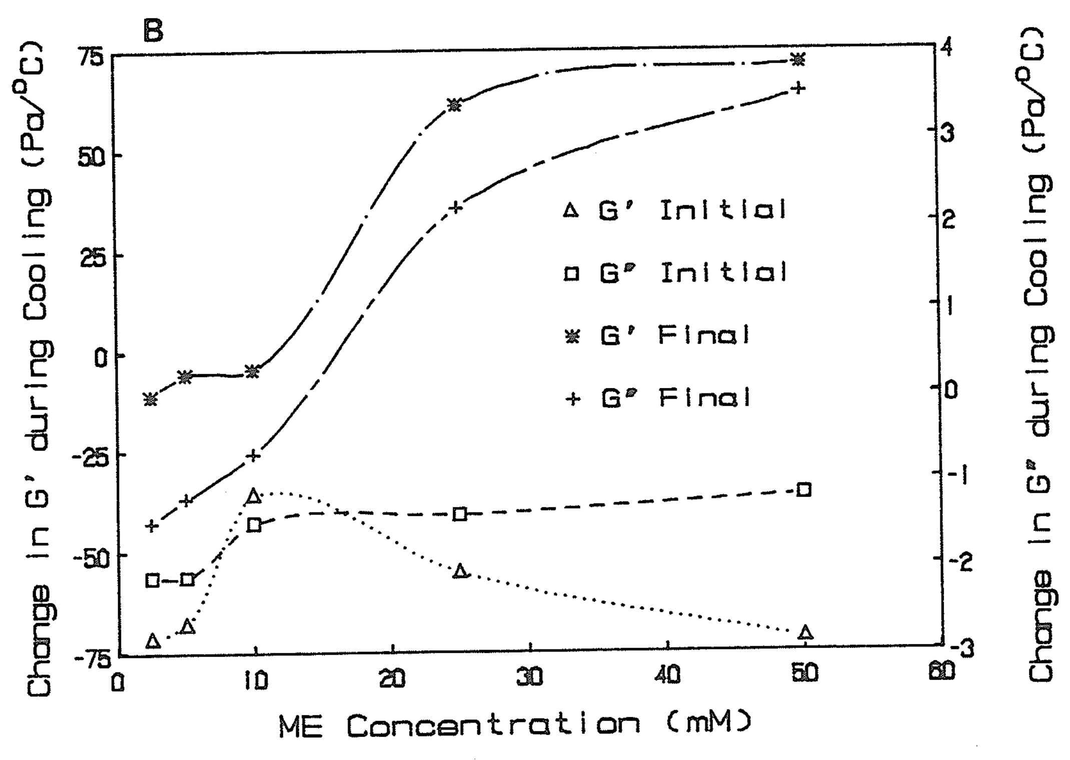

B
G' Initial
G" Initial
G' Final
G" Final
Change in G' during Cooling (Pa/°C)
Change in G" during Cooling (Pa/°C)
ME Concentration (mM)
75
50
25
0
-25
-50
-75
4
3
2
1
0
-1
-2
-3
0
10
20
30
40
50
60

Figure 43. Photomicrographs showing the effect of mercaptoethanol on heat induced networks for 10% ovalbumin in 0.15 M NaCl, pH 8.5.

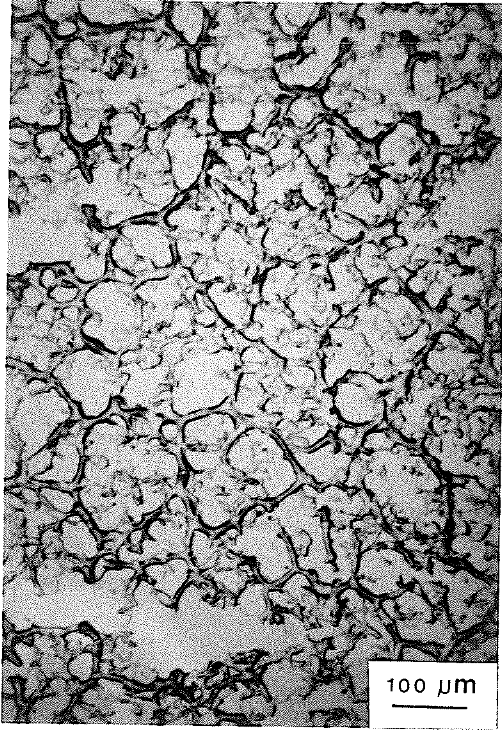
A. 2.5 mM

B. 5 mM

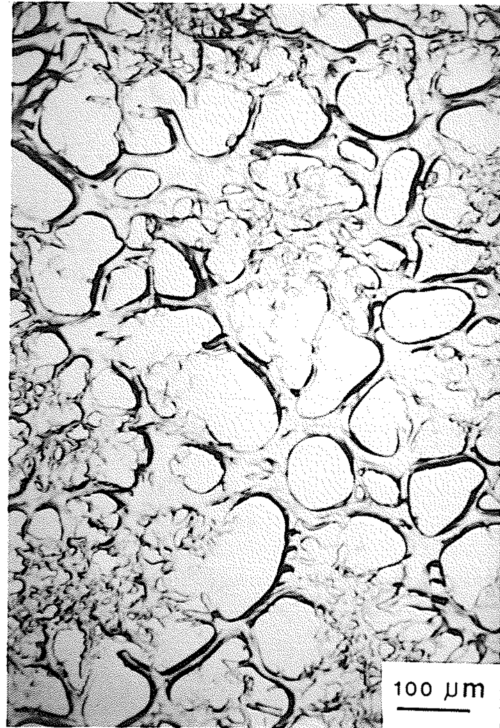
C. 25 mM

D. 50 mM

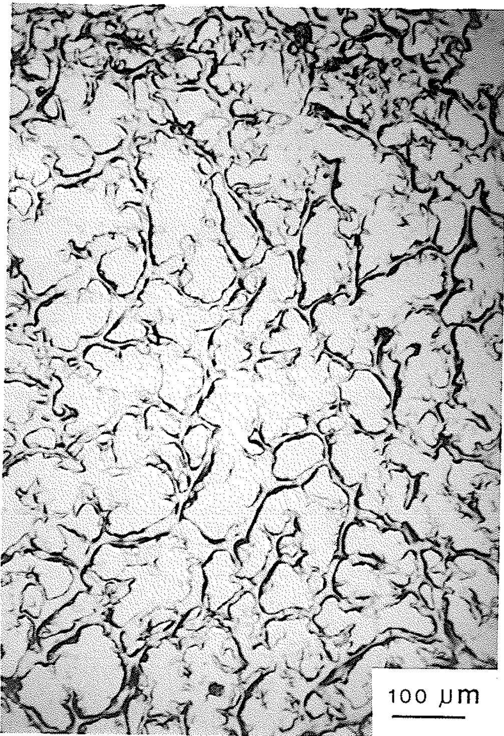
A



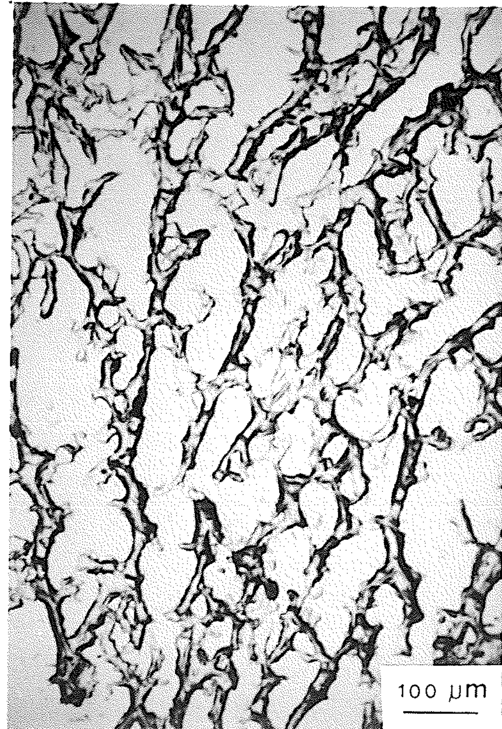
B



C



D



significantly different. In fact, at these high ME concentrations, the rates of change in the G moduli were positive, indicative of structural breakdown. While ME did not alter the conformation of the native ovalbumin, it did appear 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 crosslinking 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.

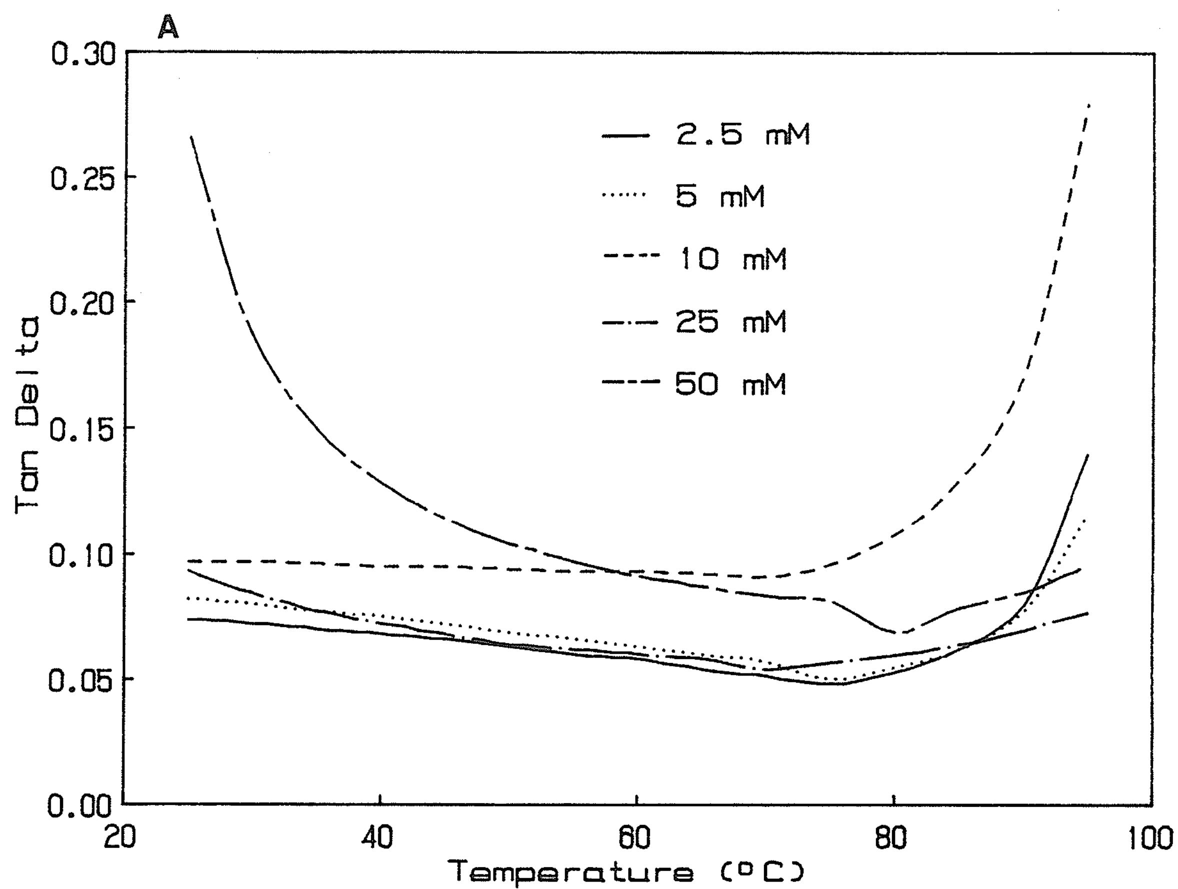
The influence of ME on gel structure was also seen in the changes in tan δ as a function of cooling temperature (Fig. 44A). As was the case with CysHCl, the cooling curves were characterized by a gradual deterioration in gel structure (increase in tan δ values) at lower cooling temperatures. With 50 mM ME, the deterioration was such that the increase in tan δ was significant. This indicated a loss in network integrity at this late stage in the cooling process.

The results with NEM were similar. Both G moduli and tan δ values were fairly constant up to a concentration of 25 mM, after which the network appeared to fall apart (Fig. 45A; Appendix 14A). The extremely low G moduli (< 20 Pa) and high tan δ value (0.473) with 50 mM NEM was indicative of complete destruction of the network. The microstructure of these networks was reasonably well crosslinked at low NEM concentrations (Fig. 46). A reduction in crosslinking was evident at 25 mM NEM and with 50 mM NEM, only short isolated protein fragments were observed. These fragments at 50 mM NEM supported the rheological data

Figure 44. Influence of mercaptoethanol on structure development during cooling for heat induced protein networks. Tan delta curves were calculated using a biphasic linear model to describe changes in G' and G".

