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# EFFECT OF PH, SALT TYPE AND DENATURANTS ON THE DENATURATION PROPERTIES, STORAGE MODULUS, SECONDARY STRUCTURE AND MICROSTRUCTURE OF HEN EGG S-OVALBUMIN HEAT-INDUCED GELS

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# EFFECT OF PH, SALT TYPE AND DENATURANTS ON THE DENATURATION PROPERTIES, STORAGE MODULUS, SECONDARY STRUCTURE AND MICROSTRUCTURE OF HEN EGG S-OVALBUMIN HEAT-INDUCED GELS

BY

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# ABSTRACT

## EFFECT OF PH, SALT TYPE AND DENATURANTS ON THE DENATURATION PROPERTIES, STORAGE MODULUS, SECONDARY STRUCTURE AND MICROSTRUCTURE OF HEN EGG S-OVALBUMIN HEAT-INDUCED GELS

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THOMAS JOSEPH HERALD

Changes in thermal properties, storage modulus, secondary structure and microstructure of S-ovalbumin as a function of pH (3.0, 7.0 or 9.0), salt type (NaCl, NaI or $Na_2SO_4$), guanidine hydrochloride (0.5, 1.0 or 2.0M) or ß-mercaptoethanol (0.5, 1.0, 2.0 and 3.0%) were studied using differential scanning calorimetry (DSC), dynamic rheological testing, Fourier transform infrared spectroscopy (FT-IR) and electron microscopy.

The DSC onset temperature of ovalbumin denaturation ($T_o$) occurred 5.8°C - 7.5°C prior to structure development ($T_s$) as measured by storage modulus. Enthalpy, $T_o$, and $T_s$ increased as pH increased. Denaturation temperature decreased in the order of the Hoffmeister series, whereas $T_s$ decreased in reverse order. Activation energies of ovalbumin denaturation and structure development were dependent on pH and salt type.

Increasing GuHCl and ß-ME decreased the denaturation temperature 10°C and 9°C, respectively. Enthalpy did not

differ ($P > 0.05$) at any of the GuHCl concentrations but decreased with increased ß-ME (62.4-44.6 kJ/mol). Onset temperature for ovalbumin structure formation decreased and storage moduli increased with increased concentrations of GuHCl and ß-ME.

Solutions of hen egg S-ovalbumin at pD 3.0, 7.0 or 9.0 were heated at temperatures between 30°C to 90°C to study changes in secondary structure by FT-IR. Second derivative infrared spectra of native ovalbumin at pD 7.0 and 9.0 revealed protein absorption bands for ß-sheet at 1626 $cm^{-1}$, $3_{10}$-helix at 1638 $cm^{-1}$, α-helix at 1656 $cm^{-1}$, and turn at 1682 $cm^{-1}$. The ß-sheet absorption band was not observed for S-ovalbumin at pD 3.0. The quantity of α-helix and ß-sheet structure decreased as heating temperature was increased.

Changes in microstructure of heat-induced hen egg S-ovalbumin gels influenced by pH, salt type and denaturants (GuHCl and ß-ME) were studied using low temperature scanning electron microscopy (LTSEM) and chemical fixation procedures with scanning electron microscopy (SEM). The LTSEM of 8% S-ovalbumin gels were honey comb in appearance, while S-ovalbumin gels prepared by chemical fixation exhibited a microstructure of grape-like clusters. S-ovalbumin gels prepared at pH 3.0 or in 0.5M $Na_2SO_4$ exhibited the smallest pore size as viewed using LTSEM. Using SEM the largest void volumes exhibited by the S-ovalbumin gels were prepared at pH 3.0 or 0.5M NaI.

This dissertation is dedicated to my wife, Christine,
and children, Jacob and Sarah

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# I. INTRODUCTION

Hen eggs contain a variety of proteins that contribute unique functional and nutritional properties to food systems. When cooked, eggs form a gel that provides a matrix for holding water, flavors, ingredients and provide texture, a critical attribute in such foods as custards. Ovalbumin is the major egg white protein and is predominately responsible for egg white gelation. Ovalbumin gels are made of a three dimensional matrix of cross-linked polypeptides. Gelation of ovalbumin is an orderly aggregation of protein. The protein may be partially or completely denatured by heating or denaturants (Hermansson, 1979). Aggregation of the unfolded ovalbumin protein form strands that interact to produce a network. Bonds giving structure to the network may be electrostatic (Beveridge et al., 1980), hydrophobic (Hayakawa and Nakai, 1985), hydrogen, covalent (Beveridge et al., 1984), van der Waals forces (Hatta et al., 1986) or a combination thereof.

From a nutritional standpoint, egg proteins are considered the best source of high quality protein and are used as a standard for protein quality determinations. Eggs contain all of the essential fatty acids, large quantity of unsaturated fatty acids, many of the minerals needed in our diet and all vitamins except vitamin C (Froning, 1988).

The present study used a multitechnique evaluation to determine changes in secondary structure, rheological properties and microstructural characteristics of ovalbumin during heat-induced gelation. It is hypothesized that perturbing the ovalbumin system by changing pH (3.0, 7.0, and 9.0), salt type (0.5M $Na_2SO_4$, 0.5M NaCl and 0.5M NaI) or by addition of chemical denaturants (0.5, 1.0, and 2.0M guanidine hydrochloride and 0.5, 1.0, 2.0 and 3.0% ß-mercaptoethanol) will provide a better understanding of the conditions needed to produce ovalbumin gels with the desired functions.

Many studies have investigated the gelation behavior of ovalbumin (van Kleef et al., 1978: Egelandsdal, 1980; Clark and Lee-Tuffnell, 1986; Hatta et al., 1986) although none have attempted to relate changes in secondary structure to heat-induced gel properties. The objectives of the present research include heating and perturbating ovalbumin (1) to monitor the denaturation temperature and quantify the enthalpy changes, (2) to characterize and quantify storage modulus of ovalbumin, (3) to monitor the changes in secondary structure and (4) to observe changes in the microstructure.

This dissertation contains 5 chapters. The first chapter is a review of the literature for the entire dissertation. Chapters 2 through 5 are the four studies which present the dissertation research. Each study was organized using the format of the Journal of Food Science and contains the following sections: Introduction, Literature Review, Materials and Methods, Results and Discussion. Chapters 7 and 8 are the overall conclusions of the research and recommendations for future research, respectively. The final chapter contains the references for the entire dissertation.

## II. LITERATURE REVIEW

### Properties of Ovalbumin

Ovalbumin is a phosphoglycoprotein and makes up 54% of the total proteins in the albumen. Ovalbumin has an isoelectric point (pI) of 4.5 and a molecular weight of 45,000 (Powrie and Nakai, 1986). The complete amino acid sequence of ovalbumin with 385 residues (Fig. 1) was determined by Nisbet et al. (1981). The N-terminal amino acid is acetylated glycine and the C-terminal amino acid is proline. Stein et al. (1990) and Wright et al. (1990) have determined the crystal structure of ovalbumin.

Ovalbumin contains a carbohydrate moiety attached to asparagine residue 292 (Lee and Montgomery, 1962; Nisbet et al., 1981). The carbohydrate moiety has a molecular weight between 1560 and 1580 daltons (Lee et al., 1964; Montgomery et al., 1965) and consists of a core of two N-acetylglucosamine units and four mannose units.

Ovalbumin exists in three forms, $A_1$, $A_2$, and $A_3$, which differ in phosphorous content. $A_1$ has 2 phosphates per molecule, $A_2$ has 1 phosphate per molecule and $A_3$ does not contain phosphate (Longsworthe et al., 1940; Cann, 1949; Perlman, 1952). The relative proportion of $A_1$, $A_2$, and $A_3$ in ovalbumin is about 85:12:3, respectively. Phosphate groups are attached to serine residues 68 and 344 (Nisbet et al., 1981). Ovalbumin has been reported to contain four

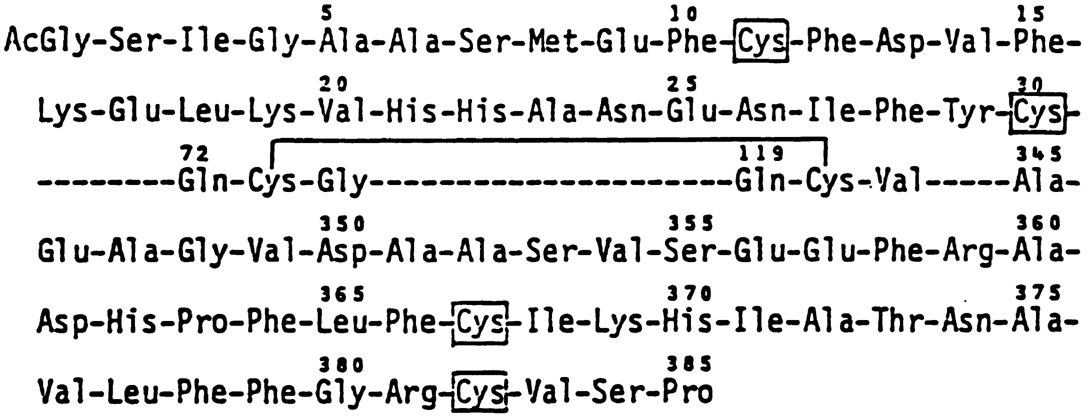


**Fig. 1-Amino acid sequence of ovalbumin. Taken from Nisbet et al. (1981).**

sulfhydryl groups and one disulfide group. Three sulfhydryl groups were observed in native ovalbumin and a fourth was observed after denaturation (Fernandez-Diez, 1964).

Optical rotary dispersion studies have indicated that ovalbumin secondary structure may be composed of 25% $\alpha$-helix and 25% $\beta$-sheet (Cho, 1970). Kato and Takagi (1988) using circular dichroism (CD) determined the secondary structure of 0.1% ovalbumin solution (67 mM sodium phosphate buffer, pH 7.0 and 0.1M NaCl) to be 49% $\alpha$-helix and 13% $\beta$-sheet.

## S-Ovalbumin

Smith (1964) and Smith and Back (1965) have shown that ovalbumin was converted to S-ovalbumin, a more heat-stable protein, during storage of eggs and ovalbumin solutions. In the latter case, the rate of conversion to S-ovalbumin increased with pH and temperature. Freeze dried ovalbumin stored in the cold for 20 years showed partial conversion to an intermediate form, but not to S-ovalbumin (Smith and Back, 1968). There are two additional ionizable carboxyl groups in S-ovalbumin that are reactive compared to ovalbumin (Nakamura et al. 1980). Kato et al. (1986) reported that one of the carboxly groups was involved in electrostatic cross-linkage with a positively charged amino acid residue in the interior of the molecule, leading to the formation of S-ovalbumin. S-ovalbumin has a slightly lower molecular weight and was more resistant to heat denaturation and to denaturation with urea and guanidine hydrochloride (GuHCl) (Smith and Back, 1965).

Smith and Back (1964) suggested sulfhydryl-disulfide interaction may be involved in the ovalbumin to S-ovalbumin transition. However, this hypothesis was rejected by analysis of peptides from tryptic and peptic digests, that showed no differences between cystine peptides from either protein (Smith and Back, 1968).

Donovan and Mapes (1976) studied the conversion of ovalbumin to S-ovalbumin in eggs using differential scanning calorimetry (DSC). The denaturation temperature ($T_d$) of ovalbumin and S-ovalbumin was 84.5°C and 92.5°C, respectively at pH 9.0 and a heating rate of 10°C/min. Shitamori et al. (1984) reported that heat-induced gel strength of S-ovalbumin was less than ovalbumin.

Kint and Tomimatsu (1979) using Raman spectroscopy observed that 3% to 4% of $\alpha$-helix changed to $\beta$-sheet configuration upon conversion from ovalbumin to S-ovalbumin.

## The Effect of Heat on Ovalbumin

Heat treatment causes denaturation and aggregation of proteins and Ferry (1948) described this mechanism as a two-step process:

```
native----> denatured protein ---->aggregated protein
              (long chains)          (associated network)
```

Heat denaturation is the process of native protein conformation changing to an unfolded conformation. Aggregation is the intermolecular protein interactions leading to higher molecular weight molecules. Kato and Takagi (1988) described that heat-induced gelation of ovalbumin is a thermally irreverisble process as measured by circular dichrosim (CD).

Knowing the rate of reaction between denaturation and aggregation help to determine gel characteristics (Gossett et al., 1984). Ferry (1948) reported a gel network occurs if the aggregation step takes place slower than the denaturation step giving the denatured protein time to orient before aggregation. Gels of this nature are higher in elasticity. If denaturation and aggregation occur simultaneously a less elastic gel results.

Tombs (1974) proposed globular protein form gels as a result of aggregation forming a "string of beads" followed by interactions of the beads to form a mesh. Koseki et al. (1989) developed a model (Fig. 2) showing high molecular weight ovalbumin linear aggregates or "string of beads" based on sedimentation analysis, viscometry and transmission microscopy. Kato et al. (1983) reported that ovalbumin aggregates were formed by hydrophobic interaction due to the presence of surface hydrophobicity of heat-denaturatured ovalbumin.

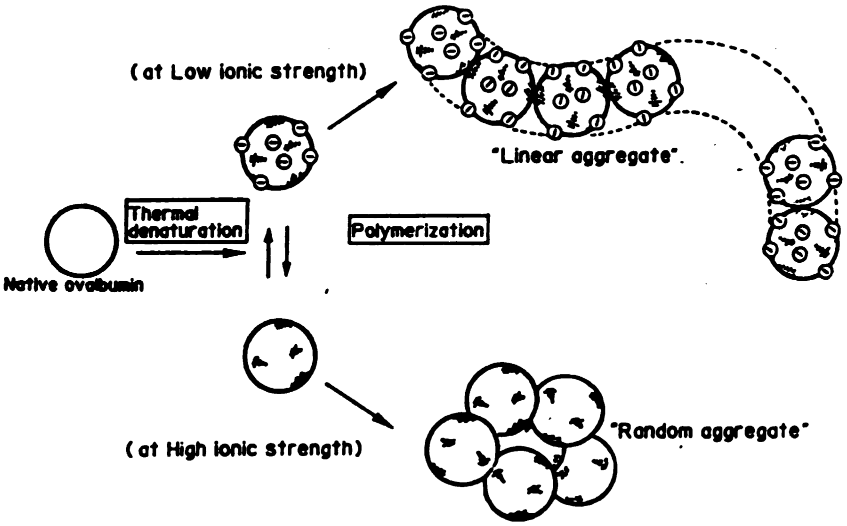


**Fig. 2-A proposed model for the thermal denaturation and aggregation behavior of ovalbumin molecules. Shadows and ⊖ indicate hydrophobic areas exposed by thermal denaturation and minus charges on the surface of ovalbumin molecules, respectively. Taken from Doi et al. (1987).**

presence of surface hydrophobicity of heat-denaturatured ovalbumin.

Protein solubility decreases with time and temperature of heat treatment. Evidence of conformational changes in protein structure as measured by CD has been related to changes in protein solubility (Neucera and Cherry, 1982). Protein functionality has been associated with protein solubility. Kato et al. (1981) reported protein functionality can improve with denaturation as long as a decrease in solubility does not occur. Surface tension decreases with denaturation as exposure of hydrophobic molecule become exposed to the surface of the protein. Surface hydrophobicity increases as denaturation procedes increasing emulsion and foam stability (Kilara and Sharkasi, 1986). The increase in surface hydrophobicity due to denaturation causes proteins to be amphiphilic. The amphiphillic nature adsorbed at the interface between oil and water or air causes reduction of surface free energy that facilitates emulsification and foaming (Kato et al., 1981).

Polyacrylamide gel electrophoresis (PAGE) showed no change in ovalbumin solubility with pasteurization temperatures of 60°C, 64°C or 68°C for 3.5 min (Herald, 1987). Heat stability of ovalbumin varies with pH. Less than 1.0% of ovalbumin was denatured at pH 6.5-7.0 when heated at 65°C for 30 min (Li-Chan and Nakai, 1989).

Hegg et al. (1979) found that ovalbumin had maximum thermal stability between pH 6.0 and 10.0. The addition of 170 mM NaCl did not affect denaturation temperature while 17 mM $CaCl_2$ caused a 2-3°C decrease in denaturation temperature.

Shimada and Matsushita (1980b) used absorbance at 340 nm to determine the effect of protein concentration on coagulation by observing turbidity changes.

Ma and Holme (1982) reported that turbidity is related to an increase in hydrophobic residue exposure.

## Effects of pH on Ovalbumin

In theory, as solution pH approaches the pI of ovalbumin, the charge on the protein is neutralized. This results in attractive forces and protein-protein interactions with minimal protein unfolding (Seideman et al., 1963; Arntfield et al., 1989).

Koseki et al. (1990) reported that ovalbumin was unstable and susceptible to denaturation at pH below the pI. The researcher reported that ovalbumin existed in a "molten globular state". The molten-globule state exists when a molecule is in a compact globular (native state) state, but the tertiary structure slowly fluctuates.

At high pH, most egg albumen proteins are negatively charged and the ability of proteins to cross-link through sulfhydryl-disulfide exchange is enhanced, possibly contributing to the structural integrity of gels (Shimada and Matsushita, 1980). At high pH the high net charge may inhibit thermal coagulation due to repulsive forces, whereas high protein concentration may overcome electrostatic forces to form networks through hydrophobic interactions (Shimada and Matsushita, 1981).

Slosberg et al. (1948) reported a greater heat stability for egg albumen at pH 6.5 than at 8.5 using whip time and angel cake volume to evaluate stability. Seideman et al. (1963) concurred using gelation scores as stability criteria.

Koseki et al. (1990) used DSC to monitor denaturation temperature ($T_d$) of ovalbumin. The researchers reported that as pH was decreased, the endothermic peak and cooperativity declined suggesting changes in ovalbumin conformation. Arntfield and Murray (1981) reported that acidic pH reduced $T_d$ and enthalpy of ovalbumin as measured by DSC. The researchers suggested a weakening of intermolecular interactions due to an increase in positive charges was responsible for the decrease in $T_d$. The decrease in enthalpy was due to an increase in the stability of the hydrophobic interactions.

Holt et al. (1984) reported that maximum strength and elasticity of ovalbumin gels were observed at pH 9.0 whereas no gel structure was evident at acid pH. At pH 9.0, the ability of the proteins to crosslink through sulfhydryl-disulfide contributed to gel formation.

Beveridge et al. (1980) measured the effect of pH between 5.5 to 9.0 on egg gel firmness using a shear press. They suggested the major mechanism responsible for ovalbumin gel strength was sulfhdryl-disulfide interchange reactions at alkaline pH. As pH decreased to 6.0, these reactions were inhibited because concentration of $S^-$ was reduced.

Arntfield et al. (1990 a) suggested 10% ovalbumin in 150 mM NaCl at pH 3.0 was denatured as indicated by $T_d$ and enthalpy. Enthalpy decreased from 15 J/g to 3.3 J/g at pH 7.0 and 3.0, respectively. Thermal denaturation temperature decreased from 83.9°C to 63.9°C at pH 7.0 and 3.0, respectively.

## Effects of Neutral Salts on Ovalbumin

Salts of the lyotropic (Hofmeister) series include $F^-$, $CH_3COO^-$, $SO_{4=}$, $Cl^-$, $Br^-$, $ClO_{4-}$, $I^-$ and $SCN^-$. Chloride salts at the same ionic strength exhibit neither salting-in or salting-out properties (Damodaran and Kinsella, 1982) and therefore, does not affect protein conformation. Protein solubility in NaCl solutions can be regarded as a reference point for comparisons with solubility in other salt solutions of equal ionic strength (Kakalis and Regenstein, 1986). The chaotropic (structure-disrupting) thiocyanate, iodide and chlorate reduce the free energy of entropy associated with the exposure of apolar residues. Chaotropic anions can reduce interprotein hydrophobic interactions and disrupt or unfold the compact native structure of proteins (Preston, 1989). Increasing concentrations of nonchaotropic anions, such as $F^-$, increase the free energy of entropy associated with the exposure of apolar residues and increase inter- and intraprotein hydrophobic interactions and stabilize native protein structure (Preston, 1989).

Salts in the lyotropic series have been used to probe hydrophobic interactions in network formation. At ionic concentrations sufficient to minimize electrostatic interactions, changes in protein properties due to anion concentration and salt type can be attributed to hydrophobic interactions (von Hippel and Schleich, 1969; Melander and Horvath, 1977). Hydrophobic interactions between amino acid side chains are recognized as the major force responsible for the stability of protein structure. The driving force for such interactions arise from the energetically unfavorable effect hydrophobic interactions have on the structure of water molecules (Damodaran and Kinsella, 1982). The reseachers suggested that chaotropic ions altered the water structure, decreasing hydrophobic interactions of the protein molecules. This action disrupted the compact native structure of the protein, favoring unfolding and destabilize water structure. Preston (1989) observed that increasing $Cl^-$ from 0.05 to 0.1M increased the free energy of entropy associated with exposure of apolar residues. The author suggested increased interprotein and intraprotein hydrophobic interactions tended to stabilize native protein structure. Protein structure can be manipulated by decreasing hydrophobic interaction by changing solvent conditions using chaotropic ions (Tanford, 1979).

Babajimopoulos et al. (1983) observed supression of electrostatic repulsive interaction between soy protein molecules at 0.5M ionic strength. Decreased electrostatic interaction allowed association and formation of hydrogen bonds and other interactions on the surface of native protein molecules. There is an optimum salt concentration for network formation. It has been suggested that low salt concentrations aid in protein solubilization before heating and provide a cross-link in the network (Kohnhorst and Mangino, 1985; Mulvihill and Kinsella, 1988). At high levels of salt concentration the net repulsive charge on the protein is masked; any further salt addition promotes aggregation. Maximum gel strength for β-lactoglobulin is between 75-300 mM NaCl (Mulvihill and Kinsella, 1988), while maximum gel strength for ovalbumin is between 50-100 mM NaCl (Egelandsdal, 1984; Holt et al., 1984; Hayakawa and Nakamura, 1986).

Salts inhibit interactions between water molecules and hydrophilic groups in protein molecules. Bull and Bresse (1970) observed that ions of higher order lyotropic series can dehydrate proteins enhancing protein-protein interaction.

Arntfield et al. (1990 b) observed that the storage modulus (G') of ovalbumin in 0.5M NaSCN increased compared to other anionic salts because of the high negative charge on the protein causing unfolding and subsequent cross-linking. Inclusion of 0.5M $Na_2SO_4$ decreased G' suggesting increased intramolecular hydrophobic interactions. The hydrophobic interaction decreased the tendency for ovalbumin to unfold and interact to form a cross-linked structure.

## Effect of Denaturants on Ovalbumin

Guanidine hydrochloride weakens hydrophobic interactions and inhibits hydrogen bonds and ionic attractions in proteins (Nandi and Robinson, 1972). Pace (1975) used optical rotation to follow changes in conformation of proteins in 6.0-8.0M GuHCl at room temperature. Globular proteins were randomly coiled with the addition of 6.0M GuHCl without residual ordered structure. High concentrations of GuHCl eliminated protein electrostatic interactions (Bismuto and Irace, 1988).

Strambini and Gonnelli (1986) worked with liver alcohol dehydrogenase and hypothesized that at predenaturational concentrations, GuHCl penetrates the protein interior. The GuHCl decreased intramolecular interactions that resulted in an increased fluidity in the interior region of the macromolecule. The researchers suggested structural organization was destroyed and decreased denaturation cooperativity.

Heertje and van Kleff (1986) observed the effect of urea on ovalbumin solutions to distinguish between formation of covalent and non-covalent cross-links during gelation. Ovalbumin gels prepared at pH 10.0 and in urea formed networks by protein unfolding, whereas at pH 5.0, the network formed by aggregation as the protein conformation was stabilized by hydrogen bonds and hydrophobic interactions. (Clark et al., 1981 a).

Ovalbumin in 6.0M GuHCl and 0.1M ß-mercaptoethanol (ß-ME) was completely unfolded as determined by intrinsic viscosity (Ansari et al., 1972; Ahmad and Salahuddin, 1974). The inclusion of 6.0M GuHCl increased protein solubility and prevented the formation of strong ovalbumin gels by reducing interactions between polypeptide chains (Egelandsdal, 1984).

Katsuta and Kinsella (1990) observed that 10% β-lactoglobulin formed gels in 6.0M urea and 6.0M GuHCl. The G' of β-lactoglobulin increased with time at 25°C indicating gel formation. Thiol groups and disulfide bonds play an important role in the heat-induced gelation of whey proteins (Hillier et al., 1980; Zirbel and Kinsella, 1989). Covalent cross-linking of protein molecules by disulfide bonds can be induced by thiol oxidation or by a thiol-induced disulfide interchange reaction that were enhanced at alkaline pH. Matsudomi et al. (1991) observed a decrease in gel hardness with bovine serum albumin (BSA) and β-lactoglobulin with increasing concentrations of N-ethylmaleimide (0-50 mM) confirming that thiol disulfide links were important in gel formation.

Hirose et al. (1986) observed the addition of up to 70 mM β-ME induced egg white gelation at room temperature. Gel hardness increased until a concentration of 70 mM β-ME then remained constant suggesting that the cleavage of the S-S bond might induce association of egg white protein molecules assisting to stabilizing the protein network.

## Differential Scanning Calorimetry

Differential scanning calorimetry is a practical technique for studying thermal behavior of food proteins. The DSC can be used to simulate denaturation of proteins during cooking by providing comparable thermal conditions (Wright, 1982). The effects of variables such as storage, pH or stabilizing treatments on the thermolability of proteins can be monitored using DSC (Donovan et al., 1975). Differential scanning calorimetry is used to measure the heat absorbed or liberated as a material changes in state (Skoog, 1985). In this technique, a sample and an inert reference are maintained at the same temperature while the temperature of both are gradually increased. Thermally induced changes occurring in the sample are recorded as a differential heat flow displayed as a peak in a thermogram. Intergration of heat flow with respect to temperature yields a value for the enthalpy change associated with the process (Skoog, 1985).

Donovan (1984) suggested the heat absorbed by the protein can be of two kinds. The first is the heat capacity ($C_p$) that produces vibrational and rotational motion of the molecules. The energy is stored in molecules pushing it to the next energy level. The second is heat that is absorbed by the molecule when it undergoes denaturation.

Based on Ferry's (1948) investigation, unfolding of a single domain protein is a two-state phenomenon in which half the molecules are folded while the other half are unfolded in a protein solution. This indicated that folding is a cooperative phenomenon, in which disruption of the folded structure leads to complete unfolding of the molecule. Therefore, the stability of each part of the protein was dependent upon the stability of all the parts. Partially folded protein must be unstable relative to being folded or unfolded (Creighton, 1984). The unfolding step was usually highly cooperative and was seen as an endothermic peak in a DSC thermogram (Kitabatake et al., 1990).

Denaturation enthalpy is associated with the amount of heat absorbed by the protein molecule that breaks hydrogen bonds and leads to unfolding of the protein (Privalov and

Khechinashvili, 1974). The denaturation enthalpy can be calculated from DSC data (Delben and Crescenzi, 1969; Delben et al., 1969). The thermogram for protein denaturation is endothermic. Arntfield and Murray, (1981) reported that differences in protein $T_d$ and enthalpy were due to hydrophobic interactions and intramolecular chemical bonds.

**Differential Scanning Calorimetry of Egg Proteins**

Three transitions (Fig. 3) were identifed in the DSC thermogram of egg white at pH 7.0 corresponding to denaturation of conalbumin, lysozyme and ovalbumin (65°C, 73°C and 85°C, respectively) (Donovan et al, 1975). Analysis of isolated egg-white proteins indicated that other minor protein constituents (ovomucoid, globulin and avidin) also contributed to the total thermogram, but were masked by the ovalbumin transition. Agreement was obtained between the observed enthalpy of denaturation for total egg white and that calculated from the enthalpies and relative amounts of the individual proteins (Donovan et al., 1975). Ovomucin, comprises about 5% of egg white proteins, exists in a random coil configuration in the native state and exhibits no denaturation transition when analyzed by DSC (Donovan et al., 1975).

Arntfield et al. (1989) observed $T_d$ for 10% ovalbumin solution of 71.2°C and 83.8°C at pH 3.0 and pH 5.0,

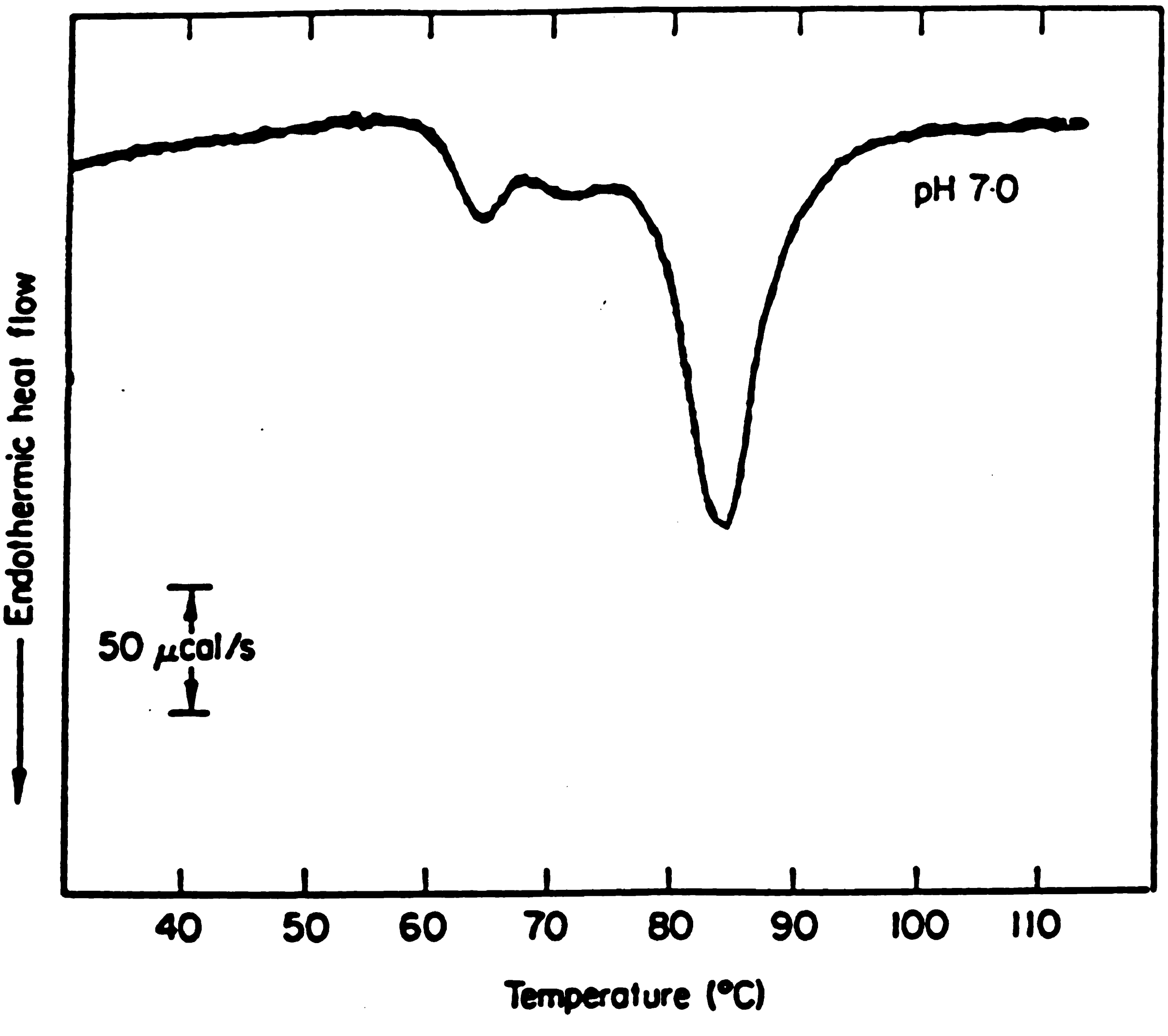


**Fig. 3-Differential scanning calorimetry thermogram of egg white at pH 7.0 heated at 10°C/min. Adapted from Donovan et al. (1975).**

respectively. No significant differences in $T_d$ of 10% ovalbumin at pH 5.0 or pH 9.0 were reported. The $T_d$ for 10% ovalbumin in 0.5M NaCl and 0.5M $Na_2SO_4$ were 89.6°C and 92.2°C, respectively. Ovalbumin displayed optimum thermal stability in the pH range 6-10 with a $T_d$ of approximately 79°C and a denaturation enthalpy of 3.64 cal/g (Donovan et al., 1975). The $T_d$ decreased to 62°C at pH 3.0 and 73°C at pH 11.0. Because of charge shielding by cations (Na or Ca) to ovalbumin solutions the temperature at which aggregation occurred is effected. Addition of 0.17M NaCl had little effect on $T_d$, compared to 17 mM $CaCl_2$ that resulted in a 2-3°C decrease in $T_d$ at pH values above the pI. Comparison of $T_d$ (measured with DSC at the pI) with the observed aggregation temperature (temperature at which 10% ovalbumin aggregated evaluated by a decrease in absorbance at 280 nm) indicated that precipitation, rather than gelation, took place when the temperature of aggregation was significantly lower than that of denaturation (Hegg et al., 1979). The reseachers suggested that by manipulating pH and salt concentration to reduce protein-protein interaction, gelation rather than aggregation was predominate.

Thermograms of ovalbumin and S-ovalbumin at pH 7.0 exhibited $T_d$ of 84.5°C and 92.0°C, respectively heated at 10°C/min. Therefore, the quality of stored or processed egg white can be monitored using DSC (Donovan and Mapes, 1976).

Factors such as concentration and heating rate have been reported to influence $T_d$ and denaturation enthalpy. Increasing soy protein solution from 6% to 70% increased the $T_d$ 80°C (Kitabatake et al., 1989). Not all proteins have exhibited different $T_d$ as a function of concentration. Heating rate has been shown to influenced $T_d$ and enthalpy in myoglobin and whey protein solutions (Hagerdal and Martens, 1976; Ruegg et al., 1977). In these cases, $T_d$ and enthalpy decreased as the heating rate decreased.

## Rheological Properties of Ovalbumin

Food systems exhibit solid (elasticity) and fluid (viscosity) properties, know as viscoelasticity. A key difference in the properties of elastic versus viscous material is the response to an applied stress (force). For an elastic material, the amount of strain (deformation) is proportional to the applied stress. For a viscous material, the rate of strain is proportional to the applied stress (Ngo and Taranto, 1986).

Dynamic testing is a rheological method to measure viscous and elastic properties of a fluid. Dynamic testing is based on different fundamental responses of the loss modulus (G"), a measure of energy dissipated as heat due to viscous flow within the sample and storage modulus (G'), a measure of energy stored due to elastic deformation of the sample (Beveridge et al., 1984 b). In dynamic testing, oscillatory movements and a pre-determined frequency between oscillation are used.

Dynamic testing does not alter the structure of the material (Ferry, 1970). Continuous measurement of rheological properties during heating gives insight into structure development in gel-forming proteins. This information contributes to an understanding of the mechanism of protein gelation and provides the basis for selecting adequate conditions for using proteins as texture building components in heat-processed foods (Tung, 1978).

van Kleef et al. (1978) used the rubbery elastic theory (Ferry, 1970) to describe gel structure in terms of the number of cross-links per molecule. The researchers reported 10% ovalbumin had 3.5 cross-links per molecule and a G' of 2,240 Pa whereas 30% ovalbumin exhibited 11.8 cross links/molecule and a G' of 133,000 Pa. Higher G' values are expected for gels made at a solution pH near the pI of ovalbumin since attractive ionic interaction will be greatest at that pH (van Kleff, 1986).

Egg protein gels are viscoelastic. When an egg protein is heated, it changes from a fluid to a gel with solid-like properties. van Kleef (1986) reported factors such as pH and ionic strength influenced G'. The G' for 25% ovalbumin solutions were higher at pH 6.1 than at pH 10. The researcher suggested that as ovalbumin moved away from it's pI the interaction between molecules decreased as was measured by a decrease in G'. Beveridge et al. (1985b) observed the $T_d$ of egg white from DSC data and G' from small amplitude oscillatory testing coincided (approximately 80°C). They concluded the formation of three-dimensional network responsible for typical gel structures of egg white did not form until ovalbumin was denatured.

Arntfield et al. (1990b) reported the G' of 10% ovalbumin decreased with increased concentration of NaCl or $Na_2SO_4$ (from 0.1 to 0.5M). Temperature of structure development ($T_s$) as determined by changes in G' decreased as pH decreased. The reseachers attributed the decrease in G' to an increase in hydrophobic interaction.

## Determination of Activation Energy

Time temperature superpositioning (TTS) was developed for use in the polymer industry. It has been demonstrated in polymers (Ferry, 1980) that viscoelastic data collected at one temperature can be superimposed upon data obtained at a different temperature by shifting of curves. The superposition principle is that the processes involved in molecular relaxation or rearrangements occur at greater rates at higher temperatures. The time over which these processes occur can be reduced by conducting the measurement at elevated temperatures and transposing the data to lower temperatures. Thus, viscoelastic changes that occur quickly at higher temperatures can be made to appear as if they occurred at longer times simply by shifting the data with respect to time (Ferry, 1980; Sichina, 1988). The degree of horizontal shifting (i.e., time) required to superimpose a given set of data upon a reference can be mathematically described as a function of temperature.

Two models are commonly used in time temperature superpositioning. The first of these relations is the Williams-Landel-Ferry (WLF) equation. The WLF equation is used to describe time-temperature behavior of polymers in the glass transition region (the change in an amorphorous region of a partially crystalline polymer from a viscous condition to a brittle one). The other model uses the Arrhenius relationship that measures a time dependent process at several temperatures.

The Arrhenius equation is as follows:

$$(1) \quad \log a_T = \frac{-E_a}{R\ (T-T_o)}$$

Where, Ea is the activation energy associated with the relaxation transition, R is the gas constant ($R = 8.314$ J/mol K), T is the measurement temperature, $T_o$ is the reference temperature and $a_T$ is the time base shift factor. Patel et al. (1990) developed a method for calculating denaturation $E_a$ of proteins from a DSC endotherm. This method is unique as the calculation is based on ramping temperature (increasing the temperature at constant rate) rather than an isothermal temperature over a specified time. The equation, reduced to linear form is:

$$(2) \quad \ln (d\alpha/dt) = \ln K_o - E_a/RT + n\,[\ln (1-\alpha)]$$

Here, the reaction rate ($d\alpha/dt$) at any temperature T is calculated as the ratio of peak height to total area, and the fraction of denatured protein ($\alpha$) is calculated as the ratio of partial area to total peak area. Values of $K_o$, $E_a$ and n were obtained by multilinear regression using $\ln (d\alpha/dt)$ as dependent variable and $1/T$ and $\ln (1-\alpha)$ as two independent variables.

Various methods have been used to determine activation energies resulting in different values for egg albumen. Donovan and Mapes (1976) using curve fitting analysis determine the Ea to be 73.3 kJ/mol, Goldsmith and Toledo (1985) used NMR data to calculate Ea in the range of 183.7 to 188.4 kJ/mol and Harte (1989) determined the denaturation Ea for 5% egg white protein (0.50M NaCl, pH7) was 158 kJ/mole using a time temperature history model.

Time temperature superposition can be used in the food industry to assist in predicting the quality of gels in foods. The ability to predict the effects of time and temperature is becoming increasingly important as lower cost proteins are being substituted into food systems. Time temperature superposition supplies a means to monitor and mathematically compare performance of protein under different processing conditions.

**Fourier Transform Infrared Spectroscopy**

Fourier transform infrared spectroscopy (FTIR) is used for the qualitative and quantitative estimation of protein secondary structure (Surewicz and Mantsch, 1988).
FTIR provides structural information about the effect of environmental conditions on bond types such as C=O, N-H and S-S.

Fourier transform infrared spectrometers are nondispersive and differ from the conventional dispersive infrared instruments (IR). Conventional IR instruments uses a grating or prism to disperse a collimated beam onto a slit that blocks out all but the desired frequeny range from reaching the detector. The entire spectrum is scanned one interval at a time by continuously changing the grating angle based on the incident light beam. The FTIR instrument uses an interferometer that simultaneously collects data from the entire spectrum (Byler and Susi, 1986). The FTIR has higher resolution, sensitivity, signal-to-noise ratio (S/N) and frequency accuracy compared to the dispersive IR instrument (Susi and Byler, 1986).

Krimm and Bandekar (1986) reported that bond types exhibited discrete vibrations and their changes can be monitored during molecular geometry variations. Therefore, different folding patterns of the polypetide backbone are assigned discrete frequencies within the mid-IR range that describe protein secondary structure.

In the mid IR region nine absorption bands characterize polypeptides and proteins. These are termed the Amide A, Amide B, and Amide I-VII bands (Table 1). The Amide A and Amide B vibrations (in solid sample) are associated with N-H stretching vibrations of hydrogen bonded groups. The Amide I band of proteins represents the vibrations of amide carbonyl groups coupled to the in-plane N-H bending and C-N stretching modes (Byler and Susi, 1986; Surewicz and Mantsch, 1988; Havel, 1989). The Amide II band is not well established for the polypeptide backbone but includes both C-N stretching and N-H bending. The Amide III band of polypeptides produce a weak IR signal. Amide III band includes C-C stretching, C=O stretching, C-N stretching, and N-H bending. The Amide III band produces a weak IR signal that can be detected by Raman spectroscopy. The amide IV vibration is complex and involves bending of the OCN angle. Little information is available on Amide V, VI and VII (Susi, 1969).

Table 2 compares Raman, FTIR and x-ray analysis for estimating protein secondary structure. Immunoglobulin G

Table 1-Frequency Range for characteristic absorption bands of secondary amides in the crystalline state (based on model compounds)[1]

| In-plane modes | Out-of-plane modes |
|---|---|
| Amide A, ≈ 3300 $cm^{-1}$ | Amide V, 640-800 $cm^{-1}$ |
| Amide B, ≈ 3100 $cm^{-1}$ | Amide VI, 537-606 $cm^{-1}$ |
| Amide I, 1620-1700 $cm^{-1}$ | Amide VII, ≈ 200 $cm^{-1}$ |
| Amide II, 1480-1575 $cm^{-1}$ | |
| Amide III, 1229-1301 $cm^{-1}$ | |
| Amide IV, 625-767 $cm^{-1}$ | |

[1] Adapted from Susi (1969)

Table 2 compares Raman, FTIR and x-ray analysis for estimating protein secondary structure. Immunoglobulin G exhibited variation between FTIR and X-ray studies for all structures. ß-lactoglobulin A showed a 10% difference in % other between Raman and x-ray studies. The overall results of the studied showed little variation between the three methods.

Protein bands assignments were established based upon model system studies using proteins with a large proportion of helix, sheet, turns or unordered structure. Hemoglobin is 80% helix (Susi and Byler, 1983), whereas immunoglobulin G (IgG) and concanavalin A are classified as all β-structure by X-ray studies (Dong et al., 1990). Unordered structure

Table 2-Estimated protein conformation by three independent methods [1]

| Protein | % α-Helix | | | % β-Structure | | | % Other[2] | | |
|---|---|---|---|---|---|---|---|---|---|
| | R[3] | FT-IR[4] | X[5]-ray | R | FT-IR | X-ray | R | FT-IR | X-ray |
| Bovine serum albumin | 39 | 47 | - | 32 | 28 | - | 29 | 25 | - |
| Carbonic anhydrase | 11 | 13 | 16 | 51 | 49 | 45 | 38 | 38 | 39 |
| Immuno-globulin G | 8 | 9 | 3 | 67 | 76 | 67 | 27 | 15 | 30 |
| α-Lactalbumin | 31 | 33 | - | 36 | 41 | - | 33 | 26 | - |
| β- Lacto globulin A | 10 | 10 | 7 | 54 | 50 | 47 | 36 | 40 | 46 |
| Lysozyme | 43 | 41 | 45 | 25 | 21 | 19 | 32 | 38 | 36 |
| Ribo-nuclease A | 21 | 21 | 22 | 50 | 50 | 46 | 31 | 29 | 32 |
| Cytochrome C | - | 51 | 49 | - | 34 | 10 | - | 15 | 41 |

[1] Taken from Byler and Susi (1988).
[2] turns and undefined segments
[3] Raman spectroscopy
[4] Fourier transform infrared spectroscopy
[5] x-ray crystallography

contains neither α-helix, β-sheet, nor turns (Richardson, 1981). Crystallography studies of α-lactalbumin (Acharya et al., 1989) support the assigned band at 1640 $cm^{-1}$ to $3_{10}$-helix (Byler and Susi, 1986; Halloway and Mantsch, 1988). Table 3 shows band assignments based up the studies of 17 proteins (Byler and Susi, 1988).

Aqueous analysis of protein structure using FTIR is difficult because of highly absorbing bands such as the O-H stretch of water (1645 $cm^{-1}$) tend to obscure the weaker conformation sensitive amide I band. Subtraction of this water band, although possible in principle, is associated with numerous problems. First, because of protein water interaction the shape of the water band is changed so adequate substraction is not always achieved. Second, signal-noise ratio is decreased in the difference spectra if a strong water band is subtracted. Finally, no criteria has been established for determining the scaling factor applied to the water spectrum that is subtracted from the solution spectrum (Surewicz and Mantsch, 1988).

Deuterium oxide has been substituted for water in protein solution preparation (Susi and Byler, 1983) to avoid the problem of highly absorbing water bands. Olinger et al. (1986) concluded that $D_2O$ solutions produce more consistent results than water. If $D_2O$ is used as a solvent, one must ensure that complete H--> D exchange has taken place in

Table 3-Characteristic infrared frequencies and assignments for amide I band components (1700-1620 $cm^{-1}$) for 19 globular proteins in $D_2O$ solution[1]

| Mean frequency ($cm^{-1}$) | Assignment |
|---|---|
| 1623 ± 3 | β-structure |
| 1630 ± 4 | β-structure |
| 1637 ± 3 | $3_{10}$-helix |
| 1645 ± 4 | unordered |
| 1653 ± 4 | α-helix |
| 1670 ± 2 | turns |
| 1675 ± 2 | turns |
| 1683 ± 2 | turns |

[1] Taken from Byler and Susi (1988).

the backbone amide groups. This is done by following the decrease in the intensity of the amide II band near 1550 $cm^{-1}$ as progressive deuteration shifts it to lower frequencies (approximately 1450 $cm^{-1}$) (Timasheff et al. 1967; Susi et al. 1967). Mathematical procedures, termed resolution-enhancement techniques, are required to resolve the component absorption bands (α-helix, β-sheet, turns and unordered structure). Derivative spectroscopy and Fourier self-deconvolution are two such resolution-enhancement methods (Prestrelski et al., 1991 a).