A. 10% ovalbumin in 0.15 M NaCl, pH 8.5.

B. 10% vicilin in 0.15 M NaCl, pH 8.5.


A
0.30
0.25
0.20
0.15
0.10
0.05
0.00
20
40
60
80
100
Tan Delta
Temperature (°C)
2.5 mM
5 mM
10 mM
25 mM
50 mM

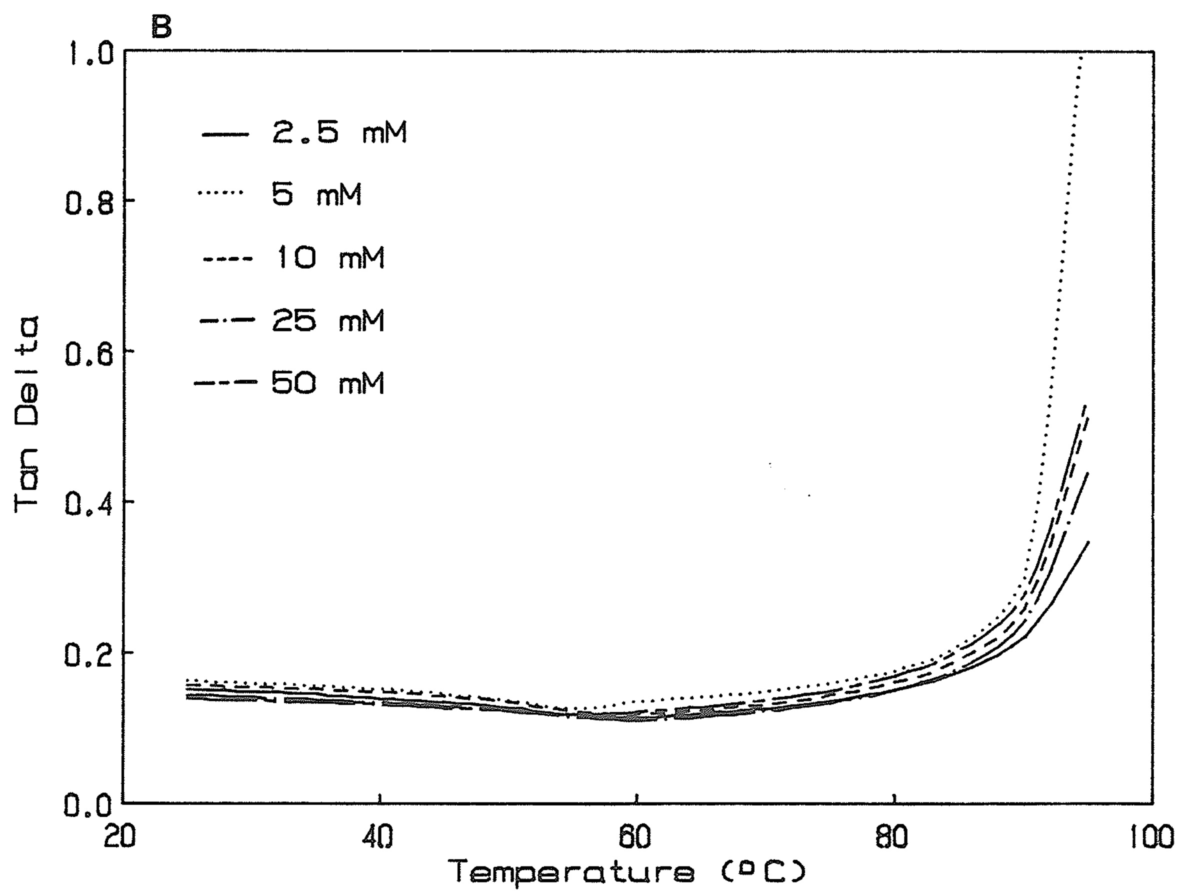
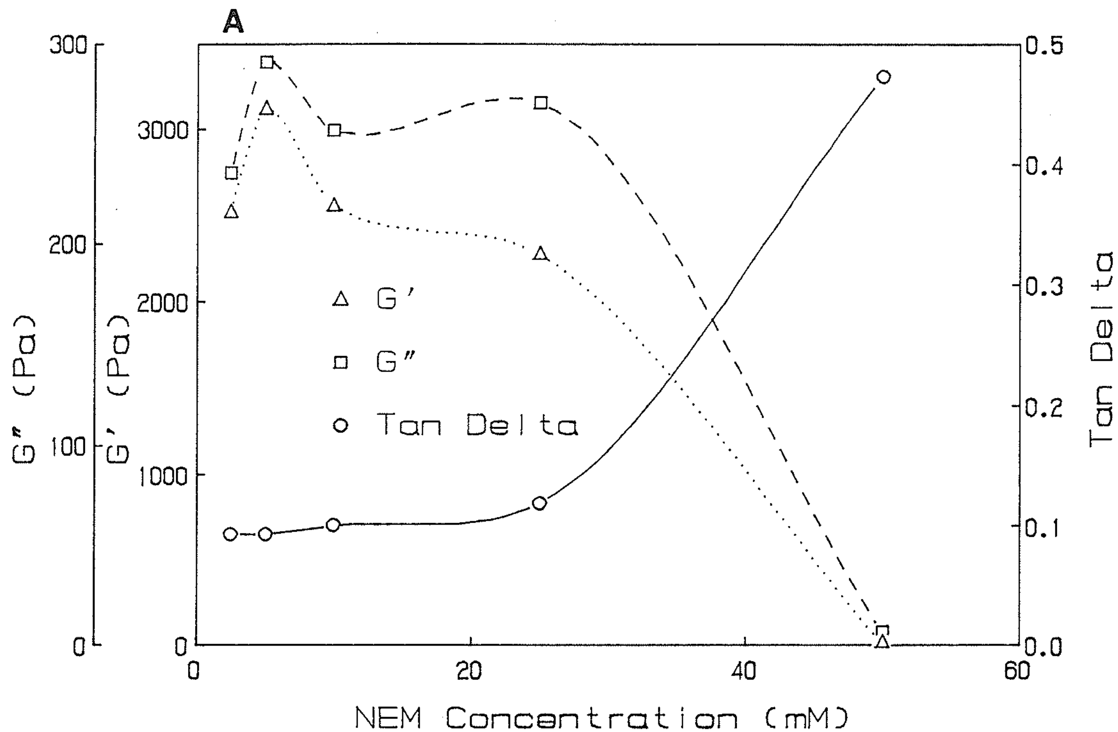

B
1.0
0.8
0.6
0.4
0.2
0.0
20
40
60
80
100
Tan Delta
Temperature (°C)
2.5 mM
5 mM
10 mM
25 mM
50 mM

Figure 45. Effect of N' - ethylmaleimide on the rheological properties (**A**) and rate of structure development (change in G') during initial and final cooling phases (**B**) for 10% ovalbumin in 0.15 M NaCl, pH 8.5.


A
G″ (Pa)
G′ (Pa)
Tan Delta
△ G′
□ G″
○ Tan Delta
NEM Concentration (mM)
300
200
100
0
3000
2000
1000
0
0.5
0.4
0.3
0.2
0.1
0.0
20
40
60

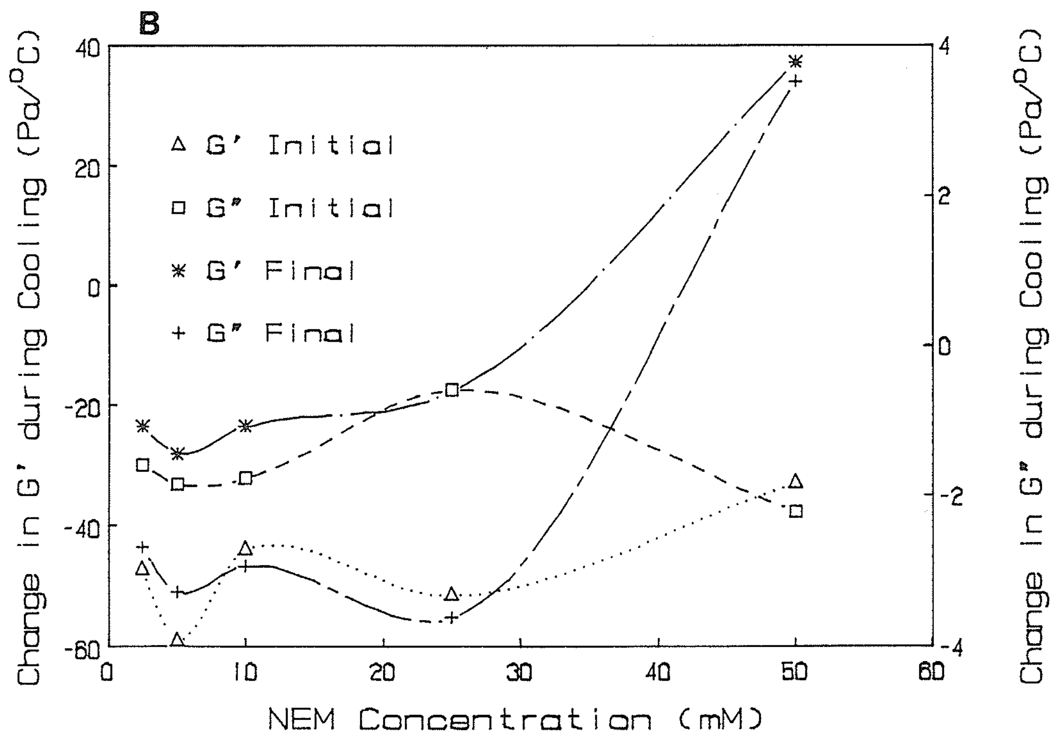

B
Change in G′ during Cooling (Pa/°C)
Change in G″ during Cooling (Pa/°C)
△ G′ Initial
□ G″ Initial
∗ G′ Final
+ G″ Final
NEM Concentration (mM)
40
20
0
-20
-40
-60
4
2
0
-2
-4
10
20
30
40
50
60

Figure 46. Photomicrographs showing the effect of N' - ethylmaleimide on heat induced networks for 10% ovalbumin in 0.15 M NaCl, pH 8.5.