## Secondary Structure of Proteins

Byler and Purcell (1989) investigated effects of heat on the secondary structure of β-lactoglobulin, bovine serum albumin (BSA) and α-lactalbumin. All three proteins changed conformation during heating as compared to their native structure at 30°C. All proteins exhibited decreased $\alpha$-helix and $\beta$-sheet contents as observed by changes in peak intensity, although, $\alpha$-lactalbumin retained more native structure than $\beta$-lactoglobulin or BSA.

Clark et al. (1981 b) used IR spectroscopy to study the secondary structure of a 10% BSA solution (pD 6.7, no added electrolytes) heated from 25°C to 90°C then cooled to 25°C (Fig. 4). A peak at 1650 $cm^{-1}$ and a shoulder at 1620 $cm^{-1}$ representing $\alpha$-helix and $\beta$-sheet, respectively were present during heating. Peak intensity of the shoulder at 1620 $cm^{-1}$ decreased when cooled to 25°C.

Surewicz and Mantsch (1988) examined the conformation of native $\beta$-lactoglobulin in aqueous solution at neutral pH and that of the protein denatured by alkaline solution or acidic methanol. The native structure contained mostly $\beta$-structure. In the spectrum of the alkali-denatured protein, all the fine structure had disappeared and the only broad band around 1640 $cm^{-1}$ was assigned to an unordered structure. The spectrum of ß-lactoglobulin in acidic methanol depicted a strong band at 1647 $cm^{-1}$ and weaker bands at 1687 $cm^{-1}$ and 1618 $cm^{-1}$.

Chen et al. (1990) observed changes in secondary

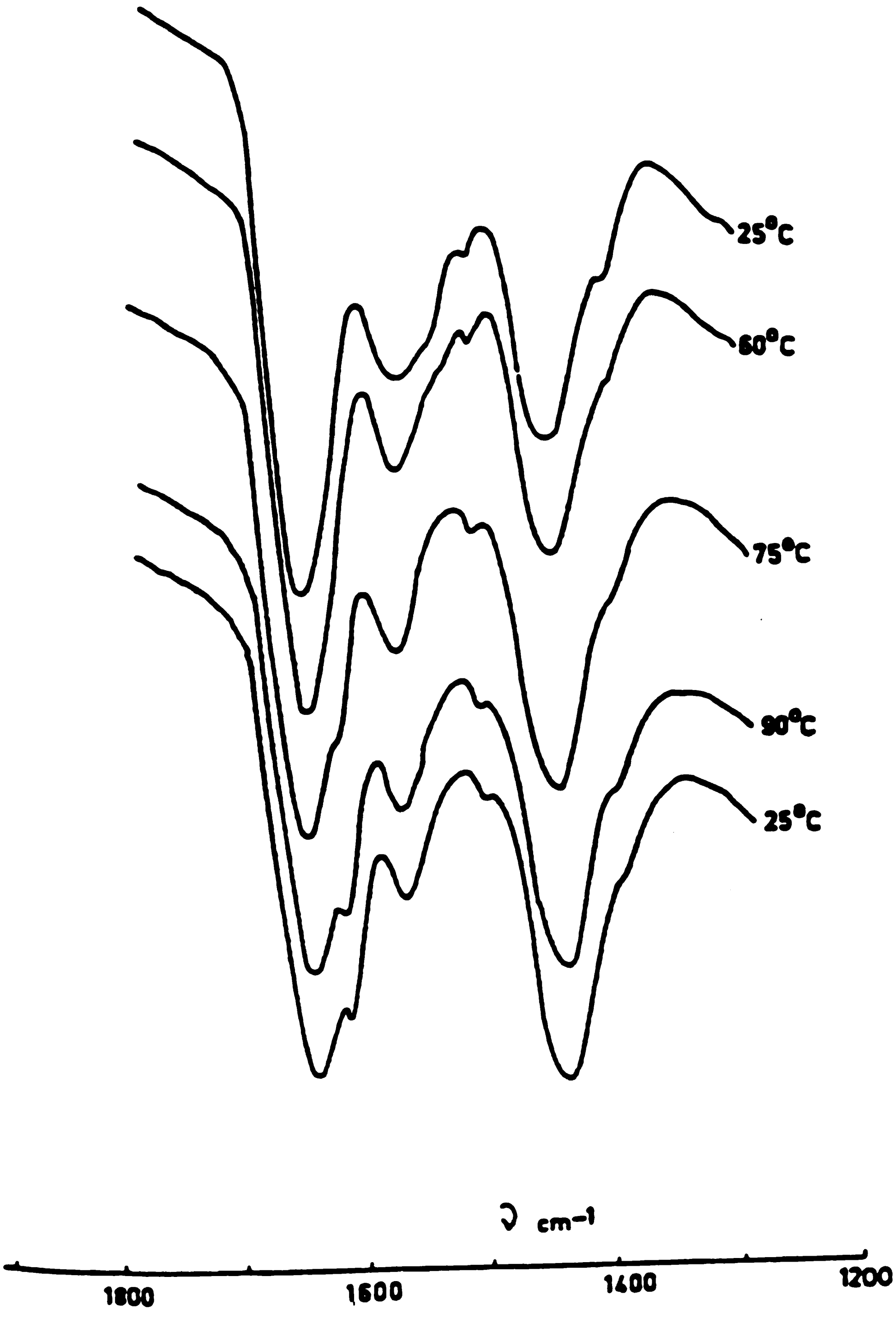


Fig. 4-Infrared spectra of bovine serum albumin (salt-free, pD 6.7) heated from 25°C to 90°C then cooled to 25°C. Taken from Clark et al. (1981).

structure of soy 11S globulin during heating and shearing. During heating of 11S globulin from 25° to 90°C α-helix increased, random coil decreased and β-sheet did not change. During shearing (25°C and 384 $sec^{-1}$) random coil increased, β-sheet decreased and α-helix remained the same as compared to the non-sheared sample.

Raman spectroscopy is another method to study conformational changes in thermally denatured proteins. The Ramam spectrum of whole egg white is similar to that of ovalbumin because ovotransferrin, the other major egg albumen, is a "poor Raman scatterer" (Painter and Koenig, 1976). Thermal denaturation of egg albumen reveals an amide III line at 1236 $cm^{-1}$ and a shift in the amide I line from 1667 to 1672 $cm^{-1}$. These changes indicate formation of regions of antiparallel β-sheet between ovalbumin molecules. Thermal denaturation of isolated ovalbumin and ovotransferrin caused formation of intermolecular β-sheet structures when monitored using Raman spectroscopy. The formation of intermolecular disulfide bridges was suggested to play only a secondary role since no new disulfide bridge formation was indicated at 500 $cm^{-1}$ in the Raman spectrum upon heating.

Circular Dichroism (CD) is an optical technique used to monitor unequal absorption of left and right circularly polarized light with chiral molecules (Yang et al., 1986).

The conformation of the protein (asymmetric and periodic arrangement of peptide units in space) gives rise to characteristic CD spectra (Alder et al. 1973). Chin et al. (1987) suggested that CD was complementary to other methods for evaluating the secondary structure of proteins. From proteins of known three-dimensional structure it was found that the helical structure gives minima in CD spectra at 222 nm and 208 nm and the β-sheet structure gives a minimum at 218 nm (Johnson, 1988). A negative CD value at 222 nm is characteristic of order structure. Therefore, the presence of disorder structure can be determned by checking for positive ellipticity at 222nm. Figure 5 shows CD spectra of native and denatured ovalbumin heated to 80°C. Native ovalbumin shows a minimum and a shoulder at 222 nm and 210 nm, respectively. Secondary structure of native and heat denatured ovalbumin has been calculated from (Table 4) CD spectra. Kato and Takagi (1988) reported that when ovalbumin was heated to 80°C, the amplitude at 222 nm decreased and the shoulder at 210 nm increased. Therefore, changes in CD spectra of ovalbumin during heat denaturation suggest that the β-sheet structure increased. The increase in β-sheet was due to the irreversible decrease in helical structure.

Kato and Takagi (1988) investigated the effects of salt concentration (20 to 160 mM NaCl, pH 7.0) on secondary

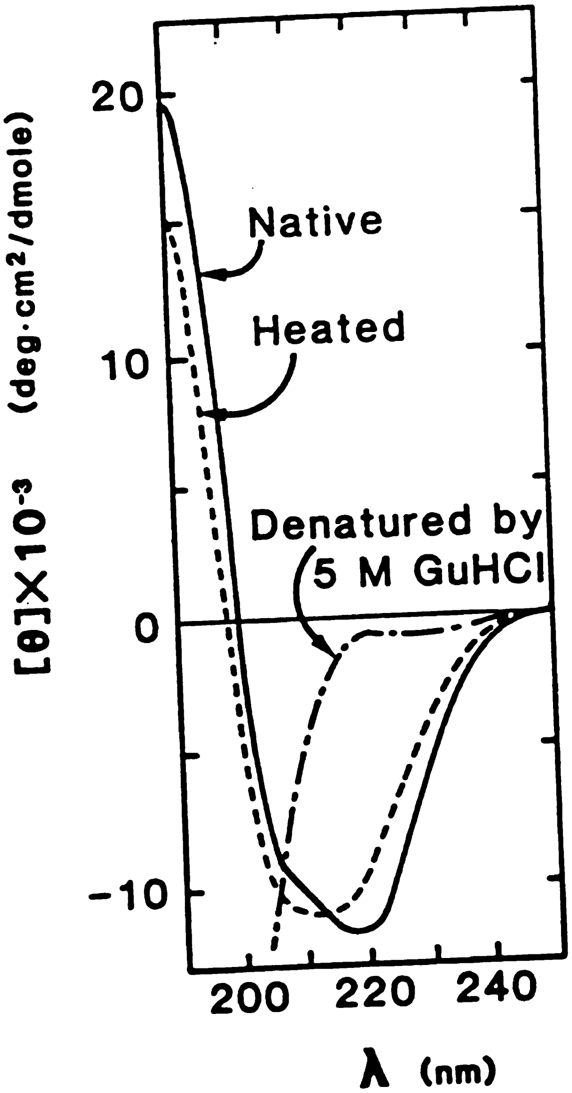


**Fig. 5-Circular dichroism spectra of native ovalbumin and ovalbumin denatured by heat or guanidine hydrochloride. Taken from Doi et al. (1987).**

Table 4-Secondary structure of native, heat-denatured and cooled ovalbumin calculated from CD spectra[1]

| | fractions of secondary structure | | | |
|---|---|---|---|---|
| ovalbumin | helix | ß-sheet | turn | unordered |
| native | 0.49 | 0.13 | 0.14 | 0.24 |
| heat-denatured | 0.16 | 0.36 | 0.15 | 0.33 |
| cooled | 0.14 | 0.46 | 0.09 | 0.31 |

1 Taken from Doi et al., 1987.

structure of ovalbumin. They reported that increasing salt concentration decreased the amount of α-helix and β-sheet. Egelandsdal (1986) reported that for salt free solutions (<10 mg/mL) of ovalbumin the α-helix content was independent of protein concentration. Clark and Lee-Tuffnell (1986) reported that the α-helixical content of BSA decreased at the expense of β-structure with increasing protein concentration up to 20 mg/mL (no salt added, pH 8.0).

## Scanning Electron Microscopy

Scanning electron microscopy (SEM) is a tool for evaluating the microstructure of protein gels at nanomeric distances (Clark et al., 1981). The greatest advantage of SEM is the large depth of focus that is about 300 times that of the light microscope (Lewis, 1979). Microstructure can provide information on the effect of attractive and repulsive molecular forces on the formation of the protein gel network (Heertje and Van Kleef, 1986). Microstructural information can assist in interpreting textural characteristics of food systems (Stanley and Tung, 1976).

Clark et al. (1981) concluded that the electron microscope can contribute to structural investigation of globular protein gels by giving a qualitative impression of prevailing distributions of pore size, strand thickness and shape. Information about how much unfolding has occurred during gel formation is not detected using an electron microscope.

van Kleef (1986) reported that 20% ovalbumin gels prepared at pH 5.0 showed a granular inhomogeneous microstructure. van Kleef (1986) suggested that at pH 5.0 network formation occurred via folded aggregates of globular protein chains. Clumps of aggregated protein were observed but without the fibrillar and sheetlike structures observed for ovalbumin/urea gels at pH 10.0 Ovalbumin gel structure at pH 5.0 is composed of individual protein aggregates (with a diameter of about 0.1 um). These aggregates form large particles (of about 0.3 um diameter), which eventually form the network (van Kleef, 1986).

At optimal pH, a fine, uniform gel matrix was formed with high gel strength. Pores are small and water-binding was improved as free water was entrapped. The key to formation of a gel with fine structure and optimum water-binding properties is the balancing of attractive and repulsive forces (Ferry, 1948). The effect of charge on microstructure is related to the net charge of the proteins in solution (Hermansson and Lucisano, 1982). Proteins tend to aggregate when heated at their pI, forming a coarse network with large pores, low gel strength and minimal water binding. Beyond the optimal pH, repulsive forces may be too strong so that fewer protein interactions are possible, and a weaker gel results (Egelandsdal, 1980). Beveridge et al. (1980) attributed increased firmness of ovalbumin gel at pH 9.0 to sulfhydryl-disulfide exchange that is accelerated at alkaline conditions.

Woodward and Cotterill (1986) evaluated the microstructure of 11% egg white gels at pH 5.0, 6.0 and 9.0 in 1.0M NaCl. At pH 5.0 and 6.0 the microstructure was in spherical clusters that were aggregated leaving large irregular void spaces. The egg white gel at pH 9.0 exhibited a uniform structure with pores being small and evenly distributed throughout the gel matrix.

Heertje and van Kleef (1986) reported that ovalbumin gels prepared at pH 10.0 in urea solution (6.0M or 8.0M) showed a uniform, homogeneous microstructure. The reseachers used NMR measurements to show protein unfolding at pH 10.0 verus partially unfolding followed by aggregation at pH 5.0. The studied concluded that complete ovalbumin unfolding at pH 10.0 occurred before network formation.

Harte (1989) reported that 5% ovalbumin gels adjusted to pH 6.0 and 7.0 before heat setting produced non-homogeneous, grape-like, aggregated protein clusters. It was suggested by Montejano et al. (1984) the spherical particles in micrographs of low pH ovalbumin gels may be caused by random aggregation that resulted in non-homogenous structure. At pH 7.0, Harte (1989) and Egelandsdal (1980) reported a smooth ovalbumin network and attributed it to the balancing of attractive and repulsive forces. The addition of salt shielded repulsive charges, thus promoting protein aggregation (Hegg et al., 1979; Hatta et al., 1986).

Low temperature scanning electron microscopy (LTSEM) is a superior method for determining ultrastructure of food products (Freeman and Shelton, 1991). The LTSEM procedure is less time-consuming, decreases induced artefacts associated with chemicals and reduces speciment shrinkage (Sargent, 1988). Harte (1989) used LTSEM for examining 5% ovalbumin gels at pH 5.0 in 3.0% NaCl. The ovalbumin gel ultrastructure showed an increase in the quantity of aggregated protein under these conditions. The reseacher observed differences between the LTSEM and a chemical fixation method (osmium-thiocarbohydraxide-osmium). The researcher reported that ovalbumin gels prepared by LTSEM exhibited a web-like appearance whereas the gels prepared by the chemical fixation method showed a grape-like cluster appearance. Other researchers (Sargent, 1988; Freeman and

Shelton, 1991) have used LTSEM for viewing a variety of food mircostructures.

Arntfield et al. (1990 a) used light microscopy to follow the formation of two dimensional network of ovalbumin at pH 8.5 in 150 mM NaCl. The investigators observed that as the concentration of ovalbumin increased from 5.5% to 15%, the intensity of the network also increased. Arntfield et al. (1990 b) reported that 10% ovalbumin gel at pH 8.5 in 0.5M NaCl formed well cross-linked strands, while 10% ovalbumin at pH 8.5 in 0.5M $Na_2SO_4$ exhibited little evidence of cross-linking. The authors also reported that increased G' and destabilizing salts correlated with decreased hydrophobic interaction in heat-induced ovalbumin gels.

Sone et al. (1983) reported 11% whey protein concentrate gels in 0.3% $Na_2SO_4$ showed higher G' and denser microstructure than gels containing 0.3% $CaCl_2$. The researchers suggested the dense microstructure was due to strong protein-water interaction, leading to less randomness and elastic gels. The $Na_2SO_4$ increases hydrophobic interaction stabilizing the protein molecule. Therefore, there is more protein-water interaction enhancing gel elasticity.

# III. Study 1

## Denaturation and Structure Development of Ovalbumin as Influenced by pH and Salt Type

## ABSTRACT

Changes in the denaturation and gelation properties of ovalbumin as a function of pH (3.0, 7.0, and 9.0) or salt type (NaCl, NaI, $Na_2SO_4$) were studied using differential scanning calorimetry (DSC) and dynamic rheological testing. The DSC onset temperature of ovalbumin denaturation ($T_O$) occurred 5.8°C - 7.5°C prior to structure development ($T_S$) as measured by storage modulus. Enthalpy, $T_O$ and $T_S$ increased as pH increased. Denaturation temperature decreased in the order of the Hoffmeister series, whereas $T_S$ decreased in reverse order. Activation energies of ovalbumin denaturation and structure development were dependent on pH and salt type.

# INTRODUCTION

Ovalbumin is the major egg white protein responsible for egg white gelation. Ovalbumin is a phosphoglycoprotein making up 54% of total proteins in the albumen. Ovalbumin has a molecular weight of 45,000 daltons and it's isoelectric point (pI) is 4.5. The complete amino acid sequence of ovalbumin includes 385 residues that have been determined by Nisbet et al. (1981).

Gelation is an important functional property of egg white proteins. Protein gels provide a matrix for holding water, flavors, ingredients and provide texture. Protein gels are made of a three-dimensional matrix of cross-linked polypeptides. Bonds giving structure to ovalbumin network may be electrostatic (Egelansdal, 1980) hydrophobic (Hayakawa and Nakai, 1985), hydrogen (Hata et al., 1986) covalent (Beveridge et al., 1984), van der Waals forces (Hatta et al., 1986) or a combination thereof. At pH's below the pI ovalbumin dissociates and unfolds at a lower temperature compared to pH 7.0 and 9.0 (Arntfield, 1989). Koseki et al. (1990) observed that the native compact globular molecule of ovalbumin remained intact while the tertiary structure fluctuated at pH conditions below the pI. At high pH ovalbumin is negatively charged and cross-links through sulfhdryl-disulfied exchange, contributing to the structural integrity of the gel (Shimada and Matsushita, 1980; 1981)

Ferry (1948) explained the protein gelation theory as a two step process:

Native ---->denatured protein-----> aggregated protein
(long chains)            (associated network)

Comparison of the rate of the denaturation step verus that of the aggregation step helps determine gel characteristics (Gossett et al., 1984). Hermansson (1979) suggested a gel network with a certain degree of order can be attained if aggregation occurs slower than denaturation; giving denatured protein molecules time to orient themselves before aggregation.

Ferry (1948) suggested that unfolding of a single domain protein in solution contain two species of molecules; those that are folded and those that are unfolded. There are no intermediate species found in a pure co-operative process. Therefore, disruption of part of the molecule leads to unfolding of the entire molecule (Creighton, 1984).

Koseki et al. (1989) developed a model showing high molecular weight ovalbumin aggregates as a "string of beads". Ovalbumin linear aggregates were formed by hydrophobic interaction forming a gel (Tung, 1974; Kato et al. 1983).

Salts in the lyotropic series were used to probe the importance of hydrophobic interactions to protein network formation (von Hippel and Schleich, 1969; Melander and Horvath, 1977). The driving force for such interactions arise from the specific effect each salt exerts on structure of the water molecules around them (Damodaran and Kinsella, 1982). By manipulating solvent conditions the structure of the protein will change due to decreasing hydrophobic interaction (Tanford, 1979).

Measurement of the rate of heat flow by differential scanning calorimetry (DSC) can determine effects of storage, pH or stabilizing treatments on protein thermolability (Donovan et al., 1975). Ovalbumin displayed optimum thermal stability in the pH range 6 to 10 with a denaturation temperature of 79°C and denaturation enthalpy of 3.64 cal/g at pH 7.0 (Donovan et al., 1975). Addition of 170 mM NaCl had little effect on denaturation temperature, but 17 mM $CaCl_2$ decreased denaturation temperature 2-3°C at pH values above the pI.

Nondestructive dynamic rheological testing is used to measure viscous and elastic properties of a protein solution during heating and gives information about structure development in gel-forming proteins (Tung, 1978). Dynamic rheological analysis was used to observe structure development measured by an increase in storage modulus (G') in ovalbumin during heating from 30°C to 95°C at 2°C/min (Arntfield et al., 1990 c). The researchers reported the G' of a 10% ovalbumin solution decreased with increased salt concentration (NaCl and $Na_2SO_4$ at 1.0M and 0.5M). Temperature of structure development as determined by changes in G' decreased with lower pH. The decrease in G' was attributed to increased intramolecular hydrophobic interaction.

The objectives of this project were to influence the heat-induced gelation mechanism of hen egg ovalbumin by changing the pH or salt type then (1) to investigate the relationship between denaturation and structure formation with DSC and dynamic rheological testing and (2) determine thermodynamic and rheological properties of the gelation process.

## Materials and Methods

### Material

Ovalbumin (Grade V, lot 19F 8105) was purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Moisture content was determined using AOAC (1984) 17.006-17.007. Solutions of 80 mg\ml ovalbumim were prepared in (1) 0.5M NaCl at pH 3.0, 7.0 and 9.0 or (2) 0.5M NaI, 0.5M NaCl and 0.5M $Na_2SO_4$ at pH 7.0. Samples were equilibrated for 30 min before testing. All solutions were prepared and tested in duplicate.

### Electrophoresis

Ovalbumin was checked for purity by sodium dodecyl sulfate polyarcylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) using a Mini-Protean II Dual slab cell (BIO-RAD, Richmond, CA) and power supply (BIO-RAD model 1000/500, Richmond, CA). A running gel of 12% acrylamide and a stacking gel 4% acrylamide were used. The protein samples were run at a constant voltage of 200. Proteins were stained with Coomassie Brilliant Blue R-250 (Bio-Rad) in 40% ethanol and 7% acetic acid and then destained in 7% acetic acid.

**Differential Scanning Calorimetry**

A Dupont 990 Thermoanalyzer equipped with a Dupont 910 Cell Base and standard DSC cell were used to determine onset denaturation temperature ($T_o$), thermal denaturation temperature ($T_d$) and enthalpy ($H_{cal}$) of ovalbumin. Approximately 15 mg of ovalbumin solution was sealed in a Dupont aluminium hermetic pan (part 900793-901) and lid (part 900794-903). Samples were heated from 30°C to 110°C at 10°C/min. Temperature calibration and calibration coefficient E for the DSC cell were determined using weighed samples of indium over a scanning range of 25-200°C. The reference pan contained an identical ovalbumin solution that was previously heat-denatured in the same temperature range as suggested by de Witt (1981 a). Rescanning the cooled ovalbumin (Patel et al., 1990) sample showed no denaturation peak, suggesting irreversible denaturation. The cell was flushed with nitrogen at 50 mL $min^{-1}$ to maintain an inert environment for all experiments. All scans showed an endothermic heat flow.

Onset denaturation temperature ($T_o$) was the temperature at which a change in slope of the curve occurred as determined by DuPont 9900 General V 2.2A software program. Ovalbumin $T_d$ was defined as the endothermic peak temperature and corresponded to 50% unfolding of ovalbumin as determined by the DuPont 9900 General V 2.2A software program. The apparent enthalpy of calorimetry ($H_{cal}$) was calculated using DuPont 9900 General V 2.2A software as follows:

(1) $H_{cal} = A/M\ (60BEqs)$

where A is the area ($cm^2$), M is the mass of the sample (mg), B is the time base (min $cm^{-1}$), E is the cell calibration coefficient, and qs is the Y axis range (mW/cm). Peak area baselines were constructed as a single straight line from the begining to the end of the endotherm. The beginning of the endotherm was determined as the initial increase in the integrating curve and endotherm termination was the integrating curve plateau.

Activation energy of ovalbumin denaturation was calculated by a method developed by Patel et al. (1990) for whey protein using DSC. The equation, reduced to linear form, was:

(2) $\ln (d\alpha/dt) = \ln K_o - E_a/RT + n\,[\ln (1-\alpha)]$

The reaction rate ($d\alpha/dt$), at any temperature, T, is calculated as the ratio of peak height to total area. The fraction of denatured protein ($\alpha$) was calculated as the ratio of partial area to total peak area. Values of the preexponential factor ($K_o$), activation energy ($E_a$), and reaction order (n) are obtained from multilinear regression (Eisensmith, 1989) performed using ln ($d\alpha/dt$) as dependent variable and 1/T and ln ($1-\alpha$) as two independent variables. The gas constant is R.

## Thermodynamic Calculations

The following two thermodynamic terms were calculated by the methods defined by Bertazzon and Tsong (1990 a). The van't Hoff enthalpy is defined as follows:

$$(3)\quad H_{VH} = 4RT^2C_p(\max) \;/\; H_{cal}$$

where R is the gas constant, T is the temperature (K), $C_p$(max) is the maximum heat capacity of the DSC excess heat capacity curve.

The cooperativity ratio is defined by:

$$(4)\quad H_{VH} \;/\; H_{cal}$$

## Dynamic Rheological Testing

Storage moduli of ovalbumin solutions during heating from 60°C to 100°C at 2°C/min and 15 min isothermal experiments were monitored using a Rheometrics Fluids Spectrometer Model 8400 (Piscataway, NJ), equipped with a 1-100 g-cm torque transducer and a silicon oil circulation system controlled by a Nelsprit Temperature Programmer. One and a half milliliters of ovalbumin solution was placed between the cone and plate geometry (radius 12.5 mm, 0.02 cone angle and 50 um gap) and equilibrated for 5 min before heating. The G' was recorded continuously at a fixed frequency of 1 rad/sec and strain of 1.0%. Limits of constant viscoelasticity were determined by conducting frequency (0.1 to 100 rad/sec) and strain sweeps (0.1 to 100 %) in preliminary experiments.

Significant rheological structure was designated when G' reached 10 Pa since it was a common point of comparison for all treatments in preliminary studies. Temperatures for isothermal heating experiments were selected based on temperatures at which G' reached at least 10 Pa between 0.1 and 15 min heating period.

Thermal scanning experiments (heating from 60°C to 100°C at 2°C/min) were used to determine the temperature at which G' equalled 10 Pa. This temperature was defined as the onset temperature for structure development ($T_s$).

Activation energy of structure formation ($Ea_s$) was calculated from isothermal heating experiments at different temperatures using time-temperature superpositioning (Ferry, 1980). Values for time (t) were taken directly from the curve of G' versus time in which the time needed to reach a G' of 10 Pa was used. The Arrenhius equation was used to calculate the activation energy of structure formation:

$$(5)\ \ln t = \ln A - Ea/RT$$

where t is the time needed to reach 10 Pa, A is the preexponential factor ($s^{-1}$), $E_a$ is the activation energy ($J\ mol^{-1}$), R is the gas constant and T is the temperature (K). Activation energy was calculated from the slope of the plot ln t versus 1/T.

## Statistics Analysis

MSTATC software (version c, East Lansing, MI) was used for basic statistics and two way analysis of variance (replication x treatment) on a complete randomized design experiment. Tukey's honestly significant difference test ($P < 0.05$) and standard error of the means were used to evaluate the significant differences between means. Multilinear regression analysis for determining $Ea_d$ and reaction order was conducted using Plotit (Eisensmith, 1985). All tests were performed in duplicate.

# RESULTS AND DISCUSSION

## Proximate Analysis

Grade V ovalbumin had a moisture content of 0.35% and the electrophoregram exhibited one band at 45,000 (Powrie and Nakai, 1986) which is the MW of ovalbumin. Donovan and Mapes (1976) observed different $T_d$ for ovalbumin and S-ovalbumin at pH 9.0 of 84.5°C and 92.5°C, respectively. In this research the $T_d$ at pH 9.0 was 90.6°C. Based on this information, S-ovalbumin was used in this experiment.

### Effect of pH on Thermal Denaturation

The $T_o$ and $T_d$ of S-ovalbumin denaturation was lower at pH 3.0 compared to pH 7.0 or 9.0 (Table 5). The decrease in $T_d$ at pH 3.0 may be explained by an increase in number of positive charges that increases repulsive forces within the molecules causing S-ovalbumin to unfold.

Luescher et al. (1974) showed that $T_d$ of ovalbumin determined by DSC was lower at pH 3.0 (63°C) than at pH 7.0 (78°C). Donovan et al. (1975) observed that ovalbumin $T_d$

Table 5 - Influence of pH on thermal denaturation of 8% (w/v) S-ovalbumin in 0.5M NaCl using differential scanning calorimetry

| pH | $T_o$ (°C) | $T_d$ (°C) | $Ea_d$ (kJ/mol) | n | $^*Ea_d$ (kJ/mol) |
|---|---|---|---|---|---|
| 3 | $68.0^a$ | $80.5^a$ | $97.1^a$ | $1.2^a$ | $16.7^a$ |
| 7 | $83.6^b$ | $90.6^b$ | $304.0^b$ | $1.3^a$ | $61.1^b$ |
| 9 | $82.4^b$ | $90.6^b$ | $278.5^b$ | $1.9^a$ | $51.3^b$ |

| | |
|---|---|
| $T_o$ | onset temperature |
| $T_d$ | thermal denaturation temperature |
| $Ea_d$ | activation energy of denaturation |
| n | reaction order |
| $^*Ea_d$ | activation energy of denaturation calculated when n=1 |

Column values with different superscripts are significantly different ($P < 0.05$).

was the same at pH 7.0 and 9.0. Arntfield et al. (1981) observed lowering pH from 7.0 to 3.0 decreased the $T_d$ from 84°C to 64°C.

S-ovalbumin $Ea_d$ was lowest at pH 3.0 and not different ($P > 0.05$) between pH 7.0 and 9.0 (Table 5). The lower S-ovalbumin $Ea_d$ at pH 3.0 indicated the energy needed to drive the reaction from native to denatured state and temperature dependence of the reaction were decreased. Donovan and Mapes (1976) assumed denaturation of ovalbumin was irreversible and reported an activation energy of 73.3 kJ/mol at pH 9.0 and 37°C. The apparent reason for the discrepancy between calculated Ea was due to the method of calculation. While Donovan and Mapes (1976) used a reaction order of one; this research used a reaction order depending on the fit of the data to the computer analysis program. Therefore, Ea was dependent on reaction order.

S-ovalbumin reaction order for denaturation was not different ($P > 0.05$) (Table 5). Cheftel et al. (1985) reported denaturation of proteins was a first order reaction. Dwek and Navon (1972) estimated the $E_a$ for denaturation of egg albumen based on thermal data to be 103 kJ/mol. Patel et al. (1990) reported reaction order of denatured whey protein (10%) at pH 6.34 to 6.38 ranged from 1.35 to 1.5. calculated using a multilinear regression equation. Dannenberg and Kessler (1988) calculated the reaction order for denaturation of α-lactalbumen and β-lactoglobulin at pH 6.6 to be 1.1 and

1.5, respectively. A reaction order greater than suggested more than one molecular event occurring such as unfolding of α-helix and β-sheet. Kokini (1991) suggested that gelation might be considered to have two reaction orders, one for denaturation and another for aggregation.

The $*Ea_d$ decreased with decreased pH (Table 5) suggesting that pH 3.0 required less thermal energy to drive the native protein to denaturation compared to pH 7.0 or pH 9.0. The $*Ea_d$ and $Ea_d$ were 5 fold different from each other indicating the concentration of S-ovalbumin may contributed to the calculation. Using a reaction order of one for the Ea calculation decreased the error due to concentration that might include aggregation. The presence of high S-ovalbumin concentration during heat-induced gelation may cause aggregation without denaturation causing inaccurate Ea calculation.

S-ovalbumin enthalpy decreased with decreased pH (Table 6). At pH 3.0 ovalbumin enthalpy was lower compared to pH 7.0 or pH 9.0. The decrease in enthalpy might be due to partially unfolded and aggregated molecules compared to completely unfolded molecules at pH 7.0 or 9.0. Because aggregation occurred without denaturation S-ovalbumin did not totally unfolded therefore, decreasing the potential heat energy absorption. Privalov and Khechinashvilli (1974) reported that aggregation is a exothermic process that will

Table 6- Effect of pH on the enthalpic contribution to the stability of 8% (w/v) S-ovalbumin in 0.5M NaCl

| pH | calorimetric enthalpy (kJ/mol) | van't Hoff enthalpy (kJ/mol) | CR |
|---|---|---|---|
| 3 | $41.2^a$ | $16.6^a$ | $0.40^a$ |
| 7 | $62.2^b$ | $52.1^b$ | $0.83^b$ |
| 9 | $77.6^c$ | $50.4^b$ | $0.65^b$ |

CR-cooperative ratio

Column values with different superscripts are significantly different ($P < 0.05$).

decrease enthalpic values. Ma et al. (1988) and Patel et al. (1990) reported a decrease in enthalpy in whey protein due to thermal denaturation at acidic pH as measured by DSC. Ovalbumin denaturation enthalpies have been reported ranging from 706.5 kJ/mol (Donovan and Mapes, 1976) to 392.9 kJ/mol (Fujita and Noda, 1981). Some variation in the calculation and instrument sensitivity might account for difference in reported values.

The unfolding step of ovalbumin is usually highly cooperative, and requires heat, which is seen as an endothermic peak in DSC thermograms (Kitabatake et al., 1990). No endothermic peak suggests a fully denatured molecule (de Wit, 1980). A cooperativity ratio (CR) below one suggests the presence of domains or intermediate steps in the melting process. A value higher than one indicates an increased cooperativity of structure and aggregation (Bertazzon and Tsong, 1990 b). The CR for S-ovalbumin was lowest at pH 3.0 and differred ($P< 0.05$) compared to pH 7.0 or 9.0 (Fig. 6). The sharpest endothermic peaks was exhibited at pH 7.0. Ovalbumin has not been identified as containing domains, therefore an intermediate form might be present.

## Effect of pH on Structure Development

The G' in Tables 7-9 were determined after 15 min isothermal heating. The highest isothermal temperature in which G' was measured was larger and differred ($P < 0.05$) from the lowest isothermal temperature. The results suggested higher temperature enhanced S-ovalbumin unfolding causing cross-linking and more structure development. The time taken to reach 10 Pa decreased with increased temperature, suggesting higher temperatures increased the rate of S-ovalbumin unfolding.

At pH 3.0 the maximum temperature used based on the time necessary to reach 10 Pa was lower than at pH 7.0 or 9.0. Suggesting pH 7.0 and 9.0 were more thermally stable than at pH 3.0. S-ovalbumin was positive charged at pH 3.0 causing electrostatic repulsion between molecules facilitating unfolding.

S-ovalbumin $T_g$ increased with pH (Table 10). At pH 3.0

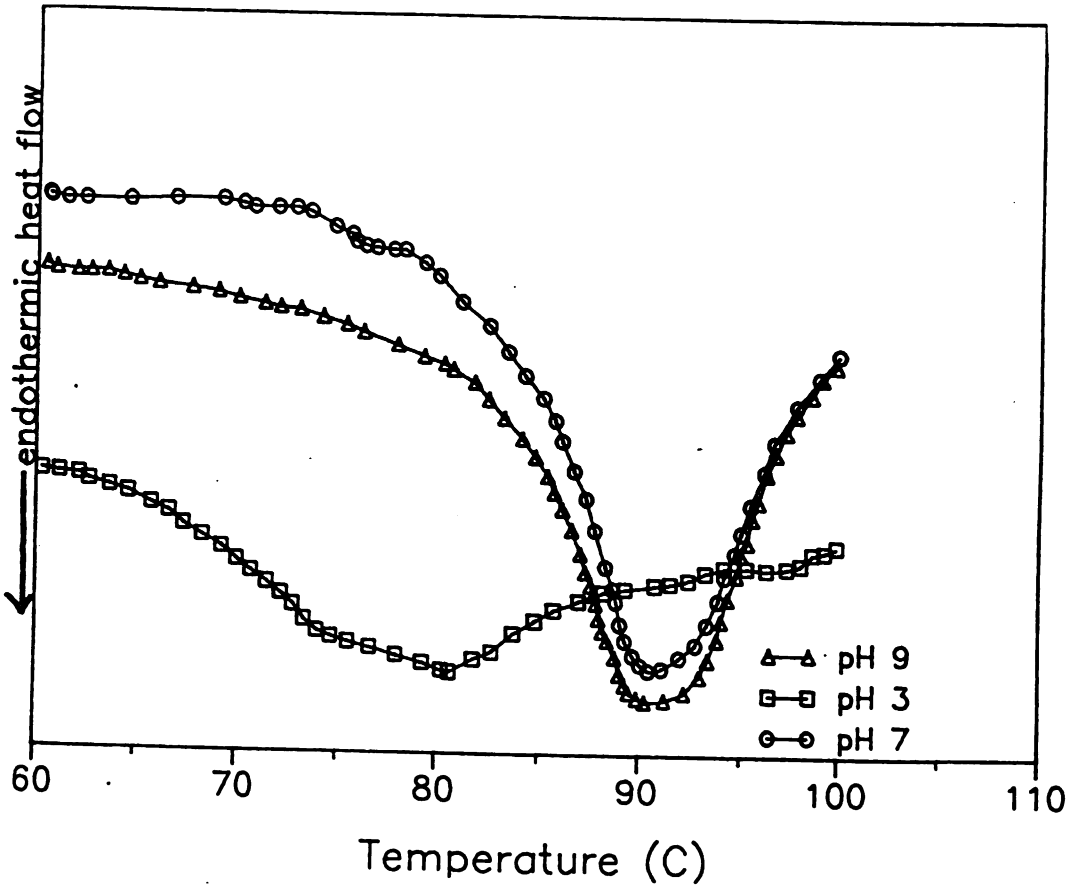


**Fig. 6-Effect of pH on the differential scanning calorimetry thermogram of 8% (w/v) S-ovalbumin in 0.5M NaCl heating at 10°C/min.**

S-ovalbumin was negatively charged and partially unfolded because of electrostatic repulsion accelerating gel structure formation. S-ovalbumin has a balance of attractive (hydrophobic interaction, S-S and hydrogen bonding) and repulsive (electrostatic) forces at pH 7.0 and 9.0, therefore higher temperatures were needed to form a G' at 10 Pa. Arntfield et al. (1989) reported the $T_s$ of ovalbumin at pH 3.0 was 72°C while at pH 7.0 and 9.0 $T_s$ was 95°C.

At each pH where thermal onset temperatures ($T_O$) from the DSC were compared to $T_s$, $T_O$ preceded $T_s$ indicating that partial unfolding of S-ovalbumin occurred before structure development. This observation was also noted by Hegg et al. (1979). Beveridge (1985 a) using small amplitude oscillatory and Arntfield et al. (1989) using dynamic rheological testing observed that development of rheological structure of ovalbumin did not begin until most of the ovalbumin had been denatured. The use of parallel plates compared to cone and plate geometry, different ovalbumin concentration and salt concentration might be responsible for the different results.

S-ovalbumin $Ea_s$ at pH 7.0 was lower than at pH 3.0 or pH 9.0 (Table 10). Higher $Ea_s$ indicated more energy was required to form cross-linked aggregates which was associated with a slower reaction. Harwalkar (1980) using

Table 7-Influence of temperature on structure development of 8% (w/v) S-ovalbumin at pH 3.0 in 0.5M NaCl during isothermal heating for 15 min

| Temperature (°C) | G' (Pa) | Time (min) |
|---|---|---|
| 65 | $14.7^{a}$ | $10.4^{a}$ |
| 70 | $32.2^{a}$ | $5.8^{b}$ |
| 75 | $125.6^{ab}$ | $3.3^{c}$ |
| 80 | $253.7^{b}$ | $1.4^{d}$ |

| | |
|---|---|
| G' | storage modulus determined from isothermal heating for 15 min |
| Time | period to reach 10 Pa during isothermal heating |

Column values with different superscripts are significantly different ($P < 0.05$).

Table 8-Influence of temperature on structure development of 8% (w/v) S-ovalbumin at pH 7.0 in 0.5M NaCl during isothermal heating for 15 min

| Temperature (°C) | G' (Pa) | Time (min) |
|---|---|---|
| 80 | $105.2^{a}$ | $6.9^{a}$ |
| 85 | $364.5^{a}$ | $4.4^{b}$ |
| 90 | $680.7^{ab}$ | $3.4^{b}$ |
| 95 | $1378.1^{ab}$ | $2.3^{bc}$ |
| 100 | $2001.3^{b}$ | $0.8^{c}$ |

| | |
|---|---|
| G' | storage modulus determined from isothermal heating for 15 min |
| Time | period taken to reach 10 Pa during isothermal heating |

Column values with different superscripts are significantly ($P < 0.05$).

Table 9-Influence of temperature on structure development of 8% (w/v) S-ovalbumin at pH 9.0 in 0.5M NaCl during isothermal heating for 15 min

| Temperature (°C) | G' (Pa) | Time (min) |
|---|---|---|
| 80 | $25.3^{a}$ | $12.7^{a}$ |
| 85 | $192.5^{b}$ | $8.9^{b}$ |
| 90 | $368.5^{c}$ | $6.4^{c}$ |
| 95 | $2218.7^{d}$ | $1.8^{d}$ |
| 100 | $1926.1^{e}$ | $0.8^{d}$ |

G' storage modulus determined from isothermal heating for 15 min
Time period of time taken to reach 10 Pa during isothermal heating

Column values with different superscripts are significantly different ($P < 0.05$).

Table 10- Influence of pH on structure development of 8% (w/v) S-ovalbumin in 0.5M NaCl during heating

| pH | $T_s$ (°C) | $Ea_s$ (kJ/mol) |
|---|---|---|
| 3 | $73.7^{a}$ | $596.3^{a}$ |
| 7 | $83.9^{b}$ | $315.7^{b}$ |
| 9 | $89.9^{c}$ | $676.2^{a}$ |

$T_s$ temperature of structure formation
$Ea_s$ activation energy for structure development

Column values with different superscripts are significantly different ($P < 0.05$).

the Arrhenius plot calculated the $E_a$ of thermal denaturation of β-lactoglobulin at pH 6.7 to be 71.6 kJ/mol. The researcher suggested that variation in $Ea_s$ due to pH or salt type, may be because of different mechanisms of thermal denaturation. Activation energies calculated from dynamic testing may have had mechanical energy transferred into the system (oscillatory motion of the geometry) that may influence the calculations. Decreasing S-ovalbumin concentration to avoid torque overload and increasing the strain might decrease the variation in $Ea_s$.
Goldsmith and Toledo (1985) used NMR data to calculate activation energies of gel strenght (structure) in the range of 183.68 to 188.44 kJ/mol. Beveridge et al. (1984) reported an $E_a$ of 138.15 kJ/mol for freeze-dried egg albumen using rate constants obtained at 85°C and 90°C.

### Effect of Salt Type on Thermal Denaturation

Thermal denaturation temperature of ovalbumin at pH 7.0 decreased as a function of salt type in the order of the Hoffmeister series $Na_2SO_4$ > NaCl > NaI (Table 11). The low $T_d$ for NaI may have resulted from the disruption of the hydrophobic interaction within the S-ovalbumin molecule producing instability of the protein structure. In contrast $Na_2SO_4$ increased intramolecular hydrophobic interaction, therefore stabilizing the native S-ovalbumin structure.

Damodaran and Kinsella (1982) suggested that hydrophobic interaction between apolar residues is the major stabilizing force of the native conformation of protein. The ions which salt-out hydrocarbons should enhance hydrophobic interaction in the protein and provide more stabilizing energy.

Arntfield et al. (1989) suggested that salts have an impact on the attractive-repulsive balance associated with network formation. At salt concentrations of 0.5M and higher the stabilizing influence was attributed to strengthened hydrophobic interactions. The reseachers reported a decrease in $T_d$ and enthalpy using 10% ovalbumin in 0.5M NaSCN as compared to 0.5M NaCl and 0.5M $Na_2SO_4$. Arntfield et al. (1989) observed a significant increase between ovalbumin $T_d$ using 0.5M $Na_2SO_4$ compared to 0.1M NaCl and 0.5M $C_2H_3NaO_2$. Damodaran (1988) observed an increase in soy isolate $T_d$ at pH 8.0 in 0.5M $Na_2SO_4$ compared to 0.5M $NaClO_4$. Arntfield et al. (1986) and Ismond et al. (1986) used DSC to rank salts in terms of their ability to stabilize faba bean proteins by monitoring changes in $T_d$. The $T_d$ values using DSC coincided with their position in the lyotropic series and reflected the importance of hydrophobic interactions to the stability of soy proteins.

Activation energies of S-ovalbumin thermal denaturation as a function of salt type did not differ (P > 0.05)

(Table 11). The reaction orders for S-ovalbumin in $Na_2SO_4$, NaCl and NaI were 1.1, 1.3 and 2.4, respectively. The reaction order might suggest the occurrance of molecular events within the ovalbumin molecules, such as unfolding of a $\alpha$-helix or $\beta$-sheet. Therefore, it was hypothesized that NaI decreases hydrophobic interaction causing uncoiling of S-ovalbumin molecules increasing the reaction order. When reaction order was one the activation energy of S-ovalbumin in 0.5M NaI differed ($P < 0.05$) compared to 0.5M NaCl or 0.5M $Na_2SO_4$. The NaI was less temperature dependent than the other two neutral salts suggesting that the energy needed to unfold the molecule might be influenced by ionic interaction that salt imparts on water structure.

Table 11- Influence of salt type on thermal denaturation of 8% (w/v) S-ovalbumin at pH 7.0 using differential scanning calorimetry

| Salt (0.5M) | $T_o$ (°C) | $T_d$ (°C) | $Ea_d$ (kJ/mol) | n | *$Ea_d$ (kJ/mol) |
|---|---|---|---|---|---|
| NaI | $79.3^a$ | $86.7^a$ | $395.3^a$ | $2.4^a$ | $38.08^a$ |
| NaCl | $83.6^b$ | $90.6^b$ | $304.0^a$ | $1.3^b$ | $61.05^b$ |
| $Na_2SO_4$ | $86.4^c$ | $94.5^c$ | $238.7^a$ | $1.1^c$ | $60.69^b$ |

| | |
|---|---|
| $T_o$ | onset temperature |
| $T_d$ | thermal denaturation temperature |
| $Ea_d$ | activation energy of denaturation |
| n | reaction order |
| *$Ea_d$ | activation energy of denaturation calculated when n=1 |

Column values with different superscripts are significantly different ($P < 0.05$).