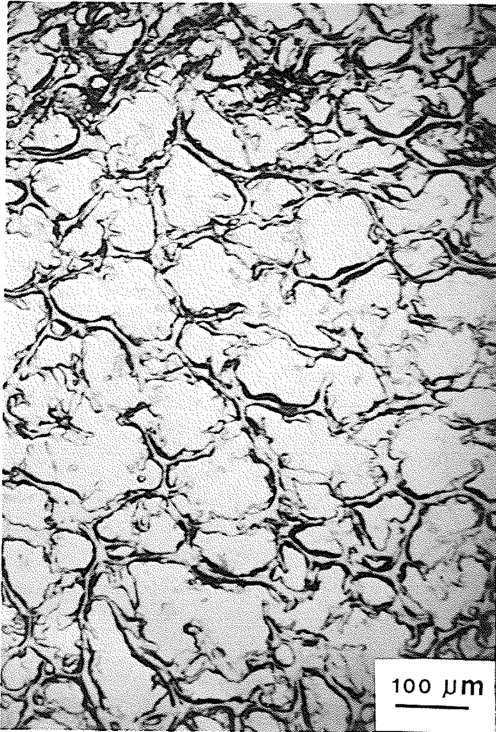
A. 2.5 mM

B. 10 mM

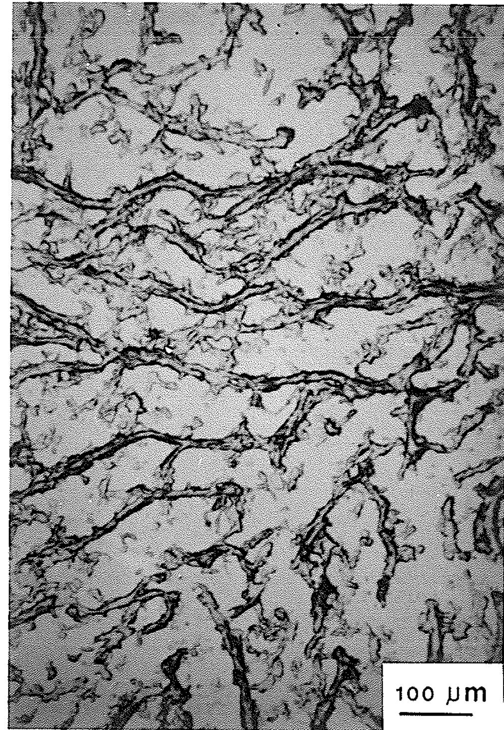
C. 25 mM

D. 50 mM

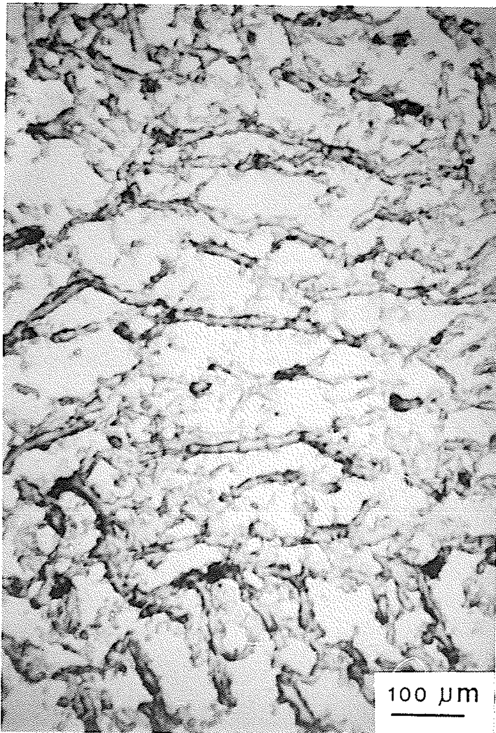
A



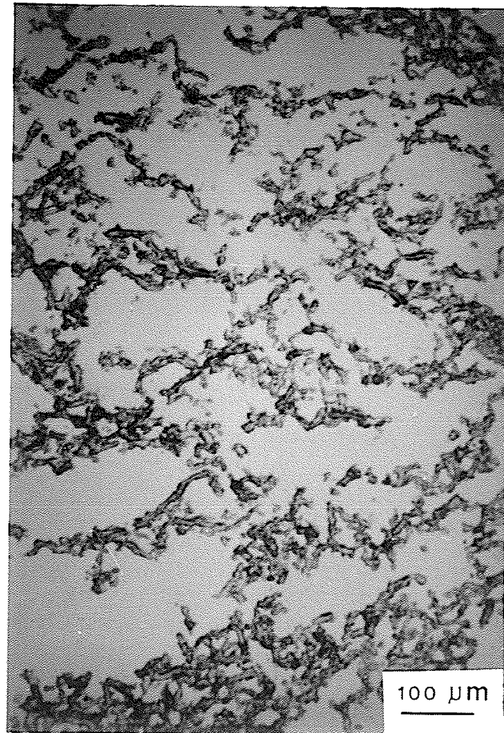
B



C



D



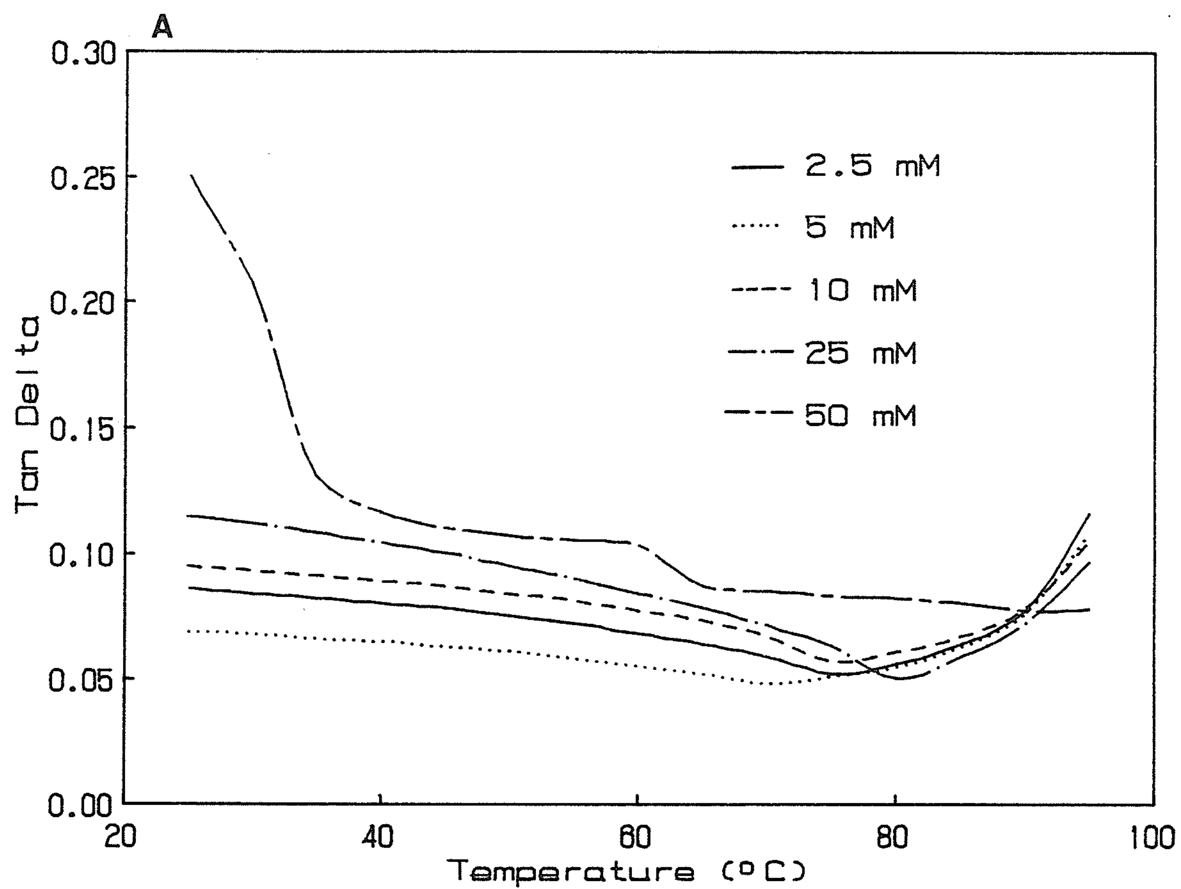
indicating no crosslinked network formation. As with ME, the dramatic change in network characteristics resulted from changes in the rate of structural development during the final cooling phase (Fig. 45B; Appendix 14B). Furthermore, the values for the rate of change in G′ and G″ in the 50 mM NEM environment were positive, indicative of structural breakdown, as was the situation at high ME concentrations. Evidence of structural breakdown was also seen in the curves monitoring tan δ as a function of cooling temperature (Fig. 47A). Up to a concentration of 25 mM NEM, the curves were similar to those for other disulfide modifying agents, but at 50 mM NEM, there was a dramatic increase in the tan δ values during the latter stages of cooling. It is interesting to note that the deterioration of network structure with NEM at a concentration of 50 mM was considerably greater than with 50 mM ME. Although NEM is used to block disulfide bond formation, it does contain two carbonyl groups which can readily participate in hydrogen bonding. It is possible that this additional influence inhibited the formation of intermolecular hydrogen bonds and thus contributed to the poor network characteristics obtained.

Vicilin: As was the case with CysHCl, the addition of ME or NEM had no impact on the rheological properties of heat induced vicilin networks (Tables 5 and 6). The microstructure, however, did indicate slight differences at higher ME and NEM concentrations. In ME, there was a noticeable thickening of the strands at 25 mM and an increase in the number of strands at 50 mM (Fig. 48). Networks formed in the presence of NEM were similar up to a concentration of 50 mM NEM, where there was a lose of uniformity and some evidence of network collapse

Figure 47. Influence of N' - ethylmaleimide on structure development during cooling for heat induced protein networks. Tan delta curves were calculated using a biphasic linear model to describe changes in G' and G".

A. 10% ovalbumin in 0.15 M NaCl, pH 8.5.

B. 10% vicilin in 0.15 M NaCl, pH 8.5.


A
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Tan Delta
20
40
60
80
100
Temperature (°C)
2.5 mM
5 mM
10 mM
25 mM
50 mM

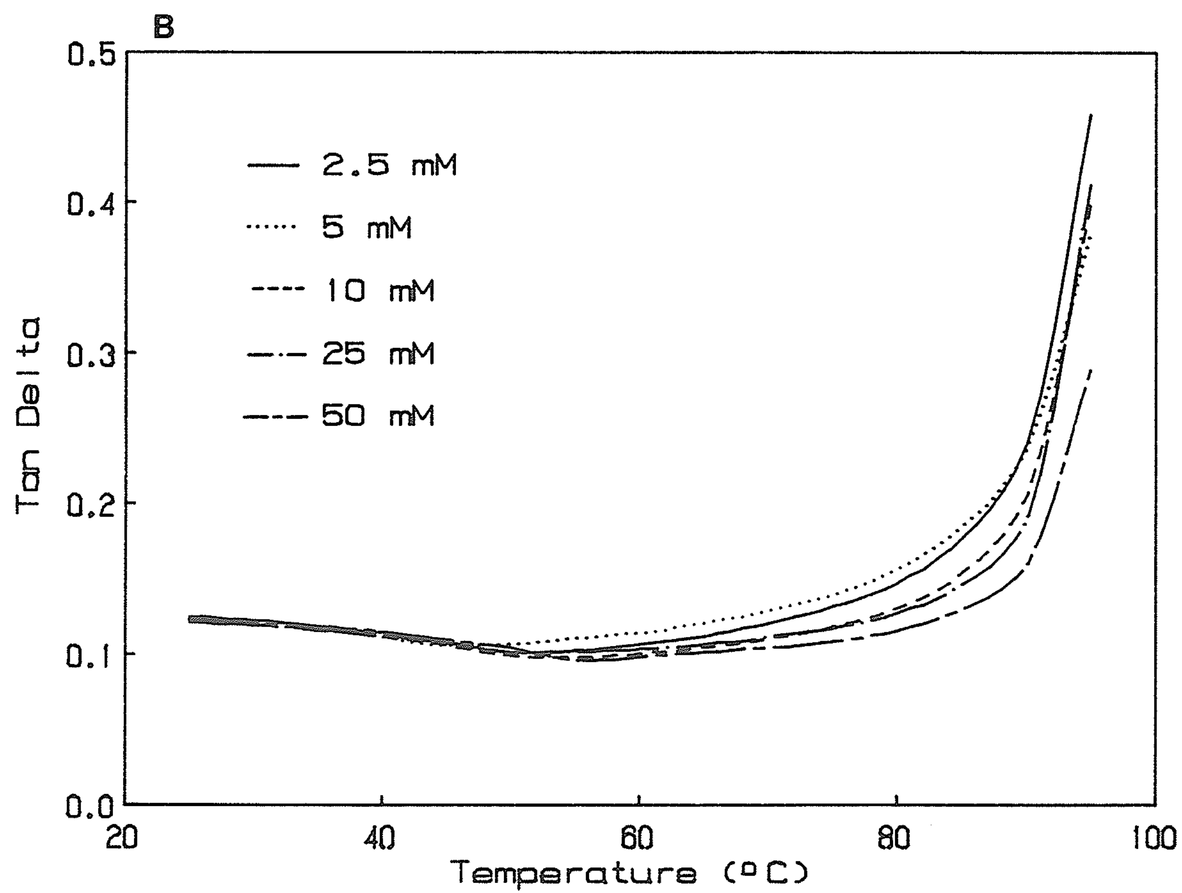

B
0.5
0.4
0.3
0.2
0.1
0.0
Tan Delta
20
40
60
80
100
Temperature (°C)
2.5 mM
5 mM
10 mM
25 mM
50 mM

TABLE 5. Effect of mercaptoethanol (ME) and N'- ethylmaleimide (NEM) on the rheological properties of heat set vicilin (10% in 0.15 M NaCl, pH 8.5).

Concentration (mM)	ME G' (Pa)	ME G" (Pa)	ME Tan δ	NEM G' (Pa)	NEM G" (Pa)	NEM Tan δ
2.5	934± 21[a]	155± 7[a]	0.166±0.004[ab]	1247± 28[a]	174± 0[a]	0.140±0.008[a]
5.0	903±144[a]	158±20[a]	0.176±0.006[a]	1223±126[a]	178±23[a]	0.145±0.004[a]
10.0	956± 44[a]	163± 8[a]	0.170±0.000[a]	1109± 13[ab]	153± 2[ab]	0.138±0.003[a]
25.0	1013±162[a]	164±23[a]	0.162±0.004[ab]	806± 65[b]	111± 4[b]	0.138±0.006[a]
50.0	1151± 80[a]	178± 8[a]	0.155±0.003[b]	1057±146[ab]	150±12[ab]	0.143±0.008[a]

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

TABLE 6. Effect of mercaptoethanol (ME) and N' - ethylmaleimide (NEM) on the rate of structure development during cooling 95°C to 25°C at 2°C/min for vicilin (10%, pH 8.5).

Conc (mM)	ME				NEM			
	Initial Cooling		Final Cooling		Initial Cooling		Final Cooling	
	G' (Pa/°C)	G" (Pa/°C)	G' (Pa/°C)	G" (Pa/°C)	G' (Pa/°C)	G" (Pa/°C)	G' (Pa/°C)	G" (Pa/°C)
2.5	-5.39±0.29[a]	-0.41±0.04[a]	-12.3±0.9[a]	-2.20±0.14[a]	-10.5±0.3[a]	-0.72±0.22[a]	-14.8±0.1[a]	-2.44±0.44[a]
5.0	-4.99±0.55[a]	-0.52±0.03[a]	-12.5±1.8[a]	-2.33±0.29[a]	-8.9±0.1[a]	-0.65±0.03[a]	-13.1±0.2[a]	-2.10±0.06[ab]
10.0	-5.15±0.26[a]	-0.42±0.02[a]	-16.2±1.6[a]	-2.78±0.00[a]	-9.2±0.5[a]	-0.68±0.08[a]	-13.6±0.2[a]	-2.14±0.16[ab]
25.0	-5.84±0.95[a]	-0.41±0.07[a]	-13.4±1.6[a]	-2.22±0.22[a]	-7.2±0.6[a]	-0.60±0.03[a]	-10.3±0.4	-1.54±0.12[b]
50.0	-6.20±0.37[a]	-0.49±0.06[a]	-15.7±1.1[a]	-2.45±0.10[a]	-9.5±2.0[a]	-0.79±0.10[a]	-13.2±0.9[a]	-2.01±0.06[ab]

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

Figure 48. Photomicrographs showing the effect of mercaptoethanol on heat induced networks for 10% vicilin in 0.15 M NaCl, pH 8.5.

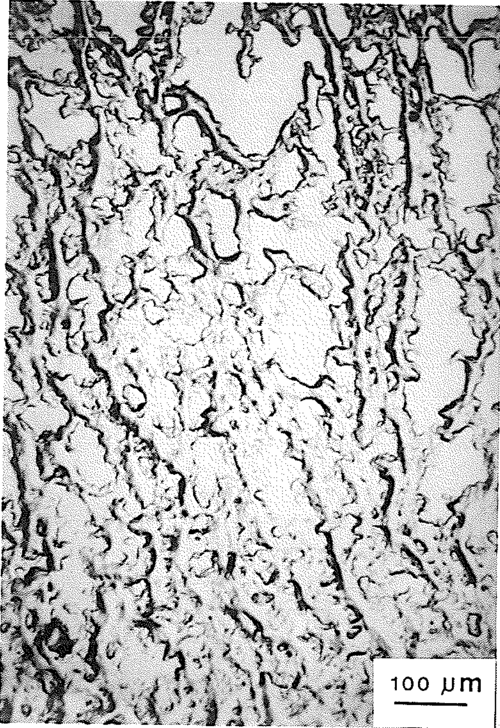
A. 5 mM

B. 10 mM

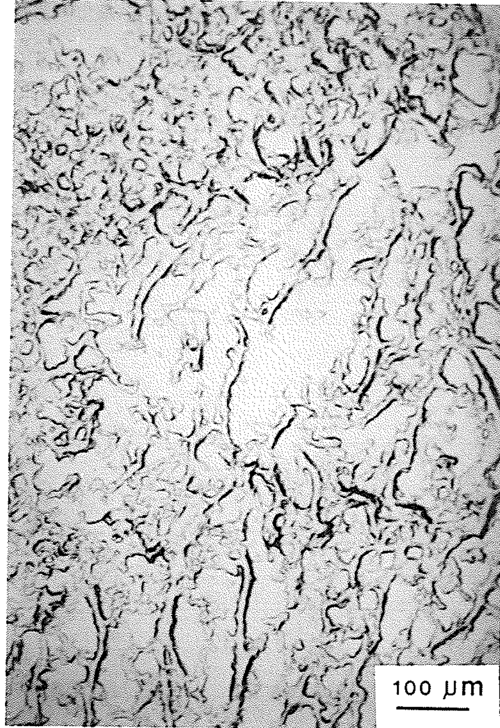
C. 25 mM

D. 50 mM

A

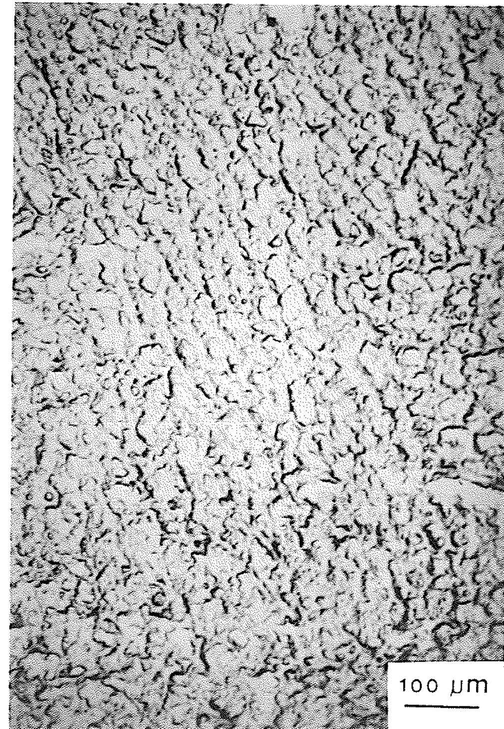


B



C

D



(Fig. 49). As these differences are indicative of some influence on disulfide bonds, it is possible that the three sulfhydryl groups per vicilin molecule may have some impact on network structure. The fact that these differences did not produce any rheological changes made the importance of this contribution questionable.