Enthalpy values for S-ovalbumin in $Na_2SO_4$, NaCl and NaI did not differ ($P < 0.05$) (Table 12). The addition of neutral salts to ovalbumin did not result in any denaturation as evidenced by the similarity in enthaply. This was supported by the CR that did not differ ($P < 0.05$) between salt types. Artifield et al. (1990a) observed no significant difference in enthalpy between neutral salts at 0.5M concentrations.

### Effect of Salt Type on Structure Development

The G' in Tables 13-15 were determined after 15 min of isothermal heating. The highest isothermal temperature in which G' was measured was larger and differred ($P < 0.05$) from the lowest isothermal temperature. The results

Table 12-Effect of salt type on the enthalpic contributions to the stability of 8% (w/v) S-ovalbumin at pH 7.0

| Salt (0.5M) | calorimetric enthalpy (kJ/mol) | van't Hoff enthalpy (kJ/mol) | CR |
|---|---|---|---|
| NaI | $62.2^a$ | $45.6^a$ | $0.73^a$ |
| NaCl | $62.4^a$ | $52.1^a$ | $0.83^a$ |
| $Na_2SO_4$ | $65.5^a$ | $56.3^a$ | $0.89^a$ |

CR-cooperativity ratio

Column values with different superscripts are significantly different ($P < 0.05$)

suggested higher temperatures facilitated the unfolding of S-ovalbumin causing cross-linking and structure development. There were large errors between each treatment temperature that might have been caused by some sample dehydration.

The time taken to reach 10 Pa had less error between treatments. Because the time take to reach 10 Pa was less than 15 min which was the time when G' was determined. All cases showeed that as isothermal temperatures increased the time decreased suggesting hihger temperatures increase the unfolding rate of S-ovalbumin. The time for NaI were higher for most time-temperature comparisons suggesting tht disrupting of the water structure and hydrophobic interaction was caused by the chaotropic activity of NaI compared to NaCl or $Na_2SO_4$.

Arntfield et al. (1990 a) reported that G' increased with the inclusion of salts at the destabilizing end of the lyotropic series. The authors concluded that the involvement of hydrophobic interactions were factors in determining the strength of heat-induced ovalbumin networks. Both NaCl and $Na_2SO_4$ stabilize protein structure by promoting hydrophobic interactions within the native structure, whereas, NaI destabilizes protein structure by weakening intramolecularhydrophobic interactions (von Hippel and Schleich, 1969; Catsimpoosas and Meyer, 1970; Babajimopoulos et al., 1983). Arntfield et al. (1989)

observed 0.5M NaCl and 0.5M $Na_2SO_4$ followed the Hofmeister series in their research with 10% ovalbumin.

S-ovalbumin $T_s$ did not differ ($P > 0.05$) (Table 15) suggesting salt type does not influence the temperature at which rheological structure was formed. Arntfield et al. (1989) noted $T_s$ for 10% ovalbumin solutions was unaffected by various salt environments. The researchers suggested it was possible that salts have an impact on attractive-repulsive balance associated with network formation.

The $Ea_s$ of S-ovalbumin in NaCl, NaI and $Na_2SO_4$ were not different ($P > 0.05$) (Table 15) suggesting salt type did not change temperature dependency for cross-linking and aggregate formation.

Harte (1989) reported an average Ea for denaturation / gelation was 183.47 kJ/mol although a value of 169.32 kJ/mol was reported using the Arrhenius kinetic theory and the temperature-time history.

## Effect of Concentration on Thermal Denaturation and Structure Development

The $T_o$ for 4.0% S-ovalbumin solution occurred 2.3°C before the $T_o$ for 8.0% S-ovalbumin solution. $T_d$ did not change with increased S-ovalbumin concentration suggesting that different concentrations of S-ovalbumin did not influence thermal denaturation (Table 16). Changes in $T_d$ due to S-ovalbumin concentration have not been discussed in literature, but Kitabatake et al. (1989; 1990) observed that increase in soy bean protein concentration increased $T_d$. The $Ea_d$ and reaction order for 4.0% S-ovalbumin was the same as 8.0% S-ovalbumin solution. The 4.0% S-ovalbumin enthalpy was less than 8.0% S-ovalbumin indicating less total heat energy was absorbed during denaturation due to the higher protein concentration (Table 17). There was no difference ($P > 0.05$) in CR between 4.0% and 8.0% S-ovalbumin solution indicating that two state unfolding theory (Ferry, 1948) was not a function of concentration.

The G' of 4% S-ovalbumin solution was not detected after 15 min isothermal heating until 90°C suggesting inadequate protein concentration for crosslinking at temperatures below 90°C (Table 18). The $T_s$ for 4.0% S-ovalbumin solution was higher compared to 8.0% S-ovalbumin solution suggesting less protein was present to form a measurable cross-linked structure. The higher $T_s$ of 4% S-ovalbumin solution was needed to detected structure formation suggesting more molecular unfolding to furhter extend the molecule for cross-linking had to occur compared to the 8.0% ovalbumin solution. The $Ea_s$ did not differ ($P > 0.05$) between 4.0% and 8.0% ovalbumin solution indicating no additional energy was needed to form a cross-linked structure due to concentration. The reaction order did not differ ($P > 0.05$) between S-ovalbumin concentrations suggesting the number of molecular events

Table 13-Influence of temperature on structure development of 8% (w/v) S-ovalbumin at pH 7.0 in 0.5M NaI during isothermal heating for 15 min

| Temperature (°C) | G' (Pa) | Time (min) |
|---|---|---|
| 75 | $62.6^{a}$ | $9.7^{a}$ |
| 80 | $441.7^{ab}$ | $3.7^{c}$ |
| 85 | $910.1^{bc}$ | $5.2^{b}$ |
| 90 | $1418.8^{cd}$ | $2.1^{d}$ |
| 95 | $1134.7^{cde}$ | $1.4^{de}$ |
| 100 | $1795.2^{e}$ | $0.4^{e}$ |

G' storage modulus determined from isothermal heating for 15 min
Time period taken to reach 10 Pa during isothermal heating for 15 min

Column values with different superscripts are significantly different ($P < 0.05$).

Table 14-Influence of temperature on structure development of 8% (w/v) S-ovalbumin at pH 7.0 in 0.5M $Na_2SO_4$during isothermal heating for 15 min

| Temperature (°C) | G' (Pa) | Time (min) |
|---|---|---|
| 80 | $43.1^{a}$ | $7.4^{a}$ |
| 85 | $86.0^{a}$ | $4.9^{bc}$ |
| 90 | $1251.4^{b}$ | $3.3^{cd}$ |
| 95 | $541.2^{a}$ | $2.0^{de}$ |

G' storage modulus determined from isothermal heating for 15 min
Time period of time taken to reach 10 Pa during isothermal heating for 15 min

Column values with different superscripts are significantly different ($P < 0.05$).

Table 15- Influence of salt type on structure development of 8% (w/v) S-ovalbumin at pH 7.0 during heating

| Salt (0.5M) | $T_s$ (°C) | $Ea_s$ (kJ/mol) |
|---|---|---|
| NaI | $83.9^a$ | $350.4^a$ |
| NaCl | $83.9^a$ | $311.5^a$ |
| $Na_2SO_4$ | $85.6^a$ | $384.1^a$ |

$T_s$ temperature of structure formation
$Ea_s$ activation energy of structure formation

Column values with different superscripts are significantly different ($P < 0.05$).

Table 16- Influence of S-ovalbumin concentration at pH 7.0 in 0.5M NaCl on thermal denaturation using differential scanning calorimetry

| concentration (%) | $T_o$ (°C) | $T_d$ (°C) | $Ea_d$ (kJ/mol) | n | $^*Ea_d$ (kJ/mol) |
|---|---|---|---|---|---|
| 8 | $83.6^a$ | $90.6^a$ | $304.0^a$ | $1.3^a$ | $66.24^a$ |
| 4 | $81.3^b$ | $90.6^a$ | $239.6^a$ | $1.0^a$ | $61.05^a$ |

$T_o$ onset temperature
$T_d$ thermal denaturation temperature
$Ea_d$ activation energy of thermal denaturation
n reaction order
$^*Ea_d$ activation energy of thermal denaturation when $n=1$

Column values with different superscripts are significantly different ($P < 0.05$)

Table 17- Effect of S-ovalbumin concentration at pH 7.0 in 0.5M NaCl on thermodynamic properties

| concentration (%) | calormetric enthalpy (kJ/mol) | van't Hoff enthalpy (kJ/mol) | CR |
|---|---|---|---|
| 8 | $62.4^a$ | $52.1^a$ | $0.83^a$ |
| 4 | $45.1^b$ | $28.6^b$ | $0.65_a$ |

CR-cooperativity ratio

Column values with different superscripts are significantly different ($P < 0.05$).

Table 18-Influence of temperature on structure development of 4% (w/v) S-ovalbumin at pH 7.0 in 0.5M NaCl

| Temperature (°C) | G' (Pa) | Time (min) |
|---|---|---|
| 90 | $46.1^a$ | $10.0^a$ |
| 95 | $131.5^{ab}$ | $6.2^b$ |
| 100 | $262.3^b$ | $3.6^{bc}$ |
| 105 | $286.8^b$ | $1.7^c$ |

| | |
|---|---|
| G' | storage modulus determined from isothermal heating for 15 min |
| Time | period taken to reach 10 Pa during isothermal heating for 15 min |

Column values with different superscripts are significantly different ($P < 0.05$).

Table 19- Effect of S-ovalbumin concentration at pH 7.0 in 0.5M NaCl on structural properties during heating

| concentration (%) | $T_s$ (°C) | $Ea_s$ (kJ/mol) |
|---|---|---|
| 8 | $83.9^a$ | $311.5^a$ |
| 4 | $91.4^b$ | $624.2^b$ |

$T_s$ temperature of structure development
$Ea_s$ activation energy of structure development

Column values with different superscripts are significantly different ($P < 0.05$).

that occur with respect to 4.0% and 8.0% S-ovalbumin were the same.

There was no difference ($P > 0.05$) between 4.0% and 8.0% S-ovalbumin $*Ea_d$ indicating that no additional energy was needed to drive the denaturation reaction due to concentration. The $Ea_d$ of 4.0% and 8.0% S-ovalbumin was approximately 4 and 5X larger, respectively than $*Ea_d$.

## Conclusion

The present study suggested that DSC and dynamic rheological testing are complementary methods in the study of ovalbumin gelation when influenced by pH and salt type. At pH 3.0 or 0.5M NaI the thermal stability of S-ovalbumin decreased due to increased electrostatic repulsion or decreased hydrophobic interaction, respectively.

At pH 9.0 or 0.5M $Na_2SO_4$ S-ovalbumin had a higher $*Ea_d$ suggesting increased thermal energy was needed to unfold the protein compared to pH 3.0 or 0.5M NaI.

Variation in $Ea_d$ was dependent on the order of the reaction. The variation might be attributed to the high concentration of ovalbumin used in the experiment or the regression routine used.

# IV. STUDY 2

## Denaturation and Structure Development of S-Ovalbumin as Influenced by Guanidine Hydrochloride and ß-Mercaptoethanol

### ABSTRACT

Effect of guanidine hydrochloride (GuHCl) and ß-mercaptoethanol (ß-ME) on the thermal denaturation and structure development of S-ovalbumin in 0.5M NaCl, pH 7.0 were studied. Increasing GuHCl and ß-ME decreased the denaturation temperature 10°C and 9°C, respectively. Enthalpy did not differ ($P > 0.05$) at any of the GuHCl concentrations but decreased with increased ß-ME (62.4-44.6 kJ/mole). Onset temperature for S-ovalbumin structure formation decreased and storage moduli increased with increased concentrations of GuHCl and ß-ME.

# INTRODUCTION

The ability to manufacture desirable egg containing products comes from understanding the behavior of proteins in different environments. Heat-induced gelation of egg proteins, particularly ovalbumin, is an important functional property in food systems. Protein gelation is a two step process involving denaturation and aggregation as defined by Ferry (1948):

$$Xp_n \;\text{<----->}\; XP_d \;\text{----->}\; (P_d)_x$$

where x is the number of native (n) or denatured (d) protein molecules (P). Several researchers have studied egg protein heat denaturation, aggregation, coagulation and gelation (Donovan and Mapes, 1976; Gossett et al., 1984; Beveridge and Arntfield, 1985; Arntfield et al., 1990 a; b; c), however more information is needed to understand the gelation mechanism. Guanidine hydrochloride (GuHCl) is a denaturant that is used for the disrupting and unfolding proteins by destabilizing hydrogen bonding and hydrophobic interactions (Shimada and Matsushita, 1981; Xiong and Kinsella, 1990). The exact nature of the interactions of guanidine with protein groups or solvent molecules is not well understood. Lee and Timasheff (1974) reported that GuHCl binds preferentially to the peptide backbone and aromatic amino acid side chains. The interaction between GuHCl and protein weakens hydrogen bonds that stabilize the globular conformation. Gordon (1972) suggested that induced changes exclusively at the surface of the protein molecule by increasing the solubility of nonpolar side chains. The increased solubility of nonpolar amino acid side chains diminish the magnitude of the hydrophobic effect by up to one third, producing unfolding of the protein (Creighton, 1984).

Strambini and Gonnelli (1986) observed that GuHCl penetrates into the interior of liver alcohol dehydrogenase decreasing intramolecular interactions resulting in an increased fluidity. Bismuto and Irace (1988) observed a decrease in structural organization and reported a decline in cooperativity of a two-state gelation process using fluorescence. The unfolding of component polypeptides and the transition from an ordered to random coiled structure allowed buried functional groups, e.g. SH of cysteine, to become exposed. Flexibility of protein molecules after unfolding enhanced the availability of some functional groups and facilitated their reactivity (Xiong and Kinsella, 1990).

Addition of ß-mercaptoethanol (β-ME) cleaved disulfide bonds disrupting the secondary and tertiary structure of proteins (Chang et al., 1978). Hirose et al. (1986) reported that the addition of 70 mM ß-ME induced egg white gelation at room temperature. The researchers concluded conalbumin (which contains 30 half-cystine residue/molecule) was involved in intramolecular disulfide linkages caused by reduction of cystine. Harwalkar and Ma (1987) and Zarins and Marshall (1990) observed the $T_d$ of oat globulin and glycinin decreased with addition of 5% β-ME.

Proteins that contain disulfide bonds between polypeptide chains will be retained after denaturation with GuHCl if reagents to break disulfide bonds are not added to the protein solution (Tanford, 1968). Denaturation of ovalbumin in 6.0M GuHCl and 0.1M β-ME yielded completely unfolded molecules (Ansari et al., 1972; Ahmad and Salahuddin, 1974). The GuHCl disrupted hydrogen bonds while β-ME reduced disulfied linkages, causing unfolding of ovalbumin.

Differential scanning calorimetry (DSC) measures the heat capacity ($C_p$) of a protein as a function of temperature and can be used to determine protein denaturation temperature ($T_d$) and denaturation enthalpy (de Wit and Swinkels, 1980). Donovon and Mapes (1976) used DSC to distinguish between ovalbumin and S-ovalbumin. The researchers determined the $T_d$ of ovalbumin and S-ovalbumin was 84.5°C and 92°C, respectively at pH 9.0.

Dynamic rheological testing is a nondestructive method used to monitor changes in viscous and elastic properties of a protein solution and thus structure formation during heating (Tung, 1978). Arntfield et al. (1989) reported the storage modulus (G') of 10% ovalbumin decreased with salt type (NaCl > $Na_2SO_4$) and salt concentration (0.1M > 0.5M).

Arntfield et al. (1990 a) observed the temperature of structure development as determined by changes in G' decreased with lower pH and was attributed to intramolecular hydrophobic interaction.

The objectives of this project were to influence the heat-induced gelation mechanism of hen egg ovalbumin by the addition of GuHCl or β-ME then (1) to investigate the relationship between denaturation and structure formation with DSC and dynamic rheological testing, respectively and (2) calculate transition temperatures and thermodynamic properties of the gelation process.

## Materials and Methods

### Material

S-ovalbumin purity, moisture determination and electrophoresis methods are described in study 1. S-ovalbumim was prepared at a concentration of 80 mg/ml at pH 7.0 in 0.5M NaCl and (1) 0.5M, 1.0M, or 2.0M GuHCl (2) 0.5%, 1.0%, 2.0% or 3.0% β-ME. All samples were allowed to equilibrate for 30 min prior to testing. All samples were prepared and tested in duplicate.

**Differential Scanning Calorimetry.** Dupont 990 Thermoanalyzer equipped with a Dupont 910 Cell Base and standard DSC cell were used to determine thermal properties of ovalbumin. Approximately 15 mg of ovalbumin solution was sealed in Dupont aluminium hermetic pan (part 900793-901) and lid (part 900794-903). Samples were heated from 30°C to 110°C at 10°C/min. Temperature calibration and calibration coefficient E for the DSC cell were determined using weighed samples of indium over a scanning range of 25-200°C. The reference pan contained an identical ovalbumin solution that had been previously heat-denatured in the same temperatue range (de Witt, 1981). Rescanning of the cooled ovalbumin (Patel et al., 1990) sample showed no denaturation peak, indicating irreversible denaturation. The cell was flushed with nitrogen at 50 mL $min^{-1}$ for all runs. All scans indicated an endothermic heat flow.

Onset denaturation temperature ($T_o$) was determined by drawing a tangent line from the $T_d$ to the base line of the endotherm performed by the DuPont General V 2.2A software program. The $T_d$ was defined as the peak temperature in the endotherm determined by the DuPont General V 2.2A software program.

The apparent enthalpy of calorimetry ($H_{cal}$):

$$(1) \quad H_{cal} = A / M \ (60BEqs)$$

where A is the area ($cm^2$), M is the mass of the sample (mg), B is the time base ($min\ cm^{-1}$), E is the cell calibration coefficient, and qs is the Y axis range (mW $cm^{-1}$).

Peak areas baselines were constructed as a single straight line from the beginning to the end of the endotherm and the area of the endotherm was integrated. The beginning of the endotherm was determined as the initial increase in the integrating curve and endotherm termination was when the integrating curve became constant.

Activation energy ($Ea_d$) was calculated by a method developed by Patel et al. (1990) for whey protein using DSC. The equation, reduced to linear form was:

$$(2)\ \ln (d\alpha / dt) = \ln K_o - E_a / RT + n [\ln (1-\alpha)]$$

The reaction rate ($d\alpha/dt$), at any temperature, T, was calculated as the ratio of peak height to total area. The fraction of denatured protein ($\alpha$) is calculated as the ratio of partial area to total peak area. Values of preexponential factor ($K_o$), activation energy ($E_a$), and reaction order (n) are obtained from multilinear regression (Eisensmith, 1989) performed using $\ln (d\alpha / dt)$ as dependent variable and $1/T$ and $\ln (1-\alpha)$ as two independent variables. The gas constant is R.

## Thermodynamic Calculations

The following two thermodynamic terms were calculated by the methods defined by Bertazzon and Tsong (1990). The van't Hoff enthalpy is defined as follows:

$$(3)\ H_{VH} = 4RT^2\ C_p(\max) / H_{cal}$$

where R is the gas constant, T is the temperature (K), $C_p$(max) is the maximum heat capacity of the DSC excess heat capacity curve.

The cooperativity ratio, is defined by

$$(4)\ H_{VH} \ /\ H_{cal}$$

**Dynamic Rheologcial Testing**

The (G') of ovalbumin solutions during heating from 60°C to 100°C at 2°C/min and 15 min isothermal experiments were monitored using a Rheometrics Fluids Spectrometer Model 8400 (Piscataway, NJ), equipped with a 1-100 g-cm torque transducer and water circulation system controlled by a Nelsprit Temperature Programmer. One and one-half milliliters of ovalbumin solution was placed between the cone and plate geometry (radius 12.5 mm, 0.02 cone angle and 50 um gap) and equilibrated for 5 min prior to heating. The G' was recorded continuously at a fixed frequency of 1 rad/sec and strain of 1.0%. Limits of constant viscoelasticity were determined by conducting frequency (0.1 to 100 $cm^{-1}$) and strain sweeps (0.1 to 100 %) in preliminary studies.

Significant rheological structure was designated when G' reached 10 Pa since it was a common point of comparison for all treatments in preliminary studies. Temperatures for isothermal heating experiments were selected based on temperatures at which G' reached at least 10 Pa between 0.1 and 15 min heating period.

Thermal scanning experiments (heating from 60°C to 100°C at 2°C/min) were used to determine the temperature at which an initial increase in G' was observed. This temperature was defined as the onset temperature for structure development ($T_s$).

Activation energy of structure formation ($Ea_s$) of ovalbumin was calculated using time-temperature superpositioning (Ferry, 1980) at temperatures between 65°C and 100°C for 15 min. Values for time (t) were taken directly from the curve of G' versus time in which time need to reach 10 Pa was used. The Arrenhius equation was used to calculate activation energy,

(5) $\ln t = \ln A - Ea/RT$

where t is the time needed to reach 10 Pa, A is the preexponential factor ($s^{-1}$), $E_a$ is the activation energy ($J/mol^{-1}$), R is the gas constant and T is the temperature (K). Activation energy was calculated from the slope of the plot ln t versus 1 / T.

## Statistics

MSTATC software (version C, East Lansing, MI) was used for basic statistics and two way analysis of variance (replication x treatments) on a complete randomized design experiment. Tukey's honestly significant difference test (P

$< 0.05$) and standard error of the means were used to evaluate the significant differences between means. Multilinear regression analysis for determining $Ea_d$ and reaction order was conducted using Plotit (Eisensmith, 1985). All treatments were tested in duplicate.

## RESULTS AND DISCUSSION

### Effect of Guanidine Hydrochloride on Thermal Denaturation

S-ovalbumin $T_o$ decreased with increased GuHCl concentration (Table 20) suggesting that denaturation occurred at a lower temperature with higher concentrations of GuHCl.

A decrease in S-ovalbumin $T_d$ at all GuHCl concentrations suggest destabilized hydrogen and hydrophobic bonds compared to the absence of the denaturant. S-ovalbumin $T_o$ was 8°C to 10°C lower than $T_d$ (Table 20) suggesting structure development occurred before $T_d$. Privalov (1979) reported lysozyme $T_d$ decreased with increased GuHCl concentrations at pH 2.3 and 4.5.

S-ovalbumin $Ea_d$ at 0.5M GuHCl was different ($P < 0.05$) from the 2.0M GuHCl treatment (Table 20). The decrease in $Ea_d$ might be due to a decrease in water structure and a decrease in hydrophobic interactions that lead to protein

Table 20- Influence of guanidine hydrochloride (GuHCl) on thermal denaturation of 8% (w/v) S-ovalbumin at pH 7.0 in 0.5M NaCl using differential scanning calorimetry

| GuHCl (M) | $T_o$ (°C) | $T_d$ (°C) | $Ea_d$ (kJ/mol) | n | $*Ea_d$ (kJ/mol) |
|---|---|---|---|---|---|
| 0.0 | $83.6^a$ | $90.6^a$ | $304.0^{ab}$ | $1.3^a$ | $61.15^a$ |
| 0.5 | $78.6^b$ | $86.2^b$ | $437.4^a$ | $2.4^b$ | $45.2^{ab}$ |
| 1.0 | $77.2^b$ | $85.2^b$ | $257.3^{ab}$ | $1.5^a$ | $40.4^b$ |
| 2.0 | $66.3^c$ | $76.2^c$ | $123.4^b$ | $1.6^a$ | $14.5^c$ |

| | |
|---|---|
| $T_o$ | onset temperature |
| $T_d$ | denaturation temperature |
| $Ea_d$ | activation energy of denaturation |
| n | reaction order |
| $*Ea_d$ | activation energy of denaturation when n=1 |

Column values with different superscripts are significantly different ($P < 0.05$).

unfolding at higher GuHCl concentration, therefore the energy needed to drive the reaction from native to denatured state was reduced. Kauzmann (1959) reported the heat capacity was strictly dependent on the ordering of water molecules around exposed hydrophobic groups and a change of heat capacity at denaturation should be connected with disruption of hydrophobic bonds.