The similarities in rheological properties were reflected in the data obtained during cooling. No significant differences were detected in the rates of structure development during either phase of the cooling regime (Table 6). Similarly, the changes in tan δ with cooling were the same for all concentrations of ME (Fig. 44B) and NEM (Fig. 47B). Despite the slight variations in microstructure, these data did not support the participation of disulfide bonds as a factor in determining the rheological properties of heat induced vicilin networks.

Disulfide Bonds in Relation to Network Characteristics

The formation of disulfide bonds is not a requirement for the production of heat induced networks. For some proteins, the number of sulfhydryl groups is too low for this type of reaction to have an impact on network formation. This was evident with vicilin where the addition of the three disulfide modifying agents had practically no effect on network characteristics. A similar lack of disulfide bond involvement in network formation has been reported for other plant proteins including sesame (Lakshmi and Nandi, 1979), oat (Ma and Harwalkar, 1988) and, in some instances, soybean (Bikbov et al., 1986; van Kleef, 1986). Not all plant protein networks show this lack of disulfide bond involvement; gels from rapeseed (Gill and Tung, 1978)

Figure 49. Photomicrographs showing the effect of N' - ethylmaleimide on heat induced networks for 10% vicilin in 0.15 M NaCl, pH 8.5.

A. 2.5 mM

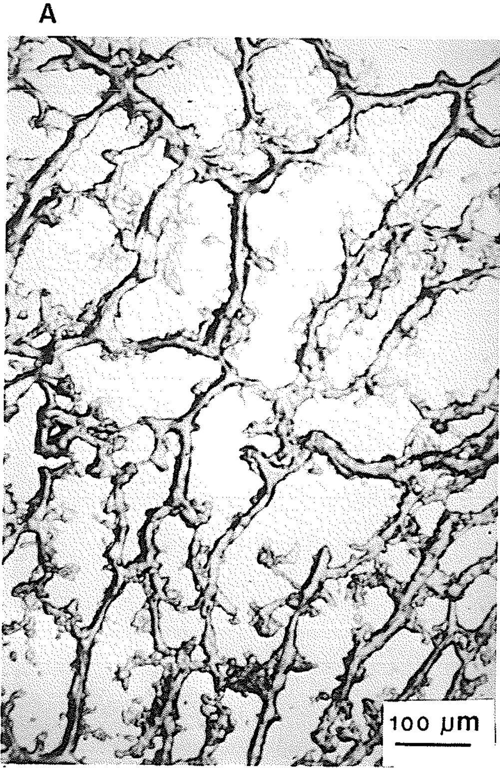
B. 5 mM

C. 25 mM

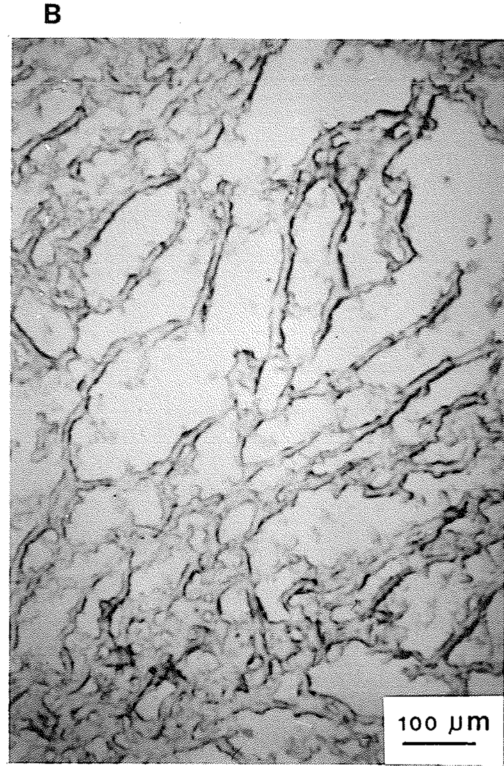
D. 50 mM

229

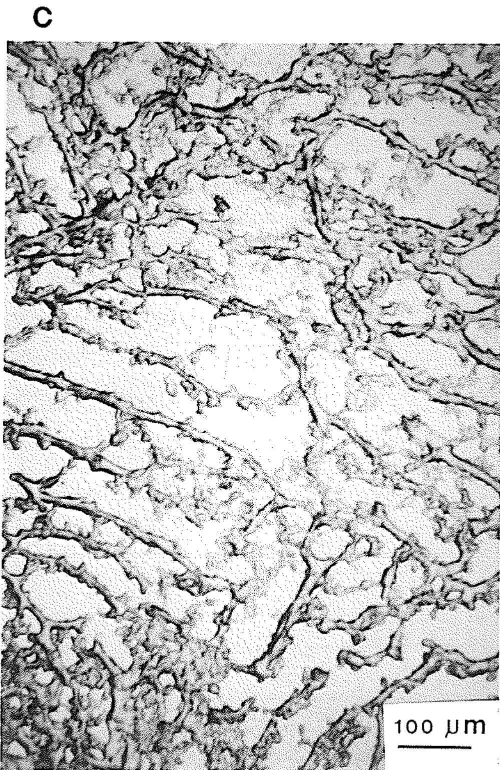
A



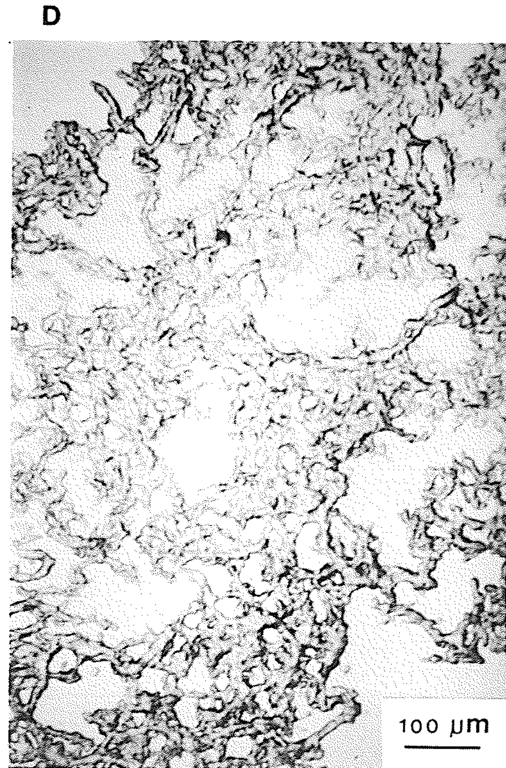
B



C



D



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. This was best demonstrated by the higher tan δ values and poor microstructure, where strands were not as well defined and the degree of crosslinking not as intense. The lack of disulfide bond involvement may contribute to this difference. This possibility was particularly evident when comparing vicilin networks in an alkaline medium to those obtained for ovalbumin at ME concentrations which appeared to inhibit disulfide bond formation.

The involvement of disulfide bonds in ovalbumin network formation was evident in this investigation. With CysHCl, the concentrations used were too low to alter final network characteristics. Changes in the rheological characteristics during cooling, however, were indicative of disulfide involvement. The decrease in the degree of crosslinking at higher concentrations of ME and NEM showed more conclusively that the disruption of disulfide bonds reduced the structural integrity of the heat induced ovalbumin networks. Both the strength (G moduli) and the nature of the network formed (tan δ values) were influenced by these environments.

The actual role of disulfide bonds has not been clarified in this study. According to the data obtained during cooling, the modifying action of these reagents occurred during the final cooling phase. As a result, it would appear that the function of the disulfide bonds was to stabilize and strengthen the network that had been established during

heating and the initial cooling phase. With this interpretation, however, there are several unanswered questions. If these bonds have no function in establishing the network, how does their disruption affect the type of network that is formed? How can the presence of these disulfide modifying agents cause structural breakdown (decreased G moduli) during the final cooling phases presumably due to the disruption of disulfide bonds, when their presence during the heat treatment was designed to prevent the formation of such bonds? Obviously, the role of disulfide bonds is complex. While not necessary for network formation, disulfide bonds do appear to aid in crosslinking within the network thus improving the rheological and microstructural characteristics. The complex behavior during cooling may be related to the influence of temperature on the disruption and reformation of disulfide bonds. This issue cannot be clarified on the basis of this study.

General Discussion

Use of Light Microscopy to Assess Network Microstructure

Light microscopy has received limited recognition as a technique for examining the microstructure of protein networks. Presumably, this is because of the higher resolution and three dimensional perspective that are possible with electron microscopy. In this study, light microscopy has proven to be a very valuable tool for examining the interactions required for network formation. Differences in network type (aggregated as opposed to crosslinked) were evident with this technique. Futhermore, in some instances where the type of network

remained the same, such as with the higher protein concentrations, differences in network intensity (strength) were also observed. In addition to the obvious benefit of requiring less expensive equipment, the lower magnification used and the ease of sample preparation make the use of light microscopy less susceptible to artifact production.

The use of light microscopy and, in particular, sample preparation by sectioning frozen material was not without problems. For good crosslinked networks, sectioning was relatively easy; however, for weak gels and aggregated products, sample preparation was more difficult and may have influenced the resulting microstructure. For vicilin at pH 3, for example, relatively poor microstructure was obtained despite rheological properties which indicated a crosslinked network. The overall strength of the network was low resulting in a delicate product which was difficult to handle. Structural collapse during sectioning may have accounted for the poor microstructure. Ice crystal formation during freezing prior to sectioning was also a possibility. Although there was no evidence of this problem in the well crosslinked networks, the alignment of protein aggregates seen with ovalbumin at 0.4 and 0.5 M $CaCl_2$ may also be attributed to this phenomenon. The potential for artifact production must, therefore, be considered when preparing network samples for light microscopy, particularly when the samples are not well structured. Nevertheless, light microscopy has been shown to be a valuable technique for examining structural differences in protein networks.

Use of Dynamic Rheology to Assess Network Characteristics

The value of using fundamental rheological techniques to evaluate

structural characteristics is in the ability to generate data which are independent of sample size and equipment used. When trying to relate these characteristics to molecular interactions, it is of utmost importance that the properties examined are functions of the material rather than the techniques used. Dynamic rheological testing represents one such technique and is particularly suited to examining properties both during network formation and in the resulting product. This technique has been applied successfully to following structure development during heating where G' or G^* were used to assess network strength (te Nijenhius, 1981; Beveridge et al., 1984, 1985; Bohlin et al., 1984; Goldsmith and Toledo, 1985a; Paulsson et al., 1986; van Kleef, 1986). Only in a few studies has the loss tangent (or tan δ) and the G moduli been used to characterize the network (Gill and Tung, 1978; Goldsmith and Toledo, 1985a; Paulson and Tung, 1989). It is clear from this study that both the G moduli and the tan δ value are necessary for the comprehensive network characterization required when examining the role of various interactions. In this respect, tan δ values reflected the type of network that formed and the G moduli were indicative of network strength. This was particularly evident in the protein concentration study where the transition from broken fragments to a crosslinked network was reflected in the tan δ values while the increase in network strength (intensity) at concentrations above the CPC resulted in increased G moduli. Any relation between the G moduli and the number of protein-protein interactions was dependent on the type of network formed. In other words, the G' modulus for an aggregated network did not necessarily represent the same number of

protein-protein interactions at a similar G' modulus for a crosslinked network. In this respect, the G moduli responded to both changes in network type and the degree of interactions for any given network.

Although changes in rheological properties have been monitored during heating in several investigations, very little information exists on the changes that occur during cooling. In the work of van Kleef (1986) on ovalbumin and soy isolates, changes in G' during cooling were negligible compared to structure development during heating and were not considered further. Paulson and Tung (1989) reported an increase in gel firmness with cooling though no values were given. In the present investigation, the change in the magnitude of the G moduli for most samples was greater during the cooling regime. Furthermore, the distinct characteristics of the cooling curves provided information as to the function of various interactions in the network. The biphasic model, used to describe the change in the G moduli during cooling allowed distinction of two mechanisms of involvement. The classification of the first phase of cooling as one involving structure development seemed reasonable in view of the large increase in G moduli in this range seen for well structured networks only. In addition, the decrease in the tan δ values for all networks occurred over this same temperature range. The nature of structure development in this region is not clear; however, due to the close association between increases in the G moduli in this region and the formation of well structured networks, the crosslinking of protein strands may be involved.

The increase in the G moduli during the final phase of cooling has

been attributed to strengthening and stabilizing of the network. The nature of this strengthening is not obvious but may involve strand thickening as has been suggested in the literature (Schmidt, 1981). Alternately, the response during this phase may simply reflect the response of the network to temperature (i.e. some interactions are stronger at lower temperatures). The similarity of the curves during this phase to a limited number of curves obtained by reheating the networks added support to this theory. As a result, changes in the G moduli during this phase would reflect the types of interactions in the networks and how they respond to temperature. In this respect, increased gel strength at lower temperatures supported the presence of hydrogen bonds as an intregal part of these networks. It is in this context that strengthening of the network during the final cooling phase has been used in this study. Exceptions to this increase in gel strength with lower temperatures were seen for ovalbumin at pH 3 and in the presence of higher concentrations of disulfide modifying agents where environmental conditions were actually disrupting network structure.

Comparison of network characteristics in this study involved the use of single frequency measurements due to the similarities in slope of the relationship between the log of the rheological parameters and the log of frequency. This simplification was used for practical purposes but it should not be forgotten that for viscoelastic networks there is variation in the rheological parameters with frequency. Although not addressed in this study, this relationship may be of importance, particularly for those networks where tan δ values are high.

Relationship between Microstructure and Rheological Characteristics

Structure - function relationships provide some insight into the role of specific structures in terms of the behavioral properties of the material. The complimentation between microstructural and rheological properties in this study was essential for a comprehensive evaluation of the interactions involved in network formation. In this respect, the relationship between the rheological parameters and the microstructure was evident when examining the effects of protein concentration and was generally true for all other experimental conditions. Essentially, the tan δ value changed in response to changes in the type of microstructure and the G moduli reflected the degree of interaction within a given network type. For those very good networks which were well crosslinked, tan δ values tended to be low (< 0.10). Good networks could be seen at slightly higher tan δ values (between 0.10 and 0.15). At still higher tan δ values, two types of microstructure were possible (aggregated or evidence of increased solubility), which on the basis of rheological data alone could not be distinguished. In this respect, the microstructural assessment provided information not available through the evaluation of rheological parameters.

Although the increase in the G moduli with increasing protein concentration was seen as an increase in network intensity, variations in the G moduli under other conditions did not always produce microstructural differences. For example, the microstructure for ovalbumin at SDS concentrations of 10 and 25 mM were essentially the same, yet both the G' and G" moduli were significantly reduced. The

benefits of evaluating networks using two different techniques is clear. In addition to the increase in confidence in the conclusions due to the supporting nature of the data, the increased sensitivity of one technique over the other, depending on the conditions examined, extended the range of effects that could be observed.