The 0.5M GuHCl reaction order was different ($P < 0.05$) compared to the other GuHCl concentrations (Table 20). At 0.5M GuHCl the solubility of ovalbumin might have been enhanced through a salting in effect. The reaction order may give information on the number of molecular events occurring in denaturation. As the number of events (uncoiling of the molecule) increased so did the reaction order suggesting that 0.5M GuHCl had more molecular events occurring compared to 2.0M GuHCl due to temperature dependency.

In addition the S-ovalbumin concentration might be too high to detect denaturation specifically and the reaction order and $Ea_d$ may included aggregation. Kokini (1991) suggested that gelation might be considered to have two reaction orders one for denaturation and another for aggregation. Further research is necessary to investigate the reaction order during gelation.

The S-ovalbumin $*Ea_d$ for the control was different ($P < 0.05$) compared to 1.0M or 2.0M GuHCl (Table 20) suggesting that the control was more temperature dependent. The $*Ea_d$ seems to be more consistent compare to $Ea_d$ with what would be expected. Since GuHCl disrupts and unfolds protein molecules one would expect a significant difference between the control and 2.0M GuHCl which is seen in $*Ea_d$ but not in $Ea_d$. S-ovalbumin enthalpy did not differ ($P > 0.05$) (Table 21) with the addition of GuHCl suggesting that no substantial unfolding occurred because of the denaturant. Privalov (1979) suggested that enthalpies associated with protein unfolding due to temperature, pH and GuHCl were the same. von Hippel and Schleich (1969) reported that guanidine salts followed the same Hofmeister

Table 21 - Effect of guanidine hydrochloride (GuHCl) on the enthalpic contributions to stability of 8% (w/v) S-ovalbumin at pH 7.0 in 0.5M NaCl

| GuHCl | calorimetric enthalpy (kJ/mol) | van't Hoff enthalpy (kJ/mol) | CR |
|---|---|---|---|
| 0.0 | $62.4^a$ | $52.1^a$ | $0.83^a$ |
| 0.5 | $56.4^a$ | $45.1^a$ | $0.80^a$ |
| 1.0 | $55.3^a$ | $38.2^{ab}$ | $0.69^a$ |
| 2.0 | $56.5^a$ | $22.4^b$ | $0.40^b$ |

CR-cooperativity ratio

Column values with different superscripts are significantly different ($P < 0.05$).

series as all neutral salts. Arntfield et al. (1989) observed addition of stabilizing salts to ovalbumin did not result in any substantial protein unfolding as indicated by the similarity in the enthalpy values.

Pfeil and Privalvo (1976) reported lysozyme enthalpy at pH 2.0 decreased with increased concentrations of GuHCl. The researchers observed no difference in enthalpy between GuHCl denatured and heat denatured lysozyme.

A cooperativity ratio (CR) equal of one is a true two step gelation process, whereas a CR < 1 suggest intermediate steps in thermal denaturation (Bertazzon and Tsong, 1990). Complete protein unfolding by GuHCl is known, although there is some doubt whether complete unfolding can be achieved by thermal denaturation (Pfeil and Privalov, 1976). Ovalbumin CR decreased with increased GuHCl concentrations (Table 21) indicating the two state unfolding theory (Ferry, 1948) may not hold true under these conditions.

Privalov (1979) observed 6.0M GuHCl unfolded lysozyme from its native structure and Tanford (1966) concluded from NMR (sharpness of transition) results indicated denaturation was highly cooperative. Pfeil (1981) suggested the cooperativity of protein folding seemed independent on the nature of the denaturing action. Cooperativity ratio was 0.7 for G-actin, suggesting that denaturation is not a simple two-state process, and therefore can be resolved into multiple steps. The higher the CR and $T_d$ the greater stability (Bertazzon et al. 1990 c). Bismuto and Irace (1988) used fluorescence intensity to monitor the effect of GuHCl on the structure of apomyoglobin. The researchers reported that apomyoglobin exhibited cooperativity with increasing GuHCl concentrations (0-4M, pH 7.0). They further suggested that GuHCl acted on specific structural regions of apomyoglobin instead of a general loosening of structure as reported with liver alcohol dehydrogenase. Doi et al. (1987) reported that ovalbumin contained residual secondary structure after heating but was mainly random coil after treatment with 5.0M GuHCl as monitored with circular dichroism (CD). For β-lactoglobulin significant differences between GuHCl and heat denaturation were found, as judged from optical rotation (Harwalkar, 1979). Suresh Chandra et al. (1984) used CD to determine the effect of GuHCl on the secondary structure of glycinin. A disordered structure was observed at 15°C that appeared to become more ordered at higher temperatures.

### Effect of Guanidine Hydrochloride on Structure Development

S-ovalbumin G' in Tables 22-24 were determined after 15 min of isothermal heating. The highest isothermal temperature in which G' was measured was larger and differred ($P < 0.05$) from the lowest isothermal temperature at all GuHCl concentrations. The results suggested higher temperatures enhanced the unfolding of S-ovalbumin casuing cross-linking and more structure development. The presence GuHCl disrupted hydrogen bonding allowing S-ovalbumin to unfold at a lower temperature compared to the control. Unfolding at a lower temperature enabled formation of higher molecular weight molecules increasing G' at higher GuHCl concentrations. S-ovalbumin was more stable at 0.5M than at the control as addition of low GuHCl concentrations might have enhanced protein solubility through a salting-in effect. The time taken to reach 10 Pa decreased with increased temperatures which suggested higher temperatures increased the rate of unfolding. Katsusta and Kinsella (1990) observed an increase in G' of 10% β-lactoglobulin in 6.0M GuHCl at 25°C during a 12 hr incubation period. The

Table 22-Influence of temperature on structure development of 8% (w/v) S-ovalbumin at pH 7.0 in 0.5M guanidine hydrochloride (GuHCl) and 0.5M NaCl during isothermal heating for 15 min

| Temperature (°C) | G' (Pa) | Time (min) |
|---|---|---|
| 80 | $66.1^{a}$ | $9.3^{a}$ |
| 85 | $285.0^{ab}$ | $4.2^{b}$ |
| 90 | $390.8^{ab}$ | $2.0^{cd}$ |
| 95 | $638.4^{b}$ | $0.8^{d}$ |

| | |
|---|---|
| G' | storage modulus determined from isothermal heating for 15 min |
| Time | period of time taken to reach 10 Pa during isothermal heating for 15 min |

Column values with different superscripts are significantly different ($P < 0.05$).

Table 23-Influence of temperature on structure development of 8% (w/v) S-ovalbumin at pH 7.0 in 1.0M guanidine hydrochloride (GuHCl) and 0.5M NaCl during isothermal heating for 15 min

| Temperature (°C) | G' (Pa) | Time (min) |
|---|---|---|
| 75 | $12.2^{a}$ | $14.9^{a}$ |
| 80 | $129.6^{a}$ | $6.5^{b}$ |
| 85 | $230.3^{a}$ | $4.9^{c}$ |
| 90 | $299.3^{a}$ | $1.4^{d}$ |
| 95 | $650.9^{b}$ | $0.3^{d}$ |

| | |
|---|---|
| G' | storage modulus determined from isothermal heating for 15 min |
| Time | period of time taken to reach 10 Pa during isothermally heating for 15 min |

Column values with different superscripts are significantly different ($P < 0.05$).

researchers suggested that 6.0M GuHCl decreased the fludity of β-lactoglobulin solution and the gel became more rigid as network-cross links were formed. S-ovalbumin $T_s$ control differred ($P < 0.05$) from 2.0M GuHCl (Table 25). Lower salt concentrations enhanced solubility, and increased S-ovalbumin heat stability compared to the control. At 2.0M GuHCl disruption of hydrogen bonds facilitated unfolding of S-ovalbumin and decreasing $T_s$. S-Ovalbumin $Ea_s$ calculated from a series of different isothermal temperatures increased with increased GuHCl concentrations (Table 25). The increase in $Ea_s$ indicates more energy is needed at 2.0M than at the control to form cross-linked aggregates. The $Ea_s$ calculated from rheological data had an inverse relationship as compared to $Ea_d$ this may be due to the rate of thermal treatment (rate) and the addition of mechanical energy input into the gel system. The data supports the idea that the control is less temperature dependent compared to 2.0M GuHCl to drive the native S-ovalbumin molecule to denaturation.

## Effects of β-Mercaptoethanol on Thermal Denaturation

The onset temperature ($T_o$) for S-ovalbumin decreased with increased β-ME concentrations (Table 26). The $T_d$ of S-ovalbumin decreased with increasing β-ME concentrations (Table 26) suggesting that disulfied bonds were not reduced. The $T_d$ should remain constant when the S-S bond is reduced.

Table 24-Influence of temperature on structure develompent of 8% (w/v) S-ovalbumin at pH 7.0 in 2.0M guanidine hydrochloride (GuHCl) and 0.5M NaCl during isothermal heating for 15 min

| Temperature (°C) | G' (Pa) | Time (min) |
|---|---|---|
| 65 | $16.5^{a}$ | $13.0^{a}$ |
| 70 | $68.5^{a}$ | $7.7^{b}$ |
| 75 | $212.4^{b}$ | $3.3^{c}$ |
| 80 | $538.2^{c}$ | $0.4^{d}$ |

| | |
|---|---|
| G' | storage modulus determined from isothermal heating for 15 min |
| Time | period of time taken to reach 10 Pa during isothermal heating for 15 min |

Column values with different superscripts are significantly different ($P < 0.05$).

Table 25- Influence of guanidine hydrochloride (GuHCl) on structural properties of 8% (w/v) S-ovalbumin at pH 7.0 in 0.5M NaCl during heating

| GuHCl (M) | $T_s$ (°C) | $Ea_s$ (kJ/mol) |
|---|---|---|
| 0.0 | $83.9^{a}$ | $311.5^{a}$ |
| 0.5 | $88.8^{b}$ | $598.9^{b}$ |
| 1.0 | $85.7^{ab}$ | $731.1^{c}$ |
| 2.0 | $75.9^{c}$ | $837.5_{d}$ |

| | |
|---|---|
| $T_s$ | temperature of structure formation |
| $Ea_s$ | activation energy of structure formation |

Column values with different superscripts are significantly different ($P < 0.05$).

Ma et al. (1987) observed the addition of 5.0% β-ME lowered $T_d$ of oat globulin suspension from 108°C to 96.7°C and caused a reduction in enthalpy. They attributed these effects to β-ME acting as a monohydric alcohol that destabilized proteins by weakening hydrophobic interactions. Zarins and Marshall (1990) concluded that the reduction in $T_d$ of soy glycinin as a consequence of increased β-ME was due to the destabilization of hydrophobic bonds in the protein interior. de Wit and Klarenbeek (1981) observed a decrease in $T_d$ of β-lactoglobulin in the presence of $1.5 \times 10^{-6}$ mol β-mercaptoethanol.

S-ovalbumin $Ea_d$ (Table 26) did not differ ($P > 0.05$) in the presence of β-ME suggesting that disulfide bonds do not have a large influence on the temperature dependence of ovalbumin unfolding. S-Ovalbumin $Ea_d$ was inconsistent suggesting the disulfide bonds was not completely reduced at any β-ME concentrations making it difficult to calculate an accurate $Ea_d$.

As discussed in the GuHCl section there seems to be more consistency in the $*Ea_d$ data than $Ea_d$. Since ß-ME cleaves disulfied bonds causing disruption and unfolding of the molecules one would expect a significant difference between the control and samples containing higher concentrations of the denaturant. There was a difference ($P < 0.05$) between the control and 3.0% ß-ME in $*Ea_d$.

Table 26-Influence of β-mercaptoethanol (β-ME) on thermal denaturation of 8% (w/v) S-ovalbumin at pH 7.0 in 0.5M NaCl using differential scanning calorimetry

| ME (%) | $T_o$ (°C) | $T_d$ (°C) | $Ea_d$ (kJ/mol) | n | $*Ea_d$ (kJ/mol) |
|---|---|---|---|---|---|
| 0.0 | $83.6^{a}$ | $90.6^{a}$ | $304.0^{a}$ | $1.3^{abc}$ | $61.15^{a}$ |
| 0.5 | $75.5^{b}$ | $86.2^{b}$ | $228.3^{a}$ | $1.6^{abc}$ | $32.8^{bc}$ |
| 1.0 | $75.7^{b}$ | $85.6^{b}$ | $310.9^{a}$ | $2.0^{b}$ | $49.1^{ab}$ |
| 2.0 | $71.3^{c}$ | $81.2^{c}$ | $146.6^{a}$ | $1.2^{c}$ | $28.8^{c}$ |
| 3.0 | $69.0^{d}$ | $77.9^{d}$ | $210.7^{a}$ | $1.5^{abc}$ | $41.9^{cb}$ |

| | |
|---|---|
| $T_o$ | onset temperature |
| $T_d$ | denaturation temperature |
| $Ea_d$ | activation energy of denaturation |
| n | reaction order |
| $*Ea_d$ | activation energy of denaturation when n=1 |

Column values with different superscripts are significantly different ($P < 0.05$).

S-ovalbumin enthalpy at 3.0% ß-ME was different ($P < 0.05$) compared to all other ß-ME concentrations (Table 27). This implied that 3.0% β-ME treated S-ovalbumin was not in its native state before enthalpy measurement, therefore partial unfolding of ovalbumin due to reduction of disulfide bond occurred. Zarins and Marshall (1990) observed that increasing β-ME concentration had little effect on the enthalpy of denaturation. S-ovalbumin CR decreased with increased β-ME concentrations (Table 27) suggesting transient states of partially unfolded S-ovalbumin. de Wit and Klarenbeek (1981) reported a broadening of the β-lactoglobulin endotherm in the presence of β-ME.

Table 27- Effect of β-mercaptoethanol (β-ME) on the enthalpic contributions to stability of 8% (w/v) S-ovalbumin at pH 7.0 in 0.5M NaCl

| ME (%) | calormetric enthalpy (kJ/mol) | van't Hoff enthalpy (kJ/mol) | CR |
|---|---|---|---|
| 0.0 | 62.4$^{a}$ | 52.1$^{a}$ | 0.83$^{a}$ |
| 0.5 | 68.0$^{a}$ | 46.7$^{a}$ | 0.69$^{ab}$ |
| 1.0 | 60.1$^{a}$ | 39.8$^{a}$ | 0.67$^{ab}$ |
| 2.0 | 66.1$^{a}$ | 43.9$^{a}$ | 0.66$^{ab}$ |
| 3.0 | 44.6$^{b}$ | 26.4$^{b}$ | 0.59$^{b}$ |

CR-cooperativity ratio

Column values with different superscripts are significantly different ($P < 0.05$).

**Effect of ß-Mercaptoethanol on Structure Development**

S-ovalbumin G'in Tables 28-31 were determined after 15 min of isothermal heating. The highest isothermal temperature in which G' was measured was larger and differred ($P < 0.05$) from the lowest isothermal temperature at all ß-ME concentrations. As the ß-ME concentration increased the S-S bond became partially reduced in ovalbumin due further unfolding of the molecules enhancing cross-linking. Exposed hydrophobic groups interacted intermolecularly increased gel structure. The time taken to reach 10 Pa decreased at higher temperatures suggesting

Table 28-Influence of temperature on structure development of 8% (w/v) S-ovalbumin at pH 7.0 in 0.5% ß-mercaptoethanol (ß-ME) and 0.5M NaCl during isothermal heating for 15 min

| Temperature (°C) | G' (Pa) | Time (min) |
|---|---|---|
| 75 | $23.0^{a}$ | $12.1^{a}$ |
| 80 | $247.2^{a}$ | $6.9^{b}$ |
| 85 | $926.5^{ab}$ | $3.9^{c}$ |
| 90 | $1664.4^{ab}$ | $2.5^{cd}$ |
| 95 | $2132.1^{ab}$ | $1.5^{de}$ |
| 100 | $4962.3^{b}$ | $0.4^{e}$ |

| | |
|---|---|
| G' | storage modulus determined from isothermal heating at for 15 min |
| Time | period taken to reach 10 Pa during isothermal heating for 15 min |

Column values with different superscripts are significanly different ($P < 0.05$).

Table 29-Influence of temperature on structure development of 8% (w/v) S-ovalbumin at pH 7.0 in 1.0% ß-mercaptoethanol (ß-ME) and 0.5M NaCl during isothermal heating for 15 min

| Temperature (°C) | G' (Pa) | Time (min) |
|---|---|---|
| 80 | $454.1^{a}$ | $5.9^{a}$ |
| 85 | $1323.3^{b}$ | $3.9^{b}$ |
| 90 | $1791.7^{bc}$ | $1.9^{cd}$ |
| 95 | $2023.6^{c}$ | $1.1^{d}$ |

| | |
|---|---|
| G' | storage modulus determined form isothermal heating for 15 min |
| Time | period taken to reach 10 Pa during isothermal heating for 15 min |

Column values with different superscripts are significantly different ($P < 0.05$).

Table 30-Influence of temperature on structure development of 8% (w/v) S-ovalbumin at pH 7.0 in 2.0% ß-mercaptoethanol (ß-ME) and 0.5M NaCl during isothermal heating for 15 min

| Temperature (°C) | G' (Pa) | Time (min) |
|---|---|---|
| 70 | $15.0^{a}$ | $13.1^{a}$ |
| 75 | $676.5^{ab}$ | $7.3^{b}$ |
| 80 | $620.5^{ab}$ | $2.2^{c}$ |
| 85 | $889.5^{ab}$ | $1.6^{c}$ |
| 90 | $1434.8^{b}$ | $0.6^{d}$ |

| | |
|---|---|
| G' | storage modulus determined from isothermal heating for 15 min |
| Time | period taken to reach 10 Pa during isothermal heating for 15 min |

Column values with different superscripts are significantly different ($P < 0.05$).

Table 31-Influence of temperature on structure development of 8%(w/v) S-ovalbumin at pH 7.0 in 3.0% ß-mercaptoethanol (ß-ME) and 0.5M NaCl during isothermal heating for 15 min

| Temperature (°C) | G' (Pa) | Time (min) |
|---|---|---|
| 65 | $13.4^{a}$ | $13.7^{a}$ |
| 70 | $133.8^{a}$ | $7.1^{b}$ |
| 75 | $693.5^{ab}$ | $2.1^{c}$ |
| 80 | $1077.2^{ab}$ | $1.4^{cd}$ |
| 85 | $2201.7^{b}$ | $0.7^{d}$ |

| | |
|---|---|
| G' | storage modulus determined from isothermal heating for 15 min |
| Time | period taken to reach 10 Pa during isothermal heating for 15 min |

Column values with different superscripts are significantly different ($P < 0.05$).

an increased rate of unfolding. Hirose et al. (1986) reported gel hardness of egg white increased with increased thiol concentration (0 to 0.2M) at 35°C over a period of 24 hr. Shimada and Cheftel (1988) suggested that partial reduction of disulfide bonds in the whey proteins enhanced interations between exposed hydrophobic regions.

Ovalbumin $T_s$ decreased with increased β-ME concentrations (Table 32). The $T_o$ occurred before $T_s$ indicating partial unfolding was needed before any structure formation in all ovalbumin treatments.

Ovalbumin $Ea_s$ increased with increased β-ME concentrations (Table 32). The increase in $Ea_s$ with β-ME concentrations, suggested that the aggregation reaction was temperature dependent. As discussed for $Ea_d$, β-ME ovalbumin system may be in a transition from oxidized to reduced state, therefore affecting the accuracy of $Ea_s$ calculations.

## Comparing DSC to Dynamic Rheological Testing

Comparing DSC to rheological data a relationship between unfolding $T_o$ and $T_s$ was exhibited. In both GuHCl and β-ME treatments $T_o$ occurred before $T_s$ indicating that partial unfolding of ovalbumin was necessary for structure development. Work by other authors investigating DSC and rheological changes induced by chemical perturbation of ovalbumin was not found. Arntfield et al. (1989) reported that $T_d$ of ovalbumin occurred before $T_s$ as with salts and pH

Table 40-Influence of β-mercaptoethanol (β-ME) on structural properties of 8% (w/v) S-ovalbumin at pH 7.0 in 0.5M NaCl during heating

| ß-ME (%) | $T_s$ (°C) | $Ea_s$ (kJ/mol) |
|---|---|---|
| 0.0 | $83.9^a$ | $311.5^a$ |
| 0.5 | $82.5^{ab}$ | $477.9^b$ |
| 1.0 | $83.5^a$ | $330.2^a$ |
| 2.0 | $79.7^b$ | $639.7^c$ |
| 3.0 | $72.9^c$ | $641.8^c$ |

G' storage modulus determined from isothermal heating at 80°C for 15 min
$T_s$ temperature of structure development
$Ea_s$ activation energy of structure development

Column values with different superscripts are significantly different ($P < 0.05$).

although Herald (1991) observed the opposite trend. This could have been do to the different geometries used (parallel plates versus cone and plate, respectively).

## Conclusion

Increased concentrations of GuHCl or ß-ME decreased the thermal stability of S-ovalbumin. Suggesting that GuHCl and ß-ME disrupted the bonding of S-ovalbumin causing unfolding of at lower temperatures. The time taken to reach 10 Pa decreased with increased denaturant concentration at all temperatures indicating S-ovalbumin unfolded due to the presence of GuHCl and ß-ME.

Differential scanning calorimetry and dynamic rheological testing was complementary in quantifying changes in thermal properties of ovalbumin.

## V. STUDY 3

## Heat-Induced Changes in the Secondary Structure of Hen Egg ß-Ovalbumin

### ABSTRACT

Solutions of hen egg S-ovalbumin at pD 3.0, 7.0 and 9.0 were heated at temperatures between 30°C to 90°C to study changes in secondary structure by Fourier transform infrared spectroscopy (FT-IR). Second derivative infrared spectra of native S-ovalbumin at pD 7.0 and 9.0 revealed protein absorption bands for ß-sheet at 1626 $cm^{-1}$, $3_{10}$-helix at 1638 $cm^{-1}$, $\alpha$-helix at 1656 $cm^{-1}$, and turns at 1682 $cm^{-1}$. The ß-sheet absorption band was not observed for ovalbumin at pD 3.0. The quantity of $\alpha$-helix and ß-sheet structure decreased as heating temperature was increased.

# INTRODUCTION

Ovalbumin is the major hen egg white protein that contributes gel properties to egg containing food systems. Conformational changes occur in ovalbumin during heating and perturbation (induced environmental change) as indicated by changes in thermal denaturation temperatures and viscoelastic properties (Arntfield et al., 1989; Herald, 1991). Monitoring heat-induced changes in the secondary structure of ovalbumin will help to understand the mechanism involved in gelation and texture formation of egg proteins. This information may be used to manipulate protein conformation to optimize textural properties for specific food systems.

The secondary structure of ovalbumin and S-ovalbumin has been determined using Raman spectroscopy. Painter and Koenig (1976) were unable to detect any conformational difference between 10% ovalbumin and S-ovalbumin in 0.05M NaCl. Both proteins contain 50% random coil, 25% α-helix or 25% β-sheet. Kint and Tomimatsu (1979) reported 7% ovalbumin solutions in 0.05M KCl, pH 8.0 contained a higher α-helix content than β-sheet. S-ovalbumin had a 3-4% higher β-sheet content than ovalbumin.

Using circular dichrosim (CD), Egelandsdal (1986) reported that a 10% ovalbumin solution at pH 9.5 contained 30% α-helix and 40% β-sheet. The α-helixical content increased when ovalbumin was solubilized in 0.04M NaCl as compared to water. Kato and Takagi (1988) observed an increase in β-sheet and a loss of α-helix when 0.28% ovalbumin solution at pH 7.0 was heated to 80°C and measured using CD. Doi et al. (1987) using CD observed more α-helix and β-sheet than unordered structure in ovalbumin. Prestrelski et al. (1991 a) and Susi and Byler (1988) reported that CD is unable to detect turns or distinguish between $3_{10}$-helix and α-helix.

The secondary structure of proteins can be examined using Fourier transform infrared spectroscopy (FT-IR) with second derivative analysis. The FT-IR is a non-dispersive technique that provides better wavelength accuracy and higher signal to noise ratio than disperive techniques (Susi and Byler, 1986). Good correlation between FT-IR and x-ray crystallography has been reported in measuring protein secondary structure of α-lactalbumin (Prestrelski, 1991 a). The FT-IR technique exhibited a higher percentage of α-helix and ß-sheet than was reported by researchers using Raman or CD (Painter and Koenig, 1976; Kint and Tomimatsu, 1979; Egelansdal 1986).

The Amide I band (1620-1700 $cm^{-1}$), caused by carbonyl stretching vibration of the peptide backbone, is detected using FT-IR and used to monitor secondary structure of proteins (Byler and Susi, 1986; Surewicz and Mantsch, 1988; Havel, 1989). Vibrational transitions associated with α-helix, $3_{10}$-helix, β-sheet, turn and unordered structure give rise to bands at specific frequencies in the Amide I region (Prestrelski et al., 1991 b). Second derivative spectra analysis allows for resolution enhancement of overlapping bands in FT-IR spectra providing a qualitative means for following subtle change in protein conformation (Byler and Susi, 1988; Byler and Purcell, 1989).

Using FT-IR spectroscopy in aqueous solution is difficult because water absorbs strongly throughout much of the mid-IR region (4000-400 $cm^{-1}$). Particular problems occur in the Amide I region because of the strong HOH bending mode that absorbs around 1644 $cm^{-1}$. Susi and Byler (1986) reported that deuterium oxide ($D_2O$) does not absorb in the Amide I region and is a good solvent to use when studying protein structure by FT-IR.

The FT-IR has been used to investigate secondary structure of whey proteins (Byler and Purcell, 1989; Prestreiski et al., 1991 b), bovine serum albumin, carbonic anhydrase, lysozyme (Byler and Susi, 1986), hemoglobin, and ribonuclease A (Susi and Byler, 1983). During heating, α-lactalbumin retained more of its native conformation compared to β-lactoglobulin or bovine serum albumin (Byler and Purcell, 1989). No research has been reported that used FT-IR with second dervative analysis to resolve the secondary structure of native ovalbumin and subsequent conformational changes due to heating and pH. The objectives of the present research were to monitor changes in secondary structure of S-ovalbumin as a function of temperature at pD 3.0, 7.0, and 9.0.

## Materials and Methods

S-ovalbumin and all reagents were stored in a vacuum dessicator with phosphorous pentoxide to absorb water vapor. Ovalbumin (Grade V, lot 19F 8105) was purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. S-ovalbumin was prepared as 1.75% (w/v) solutions in 25 mM $KD_2PO_4$ made up in $D_2O$. Samples were prepared in a glove box under an atmosphere of dry nitrogen. The 99.9% nitrogen was passed through a container of $CaSO_4$ dessicant prior to entering the glove box. The pD of the S-ovalbumin solution was adjusted with 30% DCl or 40% NaOD to pD 3.0, 7.0 or 9.0 by adding 0.4 to the pH reading (Covington et al., 1968) measured using a Corning pH meter (Model 107, Corning, NY). S-ovalbumin solutions (200 ul) were placed in capped glass vials (2 mL capacity) which were purged with dry nitrogen for 2 min before heating at a constant temperature in a Fisher Programable Isotemp Oven for 30 min (Model 230F, Pittsburg, PA). After heating samples were placed inside the glove box and allowed to stand approximately 5 min until cool.

S-ovalbumin heat treatments at pD 7.0 and 9.0 were control, 80°C, 85°C, and 90°C. Heat treatments at pH 3.0

were control, 60°C, 65°C, 70°C and 80°C. Heat treatments at pD 7.0 in 2.0M GuHCl or 3.0% ß-ME were 30°C and 80°C. Treatments were selected based upon S-ovalbumin thermal denaturation temperatures as determined by differential scanning calorimetry (Herald, 1991). The cooled sample was again purged with dry nitrogen gas for 2 min and 100 ul was transferred to a Circular Demountable Cell (Model P-E N930-1117, Perkin-Elmer, Norwalk, CT) with $CaF_2$ windows and 0teflon spacers (75 um pathlength), which had been purged with dry nitrogen gas for 10 min before sample loading.

Infrared (IR) spectra were collected at ambient temperature using a model 1800 FTIR spectrometer (Perkin-Elmer) equipped with a incandescent wire source, a potassium bromide coated beam splitter and a broad range mercury/cadmium/telluride (MCT) detector. Before the IR spectrum was recorded, the instrument sample chamber and cell were purged with dry nitrogen gas for 15 min. All spectra were scanned 1000 times and recorded at a resolution of 2 $cm^{-1}$. The sample chamber was continuously purged with dry nitrogen. Resolution enhancement by second-derivative analysis (CDS-3 Applications software, Perkin-Elmer) was performed using the Savitzky-Golay derivative routine (Savitzky and Golay, 1964) and a 13-data point (13 $cm^{-1}$) window. Spectral contributions from a $D_2O$ blank and residual water vapor were subtracted. Each treatment was tested in triplicate.

The FT-IR spectra of 19 proteins with known secondary structure (Clark et al., 1981; Byler and Susi, 1986; Susi, and Byler, 1987; Halloway and Mantsch, 1989) were used as a means to identify the ovalbumin secondary structure. For example the crystalline structure of hemoglobin contains 86% α-helix and the FT-IR spectrum of hemoglobin has it's largest peak at 1655 $cm^{-1}$. Therefore, the FT-IR band assignment for α-helix is 1655 $cm^{-1}$. Band assigments for ß-sheet are at 1624 and 1627 $cm^{-1}$ based on concanavalin A and immunoglobulin G which both contain 86% ß-sheet (Levitt and Greer, 1977; Dong et al. 1990). α-lactalbumin contains 30% $3_{10}$-helix with a band assignment at 1639 $cm^{-1}$ (Acharya et al., 1989; Halloway and Mantch, 1989). ß-lactoglobulin which contains 46% turn has FT-IR bands at 1670 and 1680 $cm^{-1}$ (Papiz et al., 1986). Since ß-helix, unordered, and ß-sheet can be assigned to other bands, the bands located at 1666, 1672, 1680 and 1688 $cm^{-1}$ were assigned to turn structures (Dong et al., 1990).

## RESULTS AND DISCUSSION

### Secondary Structure of Native S-Ovalbumin

The crystalline structure of ovalbumin is known (Stein et al., 1990; Wright, 1990). However, the FT-IR spectral bands have not been assigned. The second derivative FTIR spectra of native ovalbumin were identified at pD 3.0, 7.0, and 9.0 at 30°C.

Five Amide I component bands were observed at pD 3.0 (Fig. 7) with peaks at 1638.2 $cm^{-1}$ ($3_{10}$-helix), 1656.3 $cm^{-1}$ (α-helix), 1682.5 $cm^{-1}$ turn and two shoulders at 1625 $cm^{-1}$ (ß-sheet) and 1669.6 $cm^{-1}$ (turn).

There is strong evidence suggesting the band at 1639 $cm^{-1}$ is $3_{10}$-helix for globular proteins (Acharya et al., 1989; Byler and Purcell, 1989; Prestrelski et al. 1991 a). However, X-ray crystallograpy has not identified a $3_{10}$-helix in the secondary structure of ovalbumin (Stein et al. 1990; Wright et al. 1990). In addition the band at 1639 $cm^{-1}$ has also been assigned to ß-sheet (Dong et al. 1990). Therefore, the band at 1639 $cm^{-1}$ is still under investigation. In the presence research the band at 1639 $cm^{-1}$ was considered $3_{10}$-helix due to the strong evidence found in other globular proteins.

Second derivative FT-IR spectrum of native S-ovalbumin at pD 7.0 exhibited 4 Amide I peaks and a shoulder (Fig. 8). The spectrum was similar to that of S-ovalbumin at pD 3.0. A distinguishable peak rather than a shoulder was observed at 1627.5 $cm^{-1}$ (ß-sheet). Peak intensities were observed at pD 7.0 were larger than pD 3.0 at 1638 $cm^{-1}$ ($3_{10}$ helix). However, pD 3.0 exhibited greater peak intensities at 1656 $cm^{-1}$ (α-helix) and 1681 $cm^{-1}$ (turn).

Second derivative FT-IR spectrum of native S-ovalbumin at pD 9.0 exhibited 4 Amide I peaks and a shoulder similar to the spectrum at pD 7.0 (Fig. 9). The band representing

$3_{10}$-helix was larger at pD 9.0 than at pD 3.0 and the peak intensity representing α-helical was smaller at pD 9.0 than at pD 3.0.

**Effect of pD and Heating on Secondary Structure**

In all cases increasing the temperature shifted bands frequencies of ovalbumin by 2-3 wavenumbers. S-ovalbumin, pD 3.0, heated to 60°C (Fig. 8), exhibited an amplified band at 1614 $cm^{-1}$ (intermolecular hydrogen bonding) compared to ovalbumin at 30°C. The band intensity at 1682.5 $cm^{-1}$ (turn) increased and was broader at 60°C compared to 30°C. Intermolecular hydrogen bonding increased with higher treatment temperatures suggesting that hydrogen bonds were formed due to gel formation upon cooling. The increased hydrogen bonds might be responsible for gel rigidity. Byler and Purcell (1989) observed the appearance of sharp peaks at 1614 $cm^{-1}$ and 1684 $cm^{-1}$ prior to gelation of β-lactoglobulin and bovine serum albumin at 80°C and 75°C, respecitively. The researchers assigned the bands to intermolecular hydrogen bonds.

**Fig. 7-Subtracted second dervative FT-IR absorption spectra of 1.75% S-ovalbumin in $D_2O$ (pD 3.0) and 25 mM $KD_2PO_4$ after different heat treatments for 30 min.**

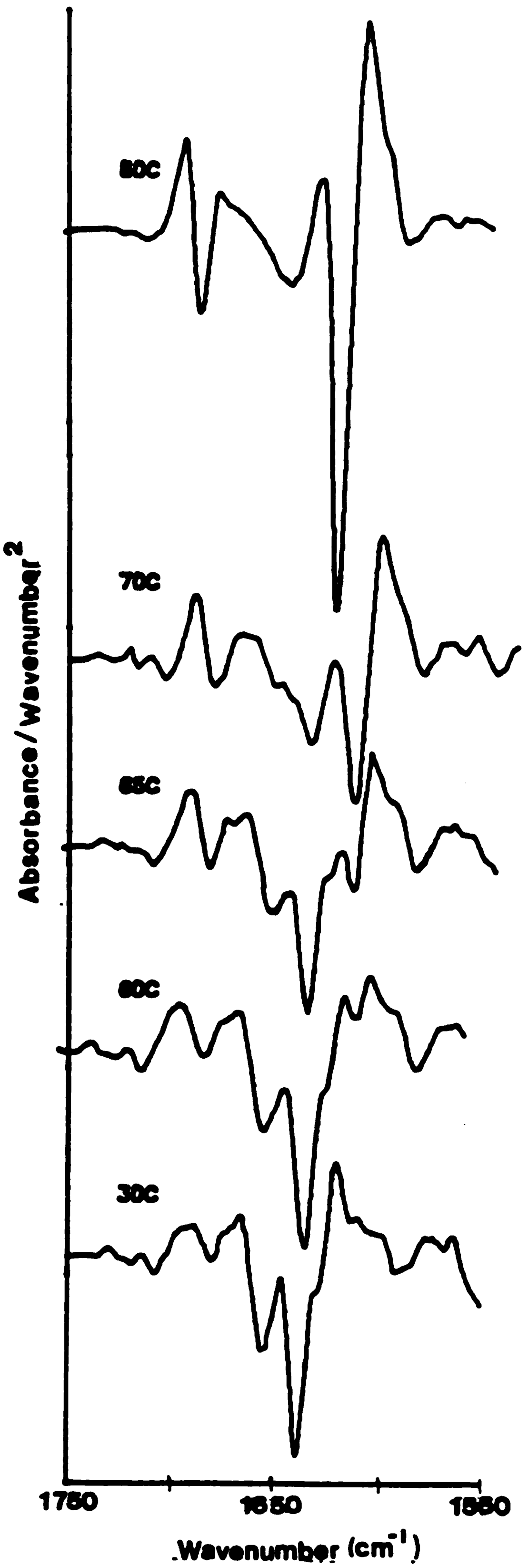

80C
70C
65C
60C
30C
Absorbance/Wavenumber$^2$
1750
1650
1550
Wavenumber (cm$^{-1}$)

**Fig. 8-Subtracted second dervative FT-IR absorption spectra of 1.75% S-ovalbumin in $D_2O$ (pD 7.0) and 25 mM $KD_2PO_4$ after different heat treatments for 30 min.**

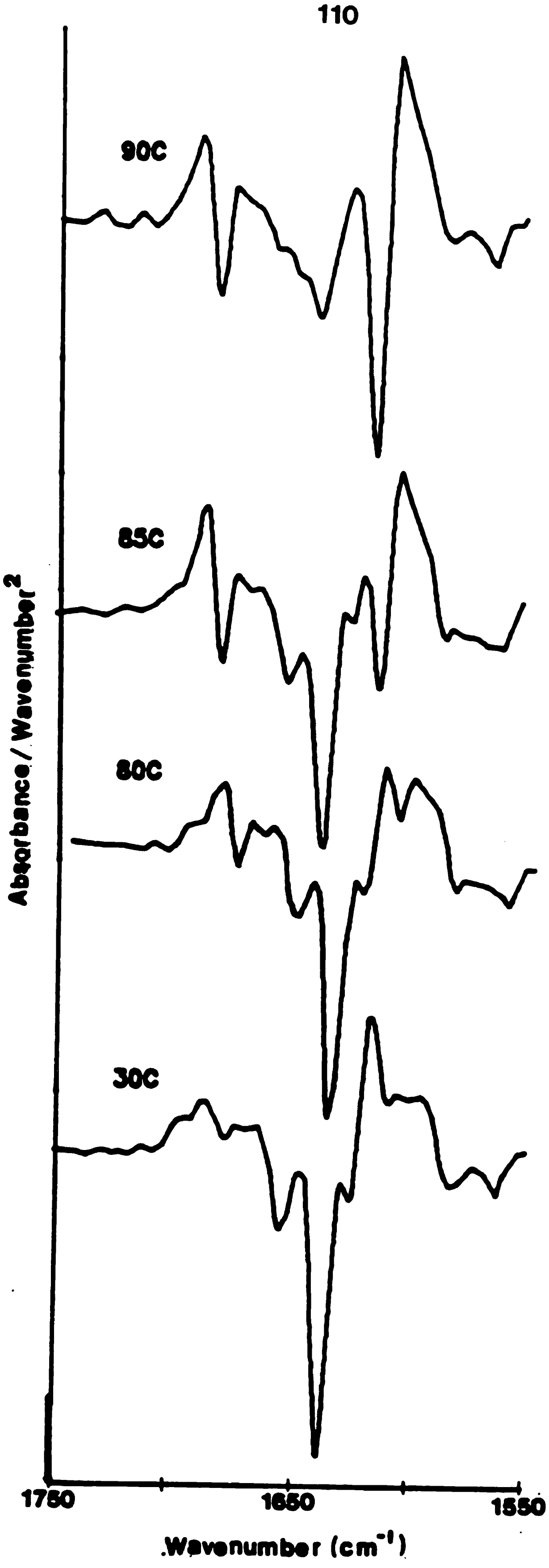
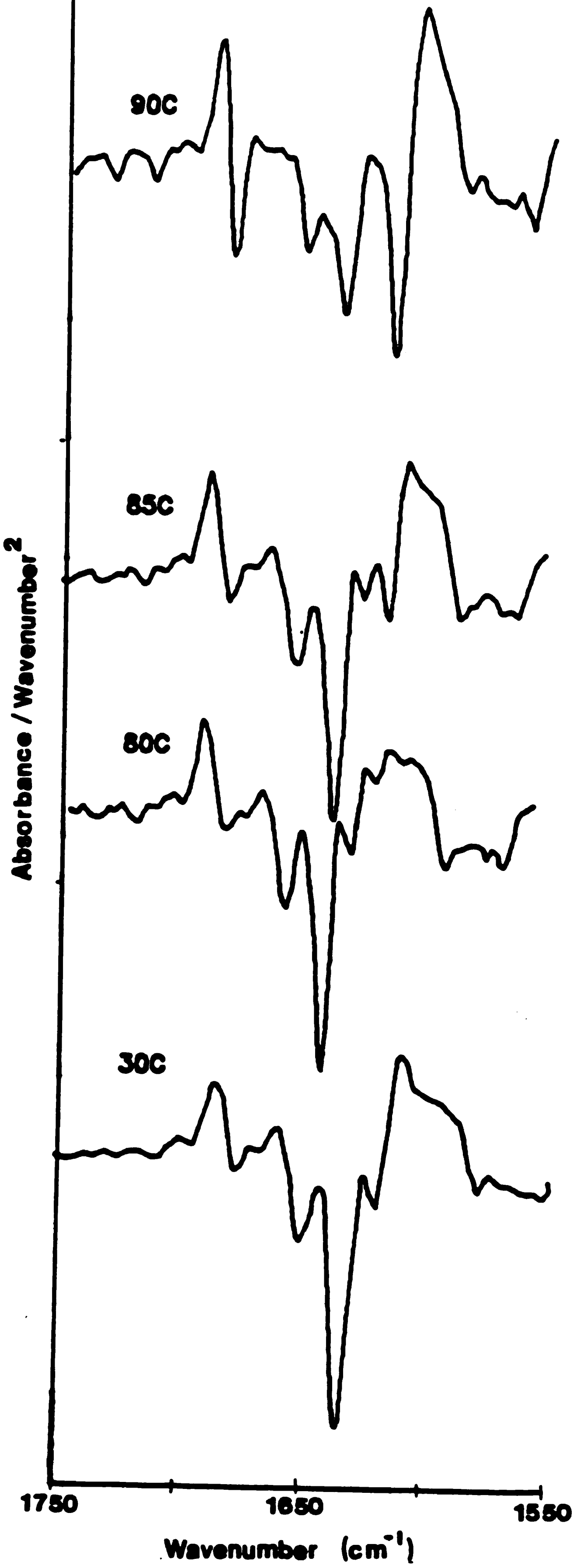

90C
85C
80C
30C
Absorbance / Wavenumber$^2$
1750
1650
1550
Wavenumber (cm$^{-1}$)

**Fig. 9-Subtracted second dervative FT-IR absorption spectra of 1.75% S-ovalbumin in $D_2O$ (pD 9.0) and 25 mM $KD_2PO_4$ after different heat treatments for 30 min.**


90C
85C
80C
30C
Absorbance / Wavenumber$^2$
1750
1650
1550
Wavenumber (cm$^{-1}$)

As the denaturation temperature of S-ovalbumin was approached the development of structure as determined by dynamic rheological testing was reported (Herald, 1991) and an increased intensity of the peaks at 1614 $cm^{-1}$ (intermolecular hydrogen bonding) and 1684 $cm^{-1}$ (turn) suggest intermolecular cross-linking between the intrmolecular hydrogen bond and turn. The turn at 1684 $cm^{-1}$ might be a type III which is a portion of a $3_{10}$-helix (Creighton, 1984). Therefore, the turn extentsion might be the result of the the $3_{10}$-helix uncoiling.

S-ovalbumin, pD 3.0, heated to 65°C exhibited an increase in band intensities at 1614.4 $cm^{-1}$ (intermolecular hydrogen bonding) and 1684.5 $cm^{-1}$ (type III turn) compared to pD 3.0 heated to 60°C. Peaks at 1637.2 $cm^{-1}$ ($3_{10}$-helix) and 1654.8 $cm^{-1}$ (α-helix ) decreased in intensity. No β-sheet structure was observed.

The spectrum of S-ovalbumin, pD 3.0, heated to 70°C exhibited an increased band intensity at 1617.3 $cm^{-1}$ as compared to the 65°C treatment indicating additional intermolecular hydrogen bonding. The quantity of 1640.2 $cm^{-1}$ ($3_{10}$-helix) and 1658.2 $cm^{-1}$ (α-helix) decreased. The 1658.2 $cm^{-1}$ peak decreased to a shoulder indicating uncoiling of helical structure. The absorption band 1684.4 $cm^{-1}$ (type III turn) increased in intensity compared to 65°C.

The pD 3.0 S-ovalbumin spectrum at 80°C was similar to the 70°C spectrum except a more intense bonds at 1615 $cm^{-1}$ (intermolecular hydrogen bonding) and 1685.5 $cm^{-1}$ (type III turn) was observed. The band at 1641.7 $cm^{-1}$ ($3_{10}$-helix) was broader and the band at 1657.8 $cm^{-1}$ ($\alpha$-helix) was not observed. Because of the broadness of the peak at 1641.7 $cm^{-1}$ smaller peaks representing disorder structure (1645 $cm^{-1}$) and $\alpha$-helix (1657.8 $cm^{-1}$) may be masked.

The thermal denaturation temperature of S-ovalbumin as determined by DSC at pH 3.0 was 80°C (Herald, 1991). Subsequently a decline in secondary structure due to uncoiling of $3_{10}$-helix and $\alpha$-helix at 80°C were observed.

Herald (1991) reported a storage modulus of 10 Pa (defined as rheologically significant) when S-ovalbumin at pH 3.0 was heated isothermally at 65°C for 15 min using dynamic rheological testing. Below 65°C no structure was evident. An increase in intermolecular hydrogen bonding and a decrease in $\alpha$-helix at 65°C correlate with dynamic rheological testing in measuring structure formation.

S-ovalbumin at pD 7.0 and heated to 80°C (Fig. 8) exhibited an increase in band intensity at 1613.4 $cm^{-1}$ (intermolecular hydrogen bonding) and 1681.6 $cm^{-1}$ (type III turn) at the expense of the bands at 1638.7 $cm^{-1}$ ($3_{10}$-helix), 1655.8 $cm^{-1}$ ($\alpha$-helix) and 1627.1 $cm^{-1}$ ($\beta$-sheet). The decrease in band intensity indicated unfolding of the $3_{10}$-helix, $\alpha$-helix and $\beta$-sheet for S-ovalbumin. A new band at 1670.9 $cm^{-1}$ was observed and was identified as a turn (Byler and Susi, 1988).