Importance of Protein Conformation during Network Formation

The currently accepted theory for the mechanism of heat induced network formation with globular protein relates the formation of protein strands to the association of the corpuscular structures formed during the heat treatment. As a result, it would seem reasonable to assume that conditions which affected the conformation of the native protein would also influence the conformation of the corpuscular structure. This conformational change should, in turn, affect network characteristics. This type of influence was observed under a number of environmental conditions.

According to DSC data, native vicilin at pH values of 3 and 4 exhibited major conformational changes. The low tan δ values for the networks at these pH values were indicative of well crosslinked structures which could result from the interaction of extended polypeptide chains. A similar influence of pH on the conformation of the corpuscular structure would account for this behavior.

Another example of a possible conformational influence was seen in the differential response of ovalbumin and vicilin to low levels of SDS. With ovalbumin, low levels of SDS, which were shown to destabilize the native protein, also resulted in improved network characteristics. The exposure of hydrophobic residues on the protein due to the

unfolding of the corpuscular structure would allow the SDS to form a crosslinking bridge between a positively charged group on one protein and a hydrophobic area on another. This bridging function would account for the improved network characteristics. The fact that no such network improvement was seen for vicilin, which was not destabilized at these SDS concentrations, implicated the conformation of the corpuscular structure as the critical factor in eliciting this SDS effect. The higher SDS levels required to denature vicilin were sufficient to increase the net charge to a point where the network was tending towards solubilization.

One other system where conformation of the corpuscular structure had a major influence on network formation was for ovalbumin at high urea concentrations. Only in conditions which promoted significant protein unfolding was the interaction between sulfhydryl groups able to produce a well crosslinked network. At lower urea concentrations, the influence of these disulfide bonds was not evident.

There are two points to be made based on these observations. First is the fact that the behavior observed in this study supported the corpuscular theory for the gelation of globular proteins. The results could only be explained if the availability of certain amino acid residues was restricted due to the structures involved. A good example of this was the decrease in the availability of the hydrophobic residues in the stabilizing salts, a situation which reduced the degree of interaction in the network and hence the G moduli. The second point is that the influence of conformation appeared to be through manipulation of the potential for interactions between proteins.

Ultimately, this influenced the balance of attractive and repulsive forces necessary for network formation.

Role of Specific Molecular Interactions in Network Formation

For both ovalbumin and vicilin, there was a CPC required for network formation as assessed by a decrease in tan δ to a constant value. Although the CPC for ovalbumin (between 5 and 7.5%) at alkaline pH values was less that for vicilin (between 7.5 and 10%), the increase in network strength as a function of protein concentration above the CPC was greater for vicilin. As a result, the disadvantage of having a higher CPC may be counteracted if stronger networks (i.e. higher protein concentrations) are required.

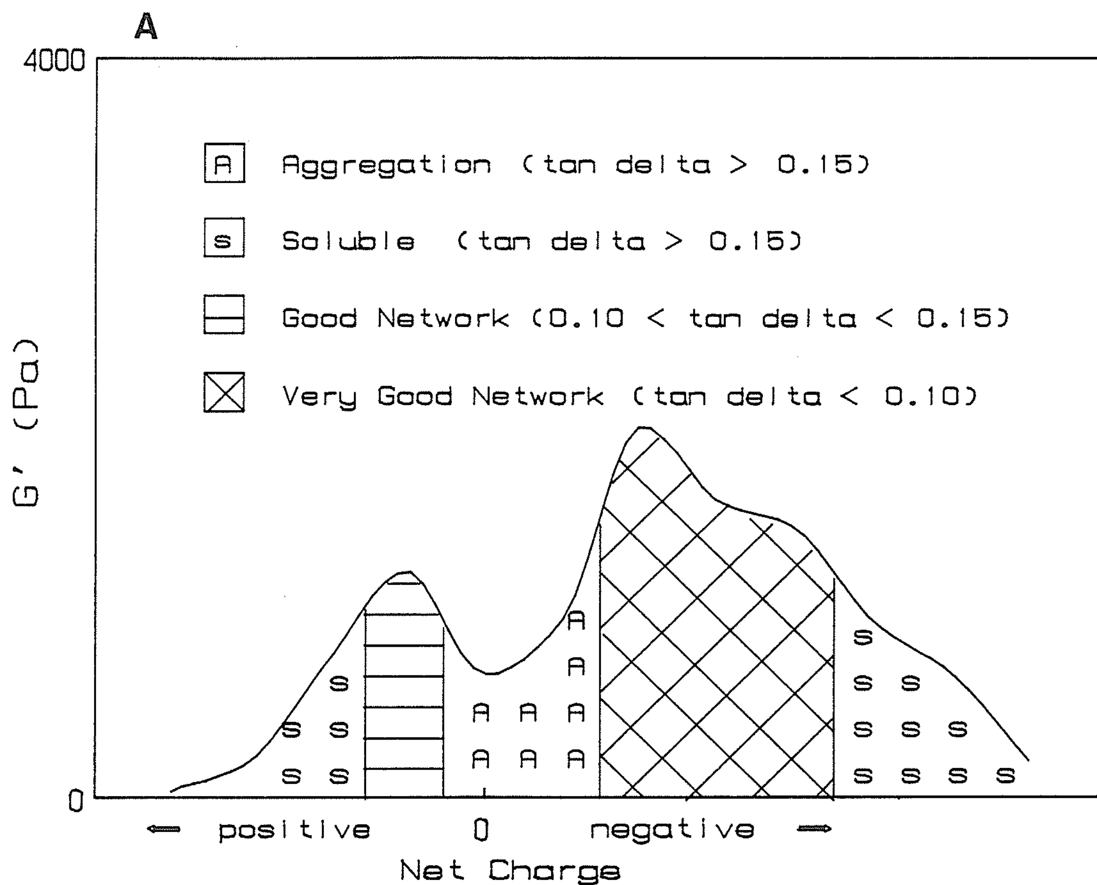
Of even more importance, however is the quality of the network that is formed. The need for a balance between attractive and repulsive forces to give good network formation was noted by Ferry (1948) in his early investigations into the mechanism of network formation. The importance of this balance has been clearly demonstrated in this study. The aim now is to understand how different molecular interactions affect this balance.

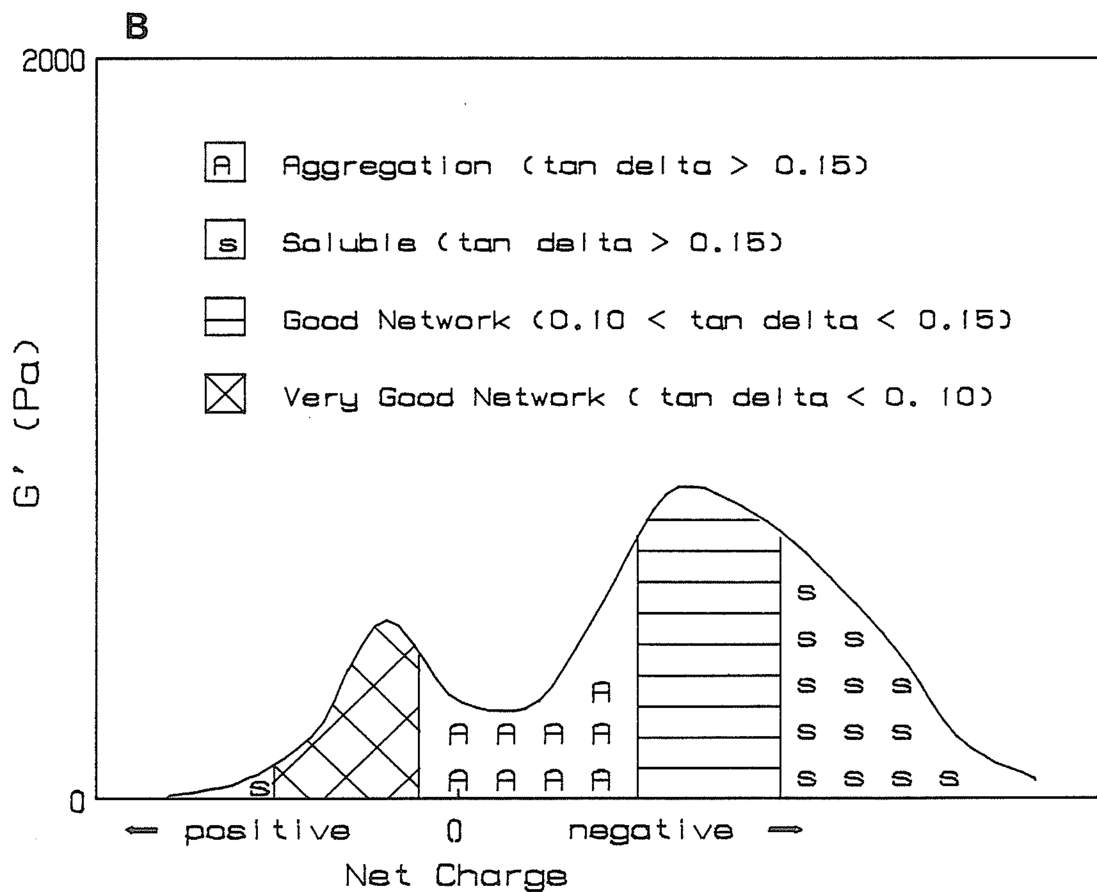
In this respect, the types of associations which can result with manipulation of this balance must be considered. As electrostatic charge appears to provide the sole repulsive force in the balance, the types of networks that form with charge manipulation can be used as a model for the networks that result from the manipulation of other attractive forces. Schematic diagrams showing changes in both type and strength of networks as a function of net charge for ovalbumin and vicilin are given in Figure 50. The divisions into network types was

Figure 50. Schematic diagram showing variability in the type and strength of heat induced networks as a function of net protein charge.

A. ovalbumin

B. vicilin


A
4000
0
G' (Pa)
A Aggregation (tan delta > 0.15)
s Soluble (tan delta > 0.15)
Good Network (0.10 < tan delta < 0.15)
Very Good Network (tan delta < 0.10)
← positive
0
negative →
Net Charge


B
2000
0
G' (Pa)
A Aggregation (tan delta > 0.15)
s Soluble (tan delta > 0.15)
Good Network (0.10 < tan delta < 0.15)
Very Good Network (tan delta < 0. 10)
← positive
0
negative →
Net Charge

based on tan δ values from pH, SDS and $CaCl_2$ data. As the protein is moved from a net charge of zero in either direction, the type of interaction goes from an aggregated state (where attractive forces dominate - tan δ values > 0.15) to good or very good network formation (where attractive and repulsive forces balance - tan δ values < 0.15) to a soluble protein (where repulsive forces dominate - tan δ values > 0.15). This trend was seen for both ovalbumin and vicilin although the quality of the networks varied as did the range over which good networks could be formed. Generally vicilin networks had lower G' moduli throughout. In addition, networks formed in the presence of a net negative charge were of a poorer quality (higher tan δ values) than those for ovalbumin, and occurred over a narrower range. The very good quality networks for vicilin in the presence of a net positive change had extremely low G moduli and were therefore of limited value. Within any given type of network, there was some variation in network strength; however, the highest G moduli were found in conditions which promoted good crosslinked network formation.

When working at a pH of 8.5, the potential for network formation based on the electrostatic balance is good for both ovalbumin and vicilin, although the quality of these networks was not the same. With this electrostatic profile, factors affecting other intermolecular interactions had the same effect as changing the charge. For example, the repression of intermolecular hydrogen bonding (in urea) removed an attractive force, thus causing the net repulsive force to increase, rendering the network soluble. This was particularly evident with vicilin, but a similar trend was also seen with ovalbumin at urea

concentrations between 0.5 and 2.0 M. Alternately, the promotion of intermolecular hydrophobic interactions at higher Na_2SO_4 concentrations increased the net attractive forces so that aggregation resulted. The deterioration in network structure (tan δ values increased from less than 0.10 to between 0.10 and 0.15) for ovalbumin in the presence of disulfide modifying agent does not quite fit into this scheme. Perhaps the transition between aggregated and very good networks should contain a division of good networks which are not as well crosslinked.

The influences of various molecular interactions on network formation with ovalbumin and vicilin have been summarized in Table 7. For both proteins, and probably all globular proteins, the net charge acts as the sole repulsive force, while hydrogen bonding and, to a lesser extent, hydrophobic interactions serve as attractive forces. The importance of these two attractive interactions has also been noted for canola protein (Paulson and Tung 1989). There is some difference in terms of the effects of disulfide bonds and electrostatic interactions. These differences may help explain why the networks formed with vicilin under any condition were not comparable to the very good networks for ovalbumin. The most obvious explanation is the role of disulfide bonds. The capacity of ovalbumin to form a highly elastic network presumably based on disulfide bonds alone, plus the similarities between the network characteristics for vicilin in an alkaline medium and ovalbumin under similar conditions but including a disulfide modifying agent, provide strong evidence that these bonds are necessary for the formation of a well crosslinked network.

The difference in terms of electrostatic interactions may also be

TABLE 7. Importance of specific molecular interactions to heat induced network formation with ovalbumin and vicilin.

Interaction	Ovalbumin	Vicilin
Electrostatic Interactions	Repulsive force required for network formation. No influence on network strength in conditions producing good networks. In aggregating conditions attractive interactions also contribute to network strength.	Repulsive force required for network formation. Attractive force also contribute to network strength.
Hydrophobic Interactions	Attractive force in network formation. Contributes to network strength. Little influence on type of structure formed.	Attractive force in network formation. Contributes to network strength. May influence type of structure if inter-molecular interactions promoted.
Hydrogen Bonds	Attractive force in network formation. Necessary for any type of structure.	Attractive force in network formation. Necessary for any type of structure.
Disulfide Bonds	Attractive force in network formation. Contribute to the type and strength of the network - mechanism unclear. Capable of forming network in the absence of noncovalent forces.	No involvement in network formation.

a factor. Theoretically, there should have been a pH value where the charge profile was such that its contribution was only as the repulsive force required for the balance. At this pH, electrostatic forces should not influence network strength as was seen with ovalbumin. With vicilin, however, electrostatic interactions represented an attractive force contributing to network strength under all conditions. As vicilin (molecular weight is 158,000) is a multidomain protein (Ismond, 1984) which is considerably larger than ovalbumin (molecular weight is 45,000), it is possible that net charge alone cannot establish this balance. The charge distribution may be a complicating factor. Furthermore, the inability of vicilin to form very good networks was reflected in relatively poor structure development during the initial cooling phase, whereas the effects on the disulfide bonds in ovalbumin were seen during the final cooling phase. This also implicated the electrostatic profile of vicilin as a factor contributing to its inferior network forming properties.

Overall, the ability to use vicilin as a substitute for ovalbumin will depend on the condition in the food product and the type of network required. In some situations, the lower gel strength for vicilin can be compensated for by increasing the protein content. The situations where substitutions can be made, however, are limited as optimum network quality does not match that attained with ovalbumin under the conditions used in this study.

CONCLUSIONS

1. Dynamic rheology represented an excellent fundamental technique for evaluating the formation and characteristics of heat induced protein networks. In order to characterize networks with respect to type and strength, both the G moduli and tan δ values had to be considered.

2. Evaluation of microstructure using light microscopy provided good complimentary data to the rheological analysis; the types of networks formed best reflected the tan δ values. An additional benefit from the microstructural data was that they provided evidence of two different network types associated with high tan δ values - aggregated and soluble - that could not be distinguished based on the rheological data alone.

3. Data with both ovalbumin and vicilin supported the corpuscular theory (Tombs, 1974) as the mechanism for network formation with globular proteins. Conditions which promoted significant protein unfolding and hence changes in the corpuscular structure had a significant impact on structure development.

4. The critical protein concentration (CPC) for network formation was detected as a significant decrease in the tan δ value. In an alkaline medium, this was less for ovalbumin (between 5 and 7.5 %) than for vicilin (between 7.5 and 10%). At concentrations above the CPC, tan δ values remained constant; the tan δ value for ovalbumin in these conditions was lower than for vicilin, indicating a more elastic

network. The increases in the G moduli with increasing protein concentration, however, were greater for vicilin.

5. A balance of attractive and repulsive forces was required for the formation of well crosslinked networks for both ovalbumin and vicilin.

6. Hydrogen bonds and, to a lesser extent, hydrophobic interactions represented attractive forces in this balance for both proteins.

7. Electrostatic repulsion was the repulsive force in this balance.

8. The contribution of electrostatic attraction to network strength was dependent on the type of interaction and the source of protein. If there was evidence of aggregation, these interactions made an important contribution. For vicilin, even with the good networks found at alkaline pH values, there was increased strength due to electrostatic attraction. Perhaps due to the large size of vicilin, the charge distribution and not just the charge impacted the potential for this type of interaction.

9. Disulfide bonds are important to the type and strength of ovalbumin networks; however, the nature of this involvement is unclear as inclusion of disulfide modifying agents disrupted structure during the final stage of cooling rather than preventing the initial crosslink formation.

10. Under conditions which inhibit the formation of noncovalent interactions (4-8 M urea), ovalbumin was able to form a strong network through disulfide bonds alone.

11. Disulfide bonds did not appear to be involved in vicilin network formation. Furthermore, vicilin did not form a network in the presence of high urea concentrations, where covalent disulfide bonds would be the sole crosslinking mechanism. Presumably these responses were due to the low sulphydryl content in vicilin. This behavior does demonstrate that disulfide bonds are not essential for network formation.

12. Vicilin networks were not as well formed as those from ovalbumin. Even at low pH values, where vicilin networks had good tan δ values, networks were weak and of questionable value. This difference may be related to the contribution of disulfide bonds; however, the size of the vicilin molecule and hence the contribution of attractive electrostatic forces may also impact network quality.

FUTURE RESEARCH CONSIDERATIONS

Future research may be considered from two distinct perspectives. The first involves extension of the molecular basis for network formation in an attempt to clarify some of the unanswered questions from this study. The second perspective involves the use of more complex systems, where the presence of other proteins and nonprotein components will influence the interactions responsible for network formation.

In terms of molecular considerations, the first issue that requires further clarification is that of protein concentration and why the curves from this study and that of van Kleef (1986) do not resemble those used to derive master concentration dependence equations. In this respect, the concentration dependence of other globular proteins should be investigated, as well as the proteins used in this study but using different environmental conditions. It is possible that under conditions which promote some protein unfolding (e.g. vicilin at low pH) the master concentration dependence relationship may be more applicable. This may help resolve the lack of conformity in terms of concentration effects. In addition, it would be interesting to examine how the type of network formed would reflect the CPC. Would the amount of protein required for network formation be lower if conformation changes in the corpuscular structures were also associated with network formation as was the case with ovalbumin in low SDS concentrations or vicilin at low pH values?

From the data in this study, it is clear that the networks formed with the plant protein vicilin were not of the same quality as those from ovalbumin. Both molecular size and the absence of disulfide linkages have been implicated in this differential response. Further research is required to clarify the importance of these two mechanisms. The use of an alternate plant protein of comparable (or larger) size, but containing an appreciable quantity of sulphydryl groups would be beneficial in this respect. In view of the implications of disulfide involvement of the 12S globulins in soybean (Utsumi and Kinsella, 1985) and rapeseed (Gill and Tung, 1978), either of these proteins would represent a good candidate for such a study.

The actual role of disulfide bonds also requires further clarification. It would be of interest to examine the network during the initial stages of development to see if the well crosslinked networks suggested by the rheological data were actually present at this stage or if the rheological data reflected a more complex relationship between temperature, the testing mechanisms and a disulfide interchange type reaction.

In order to pursue further the charge influence, in an attempt to find conditions which could optimize the repulsive forces for improved network formation, the possibility of charge modification could be considered. The net negative charge could be increased by modifying the ε- amino groups on lysine with acetic, maleic or succinic anhydride, while the net positive charge could be increased through carboxyl group modification with carbodiimide as suggested by Ma and Holme (1982).

Another consideration in terms of molecular associations, which

has not been addressed in this study, is the importance of heating time and temperature, both of which have been shown to influence network hardness (Hashizume *et al.*, 1975; Furukawa *et al.*, 1979; Hermansson, 1982a; Holt *et al.*, 1984; Woodward and Cotterill, 1986). It would be of interest to examine the impact of heating rate and maximum heating temperature on the different types of networks observed in this investigation.

On a more practical level, the use of protein networks will be determined by the actual structures developed in more complex food systems. In this respect, the presence of other proteins, particularly those with different isoelectric points, will make it difficult to attain the attractive - repulsive balance associated with good network formation. Similarly, other macromolecules, such as carbohydrates and lipids may also influence the forces involved in network formation. The nature of this type of influence and the impact of these associations on network properties warrant further consideration.

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APPENDICES

APPENDIX 1A. Effect of protein concentration on the rheological properties of heat set ovalbumin in 0.15 M NaCl (pH 8.5).

Concentration (%)	G′ (Pa)	G″ (Pa)	Tan δ
5.0	68± 2	10± 1	0.148±0.001
7.5	510±52	47± 3	0.094±0.004[a]
10.0	1006±73	94± 7	0.094±0.000[a]
12.5	1431±82	137± 8	0.096±0.000[a]
15.0	2853±99	285±10	0.100±0.000[a]

[1] Column values followed by the same letter are not significantly

APPENDIX 1B. Effect of protein concentration on the rheological properties of heat set vicilin in 0.15 M NaCl (pH 8.5).

Concentration (%)	G′ (Pa)	G″ (Pa)	Tan δ
5.0	228±24[a]	39± 5[a]	0.170±0.004[a]
7.5	519±124[a]	88±14[a]	0.170±0.008[a]
10.0	1221± 97[a]	161± 5[a]	0.132±0.006[b]
12.5	2570±381	357±41	0.140±0.005[b]
15.0	4868±503	627±65	0.129±0.006[b]

[1] Column values followed by the same letter are not significantly different (P≤0.05).

APPENDIX 2A. Effect of pH on the rheological properties of heat set ovalbumin (10% in 0.15 M NaCl).

pH	G' (Pa)	G" (Pa)	Tan δ
3	735±173[a]	133±33[a]	0.182±0.004[a]
4	1228±356[ab]	181±49[a]	0.148±0.004
5	670±201[a]	117±24[a]	0.181±0.002[a]
6	953±428[a]	178±85[a]	0.184±0.006[a]
7	2174±396[b]	230±52[a]	0.105±0.005[b]
8	1599±397[ab]	173±51[a]	0.108±0.001[b]
9	1446± 33[ab]	126± 3[a]	0.087±0.002

[1] Column values followed by the same letter are not significantly different (P≤0.05).

APPENDIX 2B. Effect of pH on the rheological properties of heat set vicilin (10% in 0.15 M NaCl).

pH	G' (Pa)	G" (Pa)	Tan δ
3	176± 9[a]	11± 1[a]	0.064±0.000
4	485± 30[b]	42± 4[a]	0.086±0.003
5	265± 40[ab]	52± 5[ab]	0.197±0.007[a]
6	251±102[ab]	48±17[a]	0.193±0.006[a]
7	532± 97[bc]	93±17[bc]	0.174±0.004
8	838±162[d]	127±22[c]	0.151±0.004[b]
9	779± 20[d]	114± 2[c]	0.147±0.001[b]

[1] Column values followed by the same letter are not significantly different (P≤0.05).

APPENDIX 3A. Effect of pH on the rate of structure development during cooling from 95°C to 25°C at 2°C/min for ovalbumin (10% in 0.15 M NaCl).

pH	Initial Cooling Phase		Final Cooling Phase	
	G' (Pa/°C)	G" (Pa/°C)	G' (Pa/°C)	G" (Pa/°C)
3	-21.7±5.8[ab]	-1.74±0.42[a]	111.9±7.3	3.33±0.06[a]
4	-15.0±0.6[a]	-2.82±0.37	12.9±5.3	1.55±0.62[a]
5	-3.2±1.1[c]	-0.52±0.11[b]	-13.8±1.5[a]	-3.09±0.91[b]
6	-0.7±0.2[c]	-0.11±0.07[b]	-15.5±5.2[a]	-3.86±1.58[b]
7	-22.5±3.9[ab]	-1.63±0.26[a]	-22.5±3.9[a]	-2.81±0.62[b]
8	-25.7±5.9[ab]	-1.73±0.30[a]	-15.5±3.8[a]	-1.92±0.53[b]
9	-32.8±2.7[b]	-1.35±0.05[a]	-12.4±1.2[a]	-1.39±0.09[b]

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

APPENDIX 3B. Effect of pH on the rate of structure development during cooling 95°C to 25°C at 2°C/min for vicilin (10% in 0.15 M NaCl).

pH	Initial Cooling Phase		Final Cooling	
	G' (Pa/°C)	G" (Pa/°C)	G' (Pa/°C)	G" (Pa/°C)
3	-2.7±5.1[ab]	-0.30±0.23	-1.8±0.1[a]	-0.07±0.03[a]
4	-10.5±1.3	-0.74±0.21[a]	-1.3±0.2[a]	-0.05±0.08[a]
5	-15.8±0.2	-1.26±0.06	21.7±1.5	1.07±0.18
6	-2.0±0.2[a]	-0.19±0.05[b]	-3.9±0.4[ac]	-1.34±0.05[b]
7	-3.4±0.9[ab]	-0.29±0.28[ab]	-12.2±4.3[b]	-2.88±0.18
8	-6.6±0.8[c]	-0.37±0.16[ab]	-11.3±1.6b	-1.88±0.34[b]
9	-4.8±0.7[bc]	-0.43±0.01[ab]	-9.7±1.1[bc]	-1.72±0.10[b]

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

APPENDIX 4A. Effect of sodium dodecylsulfate (SDS) on the rheological properties of heat set ovalbumin (10% in 0.15 M NaCl, pH 8.5).

Concentration (mM)	G′ (Pa)	G″ (Pa)	Tan δ
5	2193±151[a]	194±16	0.088±0.001[a]
10	1996± 46[a]	162± 4	0.079±0.002[a]
20	2088±183[a]	132± 3	0.064±0.007[bc]
25	1586± 37[b]	86± 1[a]	0.054±0.002[cd]
35	1449±100[b]	67± 5[a]	0.046±0.001[d]
50	163± 15	12± 2	0.076±0.005[ab]

[1] Column values followed by the same letter are not significantly different (P≤0.05).

APPENDIX 4B. Effect of sodium dodecylsulfate (SDS) on the rheological properties of heat set vicilin (10% in 0.15 M NaCl, pH 8.5).

Concentration (mM)	G′ (Pa)	G″ (Pa)	Tan δ
2.5	1131±247[ab]	190±45[a]	0.168±0.002[ab]
5.0	1387±433[a]	202±67[a]	0.145±0.002[a]
7.5	562± 83[b]	102±15[ab]	0.182±0.001[ab]
10.0	754±137[ab]	120±25[ab]	0.159±0.004[ab]
25.0	140± 5[c]	21± 1[b]	0.195±0.005[b]
50.0	0.4±0.1[c]	1.0±0.1[b]	2.370±0.030

[1] Column values followed by the same letter are not significantly different (P≤0.05).

APPENDIX 5A. Effect of sodium dodecylsulfate (SDS) on the rate of structure development during cooling from 95°C to 25°C at 2°C/min for ovalbumin (10% in 0.15 M NaCl, pH 8.5).

Concentration	Initial Cooling Phase		Final Cooling Phase	
(mM)	G′ (Pa/°C)	G″ (Pa/°C)	G′ (Pa/°C)	G″ (Pa/°C)
5	-57.6±1.3	-0.26±0.61[a]	-7.75±0.35[ab]	-1.66±0.10[a]
10	-44.1±0.6	-0.39±0.28[a]	-5.40±0.60[ac]	-1.38±0.07[a]
20	-27.9±1.1	-0.11±0.06[a]	-9.15±0.75[b]	-1.17±0.17
25	-17.6±3.1[a]	-0.08±0.06[a]	-7.47±0.43[ab]	-0.08±0.13[bc]
35	-19.6±2.8[a]	-0.29±0.01[a]	-9.98±1.81[b]	-0.34±0.13[b]
50	-1.6±0.3	-0.46±0.00[a]	-2.62±0.38[c]	-0.15±0.09[c]

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

APPENDIX 5B. Effect of sodium dodecylsulfate (SDS) on the rate of structure development during cooling 95°C to 25°C at 2°C/min for vicilin (10% in 0.15 M NaCl, pH 8.5).

Concentration	Initial Cooling Phase		Final Cooling Phase	
(mM)	G′ (Pa/°C)	G″ (Pa/°C)	G′ (Pa/°C)	G″ (Pa/°C)
2.5	-7.34±1.07[a]	-0.76±0.03[a]	-16.2±3.3[a]	-3.15±0.58[a]
5.0	-8.55±1.13[a]	-0.61±0.22[a]	-12.0±0.3[ab]	-1.93±0.16[ab]
7.5	-5.16±1.19[a]	-0.59±0.01[a]	-7.8±1.0[b]	-1.61±0.37[b]
10.0	-7.74±1.15[a]	-0.64±0.06[a]	-8.9±1.6[b]	-1.73±0.33[b]
25.0	-0.48±0.29	-0.09±0.06	-1.2±1.1	-0.20±0.18

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

APPENDIX 6A. Effect of $CaCl_2$ on the rheological properties of heat set vicilin (10%, pH 8.5).

Concentration (M)	G' (Pa)	G" (Pa)	Tan δ
0.1	380±17	71± 2[a]	0.184±0.002
0.2	135± 0	32± 1	0.237±0.008[a]
0.3	60± 6[ab]	15± 2[ab]	0.254±0.003[a]
0.4	67± 1[a]	18± 1[a]	0.263±0.012[a]
0.5	31± 1[b]	11± 1[b]	0.351±0.011

[1] Column values followed by the same letter are not significantly different (P≤0.05).

APPENDIX 6B. Effect of $CaCl_2$ on the rate of structure development during cooling from 95°C to 25°C at 2°C/min for vicilin (10%, pH 8.5).

Concentration	Initial Cooling Phase		Final Cooling Phase	
(M)	G' (Pa/°C)	G" (Pa/°C)	G' (Pa/°C)	G" (Pa/°C)
0.1	-2.01±0.17	-0.29±0.02	-5.48±0.25	-1.10±0.04
0.2	-0.58±0.02[a]	-0.12±0.00[a]	-2.23±0.07	-0.52±0.01[a]
0.3	-0.44±0.08[a]	-0.06±0.05[ab]	-1.32±0.37[a]	-0.31±0.08[a]
0.4	-0.56±0.09[a]	-0.01±0.01[b]	-1.15±0.18[ab]	-0.34±0.07[a]
0.5	-0.43±0.09[a]	-0.08±0.04[ab]	-0.35±0.14[b]	-0.09±0.05

[1] Column values followed by the same letter are not significantly different (P≤0.05).

APPENDIX 7A. Effect of NaCl on the rheological properties of heat set ovalbumin (10%, pH 8.5).

Concentration (M)	G′ (Pa)	G″ (Pa)	Tan δ
0.1	1973± 68[a]	166± 8[a]	0.084±0.001
0.2	1865±205[a]	186±16[a]	0.100±0.002
0.3	1800± 62[a]	195± 9[a]	0.108±0.001[a]
0.4	713±138[b]	80±15[b]	0.112±0.001[a]
0.5	725± 17[b]	80± 1[b]	0.111±0.001[a]

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

APPENDIX 7B. Effect of NaCl on the rheological properties of heat set vicilin (10%, pH 8.5).

Concentration (M)	G′ (Pa)	G″ (Pa)	Tan δ
0.1	1082±149[a]	171±20[a]	0.159±0.004[a]
0.2	1012± 12[a]	152± 2[a]	0.151±0.001[a]
0.3	587± 64[b]	92±15[b]	0.157±0.005[a]
0.4	502± 11[b]	77± 1[b]	0.153±0.002[a]
0.5	514± 65[b]	91±15[b]	0.176±0.002

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

APPENDIX 8A. Effect of NaCl on the rate of structure development during cooling from 95°C to 25°C at 2°C/min for ovalbumin (10%, pH 8.5).

Concentration (M)	Initial Cooling Phase		Final Cooling Phase	
	G' (Pa/°C)	G" (Pa/°C)	G' (Pa/°C)	G" (Pa/°C)
0.1	-50.5±1.4[a]	-1.77±0.09[a]	-15.1±0.7[a]	-1.71±0.07[a]
0.2	-39.6±7.8[ab]	-2.14±0.32[a]	-18.7±3.6[a]	-2.05±0.39[a]
0.3	-28.5±1.6[b]	-1.74±0.07[a]	-16.8±1.3[a]	-2.05±0.19[a]
0.4	-11.0±1.5[c]	-0.72±0.23[b]	-6.8±1.2[b]	-0.88±0.07[b]
0.5	-9.6±0.7[c]	-0.61±0.18[b]	-6.9±0.1[b]	-0.82±0.01[b]

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

APPENDIX 8B. Effect of NaCl on the rate of structure development during cooling from 95°C to 25°C at 2°C/min for vicilin (10%, pH 8.5).

Concentration (M)	Initial Cooling Phase		Final Cooling Phase	
	G' (Pa/°C)	G" (Pa/°C)	G' (Pa/°C)	G" (Pa/°C)
0.1	-5.96±0.81[a]	-0.46±0.06[a]	-14.3±1.7[ab]	-2.37±0.30[ab]
0.2	-5.11±0.12[a]	-0.33±0.09[ab]	-16.2±0.5[a]	-2.47±0.04[a]
0.3	-2.98±0.11[b]	-0.24±0.01[b]	-8.9±2.3[c]	-1.47±0.31[c]
0.4	-2.14±0.05[b]	-0.20±0.01[b]	-10.0±0.3[bc]	-1.63±0.06[bc]
0.5	-3.35±0.41[b]	-0.26±0.03[b]	-6.9±1.0[c]	-1.34±0.13[c]

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

APPENDIX 9A. Effect of various anions (sodium salts) on the rheological properties of heat set ovalbumin (10%, pH 8.5).

SALT	0.1 M			0.5 M		
	G' (Pascals)	G" (Pascals)	TAN DELTA	G' (Pascals)	G" (Pascals)	TAN DELTA
NaSCN	1837±252[a]	139±24[a]	0.075±0.003	5242±113	403± 4	0.076±0.002
$NaC_2H_3O_2$	1638±224[a]	144±18[a]	0.088±0.001[ab]	851±122[a]	113±16	0.134±0.002
NaBr	672± 70	62± 7	0.092±0.001[a]	777± 17[a]	84± 3[a]	0.109±0.001[a]
NaCl	1973± 68[a]	166± 8[a]	0.084±0.001[b]	725± 17[a]	80± 1[a]	0.111±0.001[a]
Na_2SO_4	1720±265[a]	176±28[a]	0.102±0.000	107± 1	20± 1	0.187±0.002

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

APPENDIX 9B. Effect of various anions (sodium salts) on the rheological properties of heat set vicilin (10%, pH 8.5).

SALT	0.1 M			0.5 M		
	G' (Pascals)	G" (Pascals)	TAN DELTA	G' (Pascals)	G" (Pascals)	TAN DELTA
NaSCN	8± 1	4± 1	0.556±0.007	--[2]	--	--
$NaC_2H_3O_2$	1463± 50	241±16	0.164±0.006[a]	665±248[a]	116±42[a]	0.176±0.002[a]
NaBr	1004± 92[a]	161±13[a]	0.160±0.002[a]	25± 4[b]	6± 1[b]	0.241±0.017
NaCl	1082±169[a]	171±20[a]	0.159±0.005[a]	514± 75[a]	91±14[a]	0.176±0.002[a]
Na_2SO_4	605±104	96±17	0.159±0.001[a]	7± 1b	4± 1[b]	0.524±0.035

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

[2] No values determined as no network formed.

APPENDIX 10A. Effect of various anions on the rate of structure development during cooling from 95°C to 25°C at 2°C/min for ovalbumin (10%, pH 8.5).

Salt	0.1 M				0.5 M			
	Initial Cooling		Final Cooling		Initial Cooling		Final Cooling	
	G' (Pa/°C)	G" (Pa/°C)	G' (Pa/°C)	G" (Pa/°C)	G' (Pa/°C)	G" (Pa/°C)	G' (Pa/°C)	G" (Pa/°C)
NaSCN	-56.3±10.3[a]	-0.40±0.20[a]	- 7.6±0.5[a]	-1.36±0.25[a]	-38.6±2.1	-1.59±0.46	-48.2±2.0	-6.13±0.31
$NaC_2H_3O_2$	-34.5±4.1[bc]	-1.59±0.15[b]	-14.3±2.1[b]	-1.56±0.21[a]	-7.9±0.7[a]	-0.58±0.10[a]	-10.9±2.2[a]	-1.65±0.39[a]
NaBr	-19.9±1.2[c]	-0.71±0.01[a]	-5.4±0.5[a]	-0.61±0.06	-10.7±0.0[a]	-0.74±0.05[a]	-7.6±0.2[a]	-0.88±0.01[ab]
NaCl	-50.5±1.5[ab]	-1.77±0.09[b]	-15.2±0.6[b]	-1.71±0.07[a]	-9.7±0.8[a]	-0.61±0.19[a]	-6.9±0.1[a]	-0.82±0.01[ab]
Na_2SO_4	-24.2±3.3[c]	-1.13±0.05	-15.8±2.6[b]	-1.86±0.34[a]	-1.1±0.1	-0.16±0.02[a]	-1.1±0.1	-0.16±0.02[b]

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

APPENDIX 10B. Effect of various anions on the rate of structure development during cooling 95°C to 25°C at 2°C/min for vicilin (10%, pH 8.5).

Salt	0.1 M				0.5 M			
	Initial Cooling		Final Cooling		Initial Cooling		Final Cooling	
	G' (Pa/°C)	G" (Pa/°C)	G' (Pa/°C)	G" (Pa/°C)	G' (Pa/°C)	G" (Pa/°C)	G' (Pa/°C)	G" (Pa/°C)
NaSCN	-[2]	-	-	-	-	-	-	-
$NaC_2H_3O_2$	-7.34±0.38[a]	-0.80±0.22[a]	-20.0±1.6[a]	-3.37±0.25[a]	-4.01±0.50[a]	-0.45±0.08	-13.28±3.36[a]	-2.58±0.41
NaBr	-5.48±0.41[ab]	-0.32±0.04[a]	-16.7±1.6[a]	-3.06±0.33[a]	-0.33±0.02[b]	-0.09±0.01[ab]	-0.21±0.12[b]	-0.03±0.04[a]
NaCl	-5.96±0.85[a]	-0.46±0.06[a]	-14.3±1.7[ab]	-2.37±0.30[ab]	-3.35±0.41[a]	-0.26±0.03[a]	-6.85±1.01[ab]	-1.35±0.14
Na_2SO_4	-3.45±0.66[b]	-0.29±0.10[a]	-9.5±2.1[b]	-1.54±0.42[b]	-0.13±0.06[b]	-0.05±0.04[b]	-0.45±0.06[b]	-0.12±0.06[a]

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

[2] No rheological data available as the lack of structure resulting from the NaSCN environment gave G' and G" values below the sentivity limits of the rheometer as used (92.3 g torsion bar).

APPENDIX 11A. Effect of urea on the rheological properties of heat set ovalbumin (10%, pH 8.5).

Concentration (M)	G' (Pa)	G" (Pa)	Tan δ
0.05	23± 1[a]	5.9±0.4[a]	0.255±0.020[a]
0.10	20± 5[a]	5.7±1.2[a]	0.279±0.009[a]
0.25	14± 7[a]	4.1±1.9[a]	0.285±0.004[a]
0.50	8± 3[a]	3.2±0.4[a]	0.403±0.044[b]
1.0	9± 2[a]	3.6±0.1[a]	0.487±0.050[b]
2.0	26± 2[a]	4.9±0.6[a]	0.185±0.034[a]
4.0	544± 18	14.5±1.0	0.026±0.001c
6.0	3471±160	61.9±5.7	0.018±0.001[c]
8.0	3178±158	47.4±2.7	0.015±0.014[c]

[1] Column values followed by the same letter are not significantly different (P≤0.05).

APPENDIX 11B. Effect of urea on the rheological properties of heat set vicilin (10%, pH 8.5).

Concentration (M)	G' (Pa)	G" (Pa)	Tan δ
0.05	20.6±5.8[a]	6.2±2.1[ab]	0.296±0.021[a]
0.10	21.4±4.4[a]	7.8±0.7[ac]	0.374±0.063[ab]
0.25	34.0±5.8	10.2±1.3[c]	0.316±0.091[a]
0.50	20.3±1.2[a]	6.9±0.5[abc]	0.342±0.004[ab]
1.0	13.7±0.8[a]	5.6±1.0[ab]	0.405±0.049[ab]
2.0	10.9±2.0[a]	4.4±0.7[ab]	0.407±0.010[ab]
6.0	8.3±2.4[a]	3.4±0.5[b]	0.486±0.015[b]

[1] Column values followed by the same letter are not significantly different (P≤0.05).

APPENDIX 12A. Effect of urea on the rate of structure development during cooling from 95°C to 25°C at 2°C/min for ovalbumin (10%, pH 8.5).

Concentration	Initial Cooling Phase		Final Cooling Phase	
(M)	G' (Pa/°C)	G" (Pa/°C)	G' (Pa/°C)	G" (Pa/°C)
.05	-0.14±0.05[a]	-0.03±0.00[a]	-0.26±0.02[a]	-0.04±0.01[a]
.10	-0.20±0.01[a]	-0.03±0.00[a]	-0.24±0.01[a]	-0.05±0.03[a]
.25	-0.22±0.01[a]	-0.05±0.00[a]	-0.01±0.23[a]	-0.06±0.01[a]
.50	-0.18±0.02[a]	-0.02±0.02[a]	-0.18±0.04[a]	-0.05±0.01[a]
1.0	-0.26±0.12[a]	-0.04±0.01[a]	-0.01±0.08[a]	-0.02±0.02[a]
2.0	-0.47±0.00[a]	-0.11±0.02[a]	-0.24±0.01[a]	-0.01±0.01[a]
4.0	-8.90±0.20	0.24±0.05	-1.32±0.57[a]	0.32±0.19[ab]
6.0	-31.8±0.3	2.15±0.15	-1.35±2.82[a]	0.71±0.26[b]
8.0	-29.4±0.1	2.40±0.06	4.62±0.51	2.12±0.14

[1] Column values followed by the same letter are not significantly different (P0.05).

APPENDIX 12B. Effect of urea on the rate of structure development during cooling 95°C to 25°C at 2°C/min for vicilin (10%, pH 8.5).

Concentration	Initial Cooling Phase		Final Cooling Phase	
(M)	G' (Pa/°C)	G" (Pa/°C)	G' (Pa/°C)	G" (Pa/°C)
.05	-0.30±0.07[ab]	-0.06±0.02[a]	0.09±0.06[a]	-0.01±0.01[a]
.10	-0.33±0.10[ab]	-0.10±0.05[a]	0.20±0.14[a]	0.07±0.02[a]
.25	-0.42±0.02[a]	-0.088_0.04[a]	-0.10±0.06[a]	0.04±0.13[a]
.50	-0.30±0.07[ab]	-0.08±0.04[a]	-0.03±0.17[a]	-0.01±0.01[a]
1.0	-0.32±0.01[ab]	-0.10±0.00[a]	0.25±0.07[a]	0.12±0.01[a]
2.0	-0.27±0.05[ab]	-0.07±0.01a	0.42±0.33[a]	0.14±0.06[a]
6.0	-0.14±0.04[b]	-0.04±0.02[a]	0.01±0.02[a]	-0.01±0.03[a]

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

APPENDIX 13A. Effect of cystine hydrochloride on the rheological properties of heat set ovalbumin (10% in 0.15 M NaCl, pH 8.5).

Concentration (mM)	G′ (Pascals)	G″ (Pascals)	Tan δ
2.5	1163± 67[a]	95± 6[a]	0.081±0.000[a]
5.0	1404±145[a]	110±10[a]	0.079±0.001[ab]
10.0	1205± 83[a]	110±10[a]	0.091±0.002
25.0	2541± 30[b]	204± 0	0.080±0.001[a]
50.0	1875±215[ab]	141±17[a]	0.075±0.001[b]

[1] Column values followed by the same letter are not significantly

APPENDIX 13B. Effect of cysteine hydrochloride on the rate of structure development during cooling from 95°C to 25°C at 2°C/min for ovalbumin (10% in 0.15 M NaCl, pH 8.5).

Concentration	Initial Cooling Phase		Final Cooling Phase	
(mM)	G′ (Pa/°C)	G″ (Pa/°C)	G′ (Pa/°C)	G″ (Pa/°C)
2.5	-23.8±1.2[ab]	-0.69±0.02[a]	-9.0±0.3[a]	-1.04±0.03[ab]
5.0	-27.5±0.7[a]	-0.77±0.15[a]	-11.5±1.6[a]	-1.29±0.12[a]
10.0	-21.0±1.3[b]	-0.90±0.06[ab]	-11.2±0.9[a]	-1.29±0.15
25.0	-49.5±2.5[a]	-1.21±0.15[b]	-18.2±1.8	-2.20±0.24
50.0	-72.1±0.3	-2.76±0.01	-0.4±3.1	-0.62±0.03[b]

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

APPENDIX 14A. Effect of mercaptoethanol (ME) and N'- ethylmaleimide (NEM) on the rheological properties of heat set ovalbumin (10% in 0.15 M NaCl, pH 8.5).

Concentration	ME			NEM		
(mM)	G' (Pascals)	G" (Pascals)	TAN DELTA	G' (Pascals)	G" (Pascals)	TAN DELTA
2.5	1863± 43[a]	158± 0[a]	0.085±0.002[a]	2518±178[ab]	235±10[a]	0.093±0.002[a]
5.0	1441±181[a]	130±15[a]	0.090±0.002[a]	3115±352[a]	291±34[a]	0.093_0.000[a]
10.0	907±215[b]	80±18[b]	0.087±0.000[a]	2555±264[ab]	256±26[a]	0.100±0.000[a]
25.0	520± 30[b]	68± 3[b]	0.130±0.002	2266± 78[b]	270±12[a]	0.119±0.001
50.0	449± 41[b]	61± 6[b]	0.137±0.002	15± 1	7± 1	0.473±0.006

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

APPENDIX 14B. Effect of mercaptoethanol (ME) and N' - ethylmaleimide (NEM) on the rate of structure development during cooling from 95°C to 25°C at 21°C/min for ovalbumin (10%, pH 8.5).

Conc (mM)	ME				NEM			
	Initial Cooling		Final Cooling		Initial Cooling		Final Cooling	
	G' (Pa/°C)	G" (Pa/°C)	G' (Pa/°C)	G" (Pa/°C)	G' (Pa/°C)	G" (Pa/°C)	G' (Pa/°C)	G" (Pa/°C)
2.5	-71.0±6.0[a]	-2.11±0.04[a]	-10.8±2.6[a]	-1.49±0.01[a]	-47.1±3.4[ab]	-1.59±0.09[a]	-23.4±1.3[a]	-2.66±0.15[a]
5.0	-67.4±1.8[a]	-2.10±0.40[a]	-5.5±1.6[a]	-1.20±0.19[a]	-58.9±4.7[a]	-1.83±0.03[a]	-27.9±3.4[a]	-3.28±0.34[ab]
10.0	-35.4±1.3	-1.49±0.09[a]	-4.3±2.4[a]	-0.69±0.25[a]	-43.6±5.0[bc]	-1.76±0.43[a]	-23.3±2.1[a]	-2.91±0.30[ab]
25.0	-55.1±4.5[a]	-1.40±0.12[a]	61.4±15.8[b]	2.17±1.10[b]	-51.5±2.7[ab]	-0.59±0.08	-17.6±1.0[a]	-3.61±0.24[b]
50.0	-71.5±6.2[a]	-1.16±0.83[a]	71.6±8.8[b]	3.51±1.02[b]	-32.7±0.6[c]	-2.21±0.09[a]	37.1±0.7	3.53±0.12

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

APPENDIX 15. Effect of protein concentration on the thermal denaturation of ovalbumin and vicilin in 0.15 M NaCl, pH 8.5.

Concentration	Ovalbumin		Vicilin	
(%)	Td (°C)	ΔH (J/g protein)	Td (°C)	ΔH (J/g protein)
5	85.6±0.1[a]	15.2±2.6[a]	80.8±0.4[a]	15.5±0.1[a]
7.5	85.4±0.1[ab]	13.8±2.3[a]	80.0±0.0[a]	19.3±0.2[abc]
10	85.1±0.1[c]	15.9±0.1[a]	79.1±0.9[a]	21.5±2.2[c]
12.5	85.0±0.1[c]	16.2±0.5[a]	79.0±0.7[a]	21.5±1.5[bc]
15	85.2±0.1[bc]	12.3±0.9[a]	79.0±0.9[a]	14.3±2.6[a]

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

APPENDIX 16. Effect of pH on the thermal denaturation of 10% ovalbumin and 10% vicilin in 0.15 M NaCl.

pH	Ovalbumin		Vicilin	
	Td (^{o}C)	ΔH (J/g protein)	Td (^{o}C)	ΔH (J/g protein)
3	63.9±0.9	3.3±0.1[a]	-[2]	0.0±0.0
4	71.2±0.1	6.9±1.5[a]	67.8±2.8	5.0±0.2[a]
5	83.8±0.1[a]	21.0±1.6[b]	80.3±0.1[ab]	14.4±2.4[b]
6	84.5±0.4[a]	12.3±0.3[c]	82.0±0.1[b]	11.1±1.3[bc]
7	83.9±0.1[a]	15.2±1.0[c]	79.1±0.9[ab]	13.1±1.9[b]
8	85.2±0.1[a]	14.7±2.1[c]	80.3±0.1[ab]	14.1±2.6[b]
9	85.2±0.2[a]	17.4±2.3[bc]	77.0±1.0[a]	7.4±0.5[ac]

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

APPENDIX 17. Effect of SDS (sodium dodecylsulfate) on the thermal denaturation of 10% ovalbumin and 10% vicilin in 0.15 M NaCl, pH 8.5.

Concentration	Ovalbumin		Vicilin	
(mM)	Td (°C)	ΔH (J/g protein)	Td (°C)	ΔH (J/g protein)
2.5	nd[2]	nd	78.2±0.0[a]	12.3±0.5[ab]
5.0	83.1±0.2[a]	20.2±2.9[a]	78.2±0.1[a]	11.4±0.8[a]
7.5	nd	nd	78.0±0.1[a]	12.0±0.9[ab]
10.0	81.3±0.6[a]	18.4±0.6[a]	77.4±1.0[a]	13.9±0.8[b]
20.0	74.1±0.8	10.8±0.2b	nd	nd
25.0	70.4±0.8[b]	9.5±0.3[b]	77.9±0.3[a]	8.1±0.4[c]
35.0	70.3±0.8[b]	8.6±1.2[b]	nd	nd
50.0	69.0±0.0[b]	7.4±0.2[b]	76.7±0.2[a]	6.2±0.2[c]

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

[2] nd signifies that no analysis was performed at this concentration.

APPENDIX 18. Effect of $CaCl_2$ concentration on the thermal denaturation of 10% ovalbumin and 10% vicilin, pH 8.5.

Concentration	Ovalbumin		Vicilin	
(mM)	Td (°C)	ΔH (J/g protein)	Td (°C)	ΔH (J/g protein)
0.1	83.0±0.1[a]	11.1±0.2[a]	78.3±1.4[a]	14.3±0.3[a]
0.2	82.8±0.2[a]	10.6±0.9[a]	81.6±0.1[a]	15.0±1.9[a]
0.3	81.5±0.0[b]	11.7±1.0[a]	81.8±0.5[a]	19.0±0.7[a]
0.4	81.5±0.2[b]	10.3±0.4[a]	84.7±0.3[b]	17.5±1.6[a]
0.5	81.3±0.0[b]	12.7±1.0[a]	84.8±0.1[b]	17.1±0.7[a]

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

APPENDIX 19. Effect of NaCl concentration on the thermal denaturation of 10% ovalbumin and 10% vicilin, pH 8.5.

Concentration	Ovalbumin		Vicilin	
(mM)	Td (^{o}C)	ΔH (J/g protein)	Td (^{o}C)	ΔH (J/g 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]

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

APPENDIX 20A. Effect of various anions of sodium salts (0.1 M) on the thermal denaturation of 10% ovalbumin and 10% vicilin, pH 8.5.

Salt	Ovalbumin		Vicilin	
	Td (^oC)	ΔH (J/g protein)	Td (^oC)	ΔH (J/g 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]
NaBr	84.3±0.6[a]	14.6±0.6[a]	77.9±0.1[a]	13.6±1.3[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]

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

APPENDIX 20B. Effect of various anions of sodium salts (0.5 M) on the thermal denaturation of 10% ovalbumin and 10% vicilin, pH 8.5.

Salt	Ovalbumin		Vicilin	
	Td (^oC)	ΔH (J/g protein)	Td (^oC)	ΔH (J/g 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]
NaBr	84.0±0.0	16.0±0.6[a]	81.5±0.1	12.6±1.2[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]

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

APPENDIX 21. Effect of urea on the thermal denaturation of 10% ovalbumin and 10% vicilin, pH 8.5.

Concentration	Ovalbumin		Vicilin	
(M)	Td (°C)	ΔH (J/g protein)	Td (°C)	ΔH (J/g protein)
0.05	84.7±0.5[a]	14.9±1.2[a]	74.2±0.1[a]	14.7±3.0[a]
0.1	85.1±0.1[ab]	13.9±1.0[ab]	73.2±1.0[ab]	12.1±2.1[ab]
0.25	84.7±0.2[ab]	14.2±0.7[ab]	70.5±2.1[abc]	9.2±0.7[b]
0.5	83.5±0.5[a]	16.2±1.2[a]	69.7±1.1[abc]	7.3±1.3[bc]
1.0	82.3±0.6[c]	16.5±0.7[a]	69.1±1.0[bc]	3.6±0.7[cd]
2.0	79.9±0.1	13.2±3.2[ab]	67.8±0.9[c]	2.1±1.0[d]
4.0	75.1±0.1	12.6±1.3[ab]	nd[2]	nd
6.0	69.2±0.1	10.2±0.1[b]	_[3]	0.0±0.0[d]
8.0	67.8±0.8	4.8±0.9	nd	nd

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

[2] nd signifies that no analysis was performed at this concentration.

[3] No value obtained (-) as there was no endotherm on the DSC thermal curve.

APPENDIX 22. Effect of cysteine hydrochloride on the thermal denaturation of 10% ovalbumin and 10% vicilin in 0.15 M NaCl, pH 8.5.

Concentration	Ovalbumin		Vicilin	
(mM)	Td (°C)	ΔH (J/g protein)	Td (°C)	ΔH (J/g protein)
2.5	85.0±0.1[ab]	9.8±1.2[a]	78.5±0.3[a]	15.4±1.5[a]
5.0	85.2±0.4[ab]	20.6±0.1[b]	78.4±0.1[a]	13.7±1.9[a]
10.0	84.6±0.3[ab]	23.5±2.6[b]	79.6±0.3	11.2±2.0[a]
25.0	85.6±0.1[a]	10.5±0.1[a]	78.4±0.3[a]	6.4±1.2[b]
50.0	84.3±0.3[b]	13.2±0.4[a]	78.4±0.2[a]	5.8±0.3[b]

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

APPENDIX 23A. Effect of mercaptoethanol (ME) on the thermal denaturation of 10% ovalbumin and 10% vicilin in 0.15 M NaCl, pH 8.5.

Concentration	Ovalbumin		Vicilin	
(mM)	Td (°C)	ΔH (J/g protein)	Td (°C)	ΔH (J/g protein)
2.5	85.6±0.1[a]	14.8±0.3[a]	78.8±0.0[a]	14.9±3.8[a]
5.0	85.6±0.1[a]	13.1±0.2[a]	78.4±0.1[a]	12.5±0.8[a]
10.0	83.9±0.1[b]	13.8±1.7[a]	78.8±0.7[a]	15.6±2.0[a]
25.0	84.0±0.0[b]	13.2±0.2[a]	78.1±0.0[a]	14.6±0.6[a]
50.0	82.1±0.6[a]	12.6±0.4[a]	78.3±0.1[a]	14.1±0.1[a]

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

APPENDIX 23B. Effect of N' - ethylmaleimide (NEM) on the thermal denaturation of 10% ovalbumin and 10% vicilin in 0.15 M NaCl, pH 8.5.

Concentration	Ovalbumin		Vicilin	
(mM)	Td (°C)	ΔH (J/g protein)	Td (°C)	ΔH (J/g protein)
2.5	85.3±0.2[a]	15.5±1.6[a]	79.6±0.5[a]	13.5±0.7[a]
5.0	84.5±0.6[a]	14.0±1.1[a]	81.8±0.2[ab]	10.5±1.3[a]
10.0	84.4±0.1[a]	14.0±0.8[a]	81.9±0.9[ab]	14.6±4.6[a]
25.0	82.7±0.1	12.5±1.4[a]	83.9±1.0[b]	9.2±1.2[a]
50.0	81.1±0.1	11.8±2.1[a]	81.0±0.1[a]	13.3±0.6[a]

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