S-ovalbumin at pD 7.0 and heated to 85°C exhibited an increase in band intensity at 1613.9 $cm^{-1}$ (intermolecular hydrogen bonding) and 1682.5 $cm^{-1}$ (type III turn). Compared to the 80°C S-ovalbumin treatment the band intensities at 1627.1 $cm^{1-}$ ($\beta$-sheet), 1637.8 $cm^{-1}$ ($3_{10}$ -helix) and 1653.8 $cm^{-1}$ ($\alpha$-helix) remained unchanged.

S-ovalbumin at pD 7.0 and heated to 90°C showed an increased band intensity at 1614.9 $cm^{-1}$ (intermolecular hydrogen bond) and 1682.5 $cm^{-1}$ (type III turn) and the band at 1627.1 $cm^{-1}$ ($\beta$-sheet) was not observed. A decrease in band intensity at 1639.7 $cm^{-1}$ ($3_{10}$-helix) and 1657.7 $cm^{-1}$ ($\alpha$-helix) was observed compared to the 85°C treatment. A small shoulder at 1647.5 $cm^{-1}$ was observed and was assigned to unordered structure (Byler and Susi, 1988).

Increased intermolecular hydrogen bonding and turn at 85°C correlated with rheological structure development as detected by isothermal and temperature ramping methods (Herald, 1991). Thermal denaturation temperature of S-ovalbumin as determined by DSC at pH 7.0 was 90.6°C. Uncoiling of $3_{10}$-helix, $\alpha$-helix, $\beta$-sheet and formation of unordered structure occurred during thermal denaturation. Intermolecular hydrogen bond formation occurred before thermal denaturation of ovalbumin at pH 7.0 as determined by DSC (Herald, 1991) suggesting structure formation occurred before thermal denaturation. Herald (1991) reported that temperature in which S-ovalbumin structure was formed as measured by dynamic rheological testing occurred before thermal denaturation temperature. This is supported by FT-IR in which changes in secondary structure occurred before $T_d$.

S-ovalbumin, pD 9.0, heated to 80°C exhibited a new band at 1614.4 $cm^{-1}$ associated with intermolecular hydrogen bonding (Byler and Purcell, 1989). A slight decrease was observed in the band at 1653.8 $cm^{-1}$ (α-helix) and no changes in band intensities were exhibited at 1627.1 $cm^{-1}$ (ß-sheet), 1636.8 $cm^{-1}$ ($3_{10}$-helix), 1670.9 $cm^{-1}$ (turn) and 1680.6 $cm^{-1}$ (type III turn).

S-ovalbumin, pD 9.0, heated to 85°C an increase in band intensity at 1614.9 $cm^{-1}$ (intermolecular hydrogen bonding increased). No other changes were exhibited between the 80°C and 85°C treatments.

S-ovalbumin at pD 9.0 and heated to 90°C exhibited conformational changes compared to the 85°C treatment. Increase in band intensity 1615.4 $cm^{-1}$ (intermolecular hydrogen bonding) and 1683.5 $cm^{-1}$ (type III turn) were observed. The band intensity at 1636.3 $cm^{-1}$ ($3_{10}$-helix) and 1653.8 $cm^{-1}$ (α-helix) decreased. The bands at 1624.6 $cm^{-1}$ (ß-sheet) and 1670.9 $cm^{-1}$ (turn) were not present.

Herald (1991) reported 8% S-ovalbumin heated at 80°C for 15 min had a G' of 25 Pa at pH 9.0 using dynamic rheological testing. Both dynamic rheological testing and FT-IR support the theory that conformational changes occur before structure development takes place as indicated by increase in intermolecular hydrogen bonding and an increase in G'. Thermal denaturation temperature of S-ovalbumin as determined by DSC at pH 9.0 was 90.6°C (Herald, 1991). Therefore, uncoiling of $3_{10}$-helix, $\alpha$-helix and loss of $\beta$-sheet were necessary before thermal denaturation occurred. The most stable of all pD treatments observed was pD 9.0 as indicated by the amount of original structure left after heating at 90°C.

At 30°C native S-ovalbumin's secondary structure did not change at pD 3.0, 7.0 or 9.0 as measured by FT-IR. Conformational changes were observed after heating suggesting pD alone does not influence secondary structure of S-ovalbumin. At pH 9.0 more $3_{10}$-helix and $\alpha$-helix were retained than either at pD 3.0 or pD 7.0 suggesting a more stable environment.

S-ovalbumin activation energy ($E_a$) calculated from DSC data was less at pH 3.0 than at pH 7.0 or pH 9.0 (Herald, 1991), suggesting that energy needed to drive the reaction from the native to denatured state and temperature dependence of the reaction was over lower at pH 3.0. S-ovalbumin at pD 3.0 did not contain $\beta$-sheet at 30°C and there was a decrease in intensity of $3_{10}$-helix at 80°C compared at pD 7.0 or 9.0. Therefore, $E_a$ can be related to conformational changes of S-ovalbumin due to pD.

## Effect of Denaturants on Secondary Structure

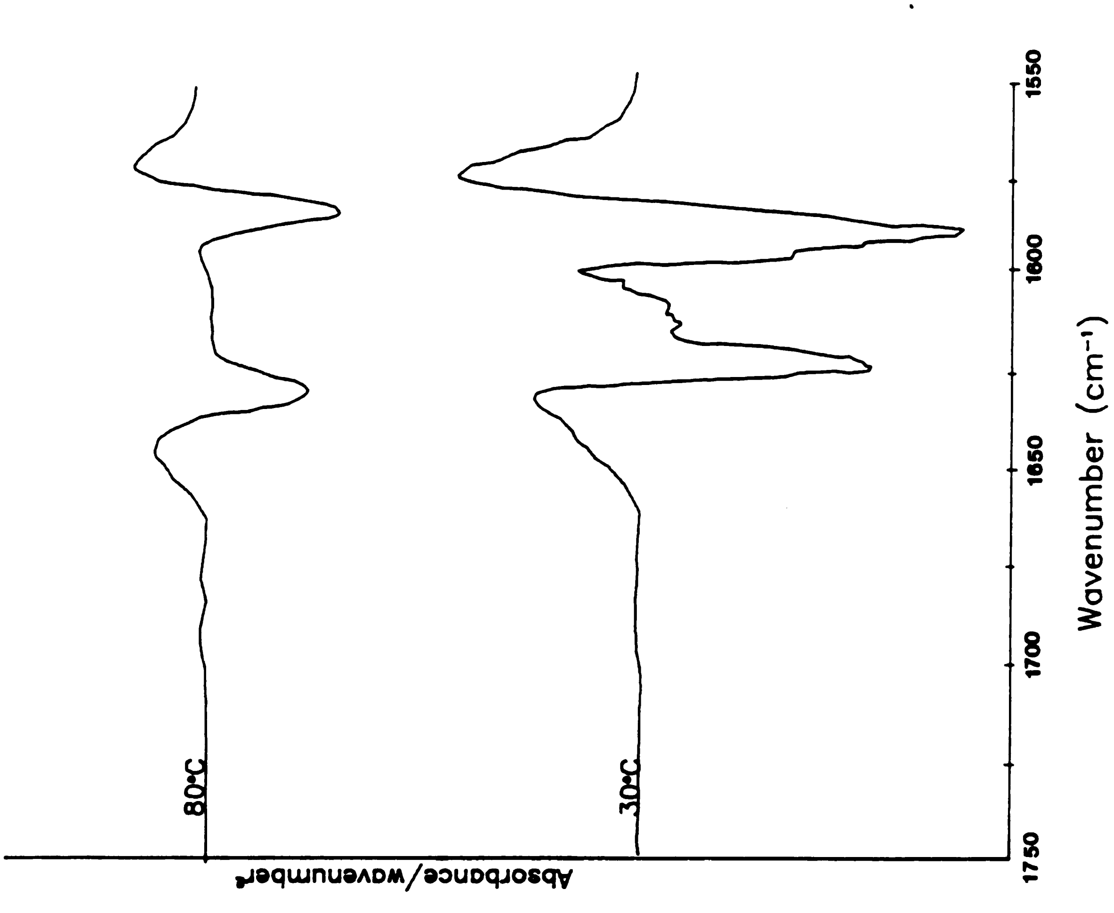
Second derivative FT-IR spectrum of S-ovalbumin at 30°C in 2.0M GuHCl (Fig. 10) exhibited one band at 1624 $cm^{-1}$ inthe Amide I region. This band was attributed to ß-sheet based on model systems (Susi and Byler, 1983). One other band at 1582 $cm^{-1}$ outside the Amide I region was observed. The $3_{10}$-helix, α-helix and turn structure were not observed in the 2.0M GuHCl ovalbumin sample as compared to the native ovalbumin, suggesting that GuHCl disrupted most of the secondary structure of native S-ovalbumin.

There was a decrease in intensity in the second derivative FT-IR spectrum of ovalbumin heated to 80°C in 2.0M GuHCl. A peak at 1628 $cm^{-1}$ was exhibited in the Amide I region. Temperature influenced the S-ovalbumin FT-IR spectrum in the presence of 2.0M GuHCl. The few small peaks that are observable are believe to be incomplete H-D exchange between $D_2O$ and GuHCl.

## Conclusion

The present study suggested that FT-IR using second derivative analysis can be used to follow changes in secondary structure of ovalbumin. Changes in ovalbumin

Fig. 10-Subtracted second dervative FT-IR absorption spectra of 1.75% S-ovalbumin in $D_2O$ (pD 7.0), 25 mM $KD_2PO_4$ and 2.0M guanidine hydrochloride after different heat treatments for 30 min.


80°C
30°C
Absorbance/wavenumber²
1750
1700
1650
1600
1550
Wavenumber (cm⁻¹)

conformation using FT-IR occurred prior to structural changes reported using DSC and dynamic rheological testing. Unfolding of S-ovalbumin was related to thermal denaturation temperatures and an increase in storage modulus at different isothermal temperatures.

# VI. STUDY 4

## Effects of pH, Salt Type and Denaturants on the Microstructure of Hen Egg S-Ovalbumin as Determined by Scanning Electron Microscopy

### ABSTRACT

Changes in microstructure of heat-induced hen egg S-ovalbumin gels influenced by pH (3.0, 7.0, 9.0), salt type (NaCl, NaI, $Na_2SO_4$) and denaturants (guanidine hydrochloride and ß-mercaptoethanol) were studied using low temperature scanning electron microscopy (LTSEM) and chemical fixation procedures with scanning electron microscopy (SEM). The LTSEM of 8% S-ovalbumin gels were honey comb in appearance, while ovalbumin gels prepared by chemical fixation exhibited a microstructure of grape-like clusters. S-ovalbumin gels prepared at pH 3.0 or in 0.5M $Na_2SO_4$ exhibited the smallest pore size as viewed using LTSEM. Using SEM the largest void volumes exhibited by the S-ovalbumin gels were prepared at pH 3.0 or 0.5M NaI.

# INTRODUCTION

Protein-protein interactions are responsible for providing structural integrity to gel systems, while entrapping other food ingredients such as water and flavors (Arntfield et al., 1990 a). Ovalbumin is the major egg white protein and is responsible for structure development in egg containing products. Herald (1991) characterized ovalbumin properties due to heating and perturbation with pH, salt type and denaturants. The researcher reported that structure development, unfolding and secondary structure were all influenced by perturabation of environmental conditions.

Scanning electron microscopy (SEM) has provided information on microstructure of heat-induced egg white gels that assist in interpreting textural characteristics of food systems (Stanely and Tung, 1976; Heertje and van Kleef, 1986). Microstructure can illustrate the effect of attractive and repulsive molecular forces on the formation of the protein gel network (Heertje and Van Kleef, 1986) by visual analysis of pore size, strand thickness and shape (Clark et al., 1981). Ferry (1948) suggested that an optimal pH balance between attractive and repulsive forces would provide a uniform gel matrix possessing high gel strength and optimum water-binding properties. Beyond optimal pH repulsive forces may be too strong resulting in

1980). Harte (1989) reported the balancing of attractive and repulsive forces were responsible for an optimal ovalbumin network at pH 6.0 and 7.0.

Although SEM micrographs of egg white gels have been published using chemical fixation and critical point drying (CPD) little information is available on the low temperature scanning electron microscopy (LTSEM) method. The LTSEM procedure is less time-consuming, decrease induced artefacts associated with chemicals and reduces specimen shrinkage (Sargent, 1988). Freeman and Shelton (1991) and Sargent (1988) have reviewed the advantages of LTSEM for determining ultrastructure of food products.

The objectives of this investigation were (1) to compare chemical fixation and low temperature methods for ovalbumin gels examined by SEM and (2) to evaluated network structure of heat-induced ovalbumin gels prepared under different conditions by varying pH, salt type, and denaturants.

## MATERIALS AND METHODS

### Materials

Pure ovalbumin (grade V, lot 19F 8105) was purchased from Sigma Chemical Co.( St. Louis, MO) and used without further purification. Ovalbumin was prepared at a concentration of 80 mg/ml in the following aqueous environments (1) pH 3.0, 7.0 and 9.0 in 0.5M NaCl, (2) 0.5M environments (1) pH 3.0, 7.0 and 9.0 in 0.5M NaCl, (2) 0.5M NaI, NaCl and $Na_2SO_4$ at pH 7.0 (3) 0.5M, 1.0M and 2.0M guanidine hydrocholride (GuHCl) at pH 7.0 and 0.5M NaCl (4) 0.5%, 1.0%, 2.0% or 3.0% β-mercaptoethanol (β-ME) at pH 7.0 and 0.5M NaCl. All samples were rechecked after 30 min to ensure pH stability. The ovalbumin solutions were deaerated prior to heating at 80°C for 30 min. The 80°C temperature was selected based on the other studies used in the dissertation. Two representative micrographs of each sample were obtained for comparison.

## Electron Microscopy

Two scanning electron microscopy techniques were used to examine ovalbumin gel morphology. The first was LTSEM that involved examination of ovalbumin gels at low temperature and high vaccuum. The second technique involved chemical fixation in which four methods of ovalbumin gel preparation were compared (1) gluteraldehyde (2) osmuim tetroxide (3) osmium-thiocarohydrazide osmium (OTO) (4) osmium-tannic acid-uranyl acetate (OTU).

## LTSEM

A portion of S-ovalbumin gel (less than 5 $mm^2$) was mounted on a copper stub and submerged into a nitrogen slush maintained at -190°C to -160°C and held under vacuum of $10^{-2}$ - $10^{-3}$ Torr (EMScope SP-2000 Sputter-Cryo, Ashford, Kent, sample was then transferred to the preparation chamber ( < -160°C) and carefully fractured using a knife maintained at the same low temperature. The ovalbumin gel was transferred to the cold stage of the SEM (Model JSM-35C, Japan Electron Optics Limited, Tokyo, Japan) and etched for approximately 8 min at -65°C to remove the top layer of water which, may obscure surface detail. After the ovalbumin gel temperature was lowered to -165°C, it was transferred back to EMScope SP2000 Sputter-Cryo and gold sputter-coated at 40 mA for 6 min. The prepared ovalbumin gel was transferred to the cold stage of the microscope and viewed using a 15kV beam, working distance of 39 mm and condenser lens setting of 600

### Chemical Fixation Methods

**(1) Glutaraldehyde fixation method.** Portions of ovalbumin gel (5 mm x 2 mm) were cut with a razor blade and fixed for 6 hr in 2% glutaraldehyde in 0.1M $Na_2PO_4$ buffer pH 6.0
**(2) $OSO_4$ procedure** (Beveridge and Ko, 1984) ovalbumin gels were fixed in gluteraldehyde then postfixed in 1% $OSO_4$ for 2 hr at 23°C **(3) OTO procedure** (Woodward and Cotterill, 1985) Ovalbumin gels were fixed in gluteraldehyde and were post-fixed in 1% $OsO_4$ for 2 hr at 23°C. Samples were rinsed three times in deionized water ($dH_2O$) and placed in 1% thiocarbohydrazide solution for 30 min. After rinsing six times in $dH_2O$, samples were placed in 2% $OsO_4$ for 1 hr

**(4) OTU procedure** (Woodward and Cotterill, 1985) S-ovalbumin gels fixed in glutaraldehyde and post-fixed in $OsO_4$ were washed in $dH_2O$ and placed in 1% tannic acid for 1 hr at 23°C. After three rinses with $dH_2O$, S-ovalbumin gels were placed in 0.5% uranyl acetate for 1 hr at 23°C and then rinsed again.

Following fixation using one of the four above mentioned procedures, all samples were stored overnight in $dH_2O$ at 4°C and dehydrated sequentially in 25%, 50%, 75%, 95% and three changes of 100% ethanol (v/v) 15 min/step. Fixed S-ovalbumin gels were placed in critical point drying (CPD) baskets in 100% ethanol and then transferred immediately to the CPD chamber (Balazer FL 9496 Critical Point Dryer, Balzer's Union, Furstentum, Liechtenstein, West Germany) and dried using $CO_2$ as the transitional fluid. Following CPD with $CO_2$, the fixed and dried S-ovalbumin gels were mounted to aluminum mounts (Electron Microscopy Sciences, Ft. Washington, PA). A fine line of Conducting Graphite Paint (Ladd 60780, Burlington, VT) was applied from the edge of the sample over the edge of the metal stub to prevent charging. The mounted samples were sputter coated with gold (Emscope SC 500, Emscope, Ashord, Kent, England) under a vacuum of 0.006 Torr for 6 min at 30 mA. Dried and gold coated samples were stored in a vacuum desiccator jar. Gels were examined at 10,000 X magnification in a JEOL JSM-35 scanning electron microscope equippied with a tungsten electron gun at 15 kV accelarating voltage. Working distance was 15 mm with a condenser lens of 600. Micrographs were made using Polaroid film (665P/N, Polariod Corp., Cambridge, MA).

## RESULTS AND DISCUSSION

### LTSEM

At 2000 X magnification the S-ovalbumin gel network at pH 3.0, 7.0 and 9.0 exhibited a honey comb appearance (Fig. 11). The interspaces of ovalbumin gel at pH 9.0 (Fig. 11c) were 15 um compared to 10 um in diameter for pH 3.0. The large interspaces between cells might be explained by ice crystal formation (Harte, 1989). Davis and Gordon (1984) decreased the interspaces by adding 30% surcrose to a 10% collagen gel providing a cryoprotectant effect. The researchers noted that caution should be taken when evaluating gel microstructure in the presence of a cryoprotectant. Harte (1989) reported similar structure with 5% ovalbumin gels at pH 7.0. Herald (1991) reported the storage modulus (G') of S-ovalbumin decreased with increased pH suggesting that at higher pH S-ovalbumin gels were weak and that less structure development had occurred. Therefore, this accounts for a more open microstructure at pH 9.0 compare to pH 3.0.

The S-ovalbumin gel prepared in 0.5M NaI (Fig. 12a)

Fig. 11-The use of low temperature scanning electron microscopy in evaluating the influence of pH on the microstructure of 8% (w/v) S-ovalbumin gel in 0.5M NaCl heated at 80°C for 30 min A. pH 3.0 B. pH 7.0 C. pH 9.0.

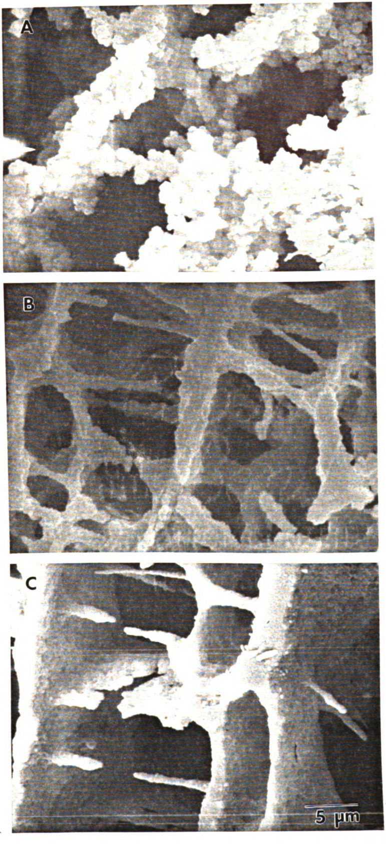
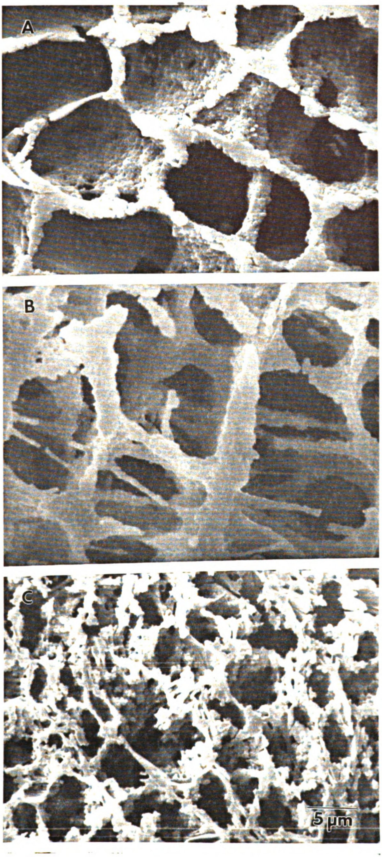

A
B
C
5 µm

**Fig. 12-The use of low temperature scanning electron microscopy in evaluating the influence of denaturants on the microstructure of 8% (w/v) S-ovalbumin gel at pH 7.0 heated at 80°C for 30 min A. 0.5M NaI B. 0.5M NaCl C. 0.5M $Na_2SO_4$.**


A
B
C
5 µm

exhibited the same honey comb structure as 0.5M NaCl (Fig. 11b), although the pore size was larger. The 0.5M $Na_2SO_4$ (Fig. 12c) S-ovalbumin gel structure was denser with smaller diameter strands than either gel prepared in 0.5M NaCl or 0.5M NaI. The microstructure suggested that the stabilizing salt ($Na_2SO_4$) produced a weaker protein network than the destabilizing salt types under the conditions measured. Ovalbumin gels compared at pH 7.0 in 2.0M GuHCl (Fig. 13a) or 3.0% ß-ME (Fig. 14b) at 2000 X magnification exhibited similar network structure. Therefore, it was difficult to compare the effects of denaturants on the microstructure of S-ovalbumin at 2000 X magnification.

### Comparison of Chemical Fixation Techniques

S-ovalbumin gels fixed in gluteraldehyde did not exhibit grape-like clusters as described by Clark et al. (1981) but is composed of small globular aggregates. S-ovalbumin gels prepared in glutaraldehyde appeared ruptured (Fig. 14a). This may have been due to high pressure imposed by CPD procedure that can shrink and collapse the spherical structure of the egg protein molecules (Woodward and Cotterill, 1986). The gluteraldehyde fixation procedure produced a S-ovalbumin gel which was fragile to handle therefore difficult to mount onto stubs. The $OsO_4$, OTO and OTU fixations provided good

Fig. 13-The use of low temperature scanning electron microscopy in evaluating the influence of denaturants on the microstructure of 8% (w/v) S-ovalbumin gel at pH 7.0 in 0.5M NaCl heated at 80°C for 30 min A. 2.0M guanidine hydrochloride B. 3.0% β-mercaptoethanol.

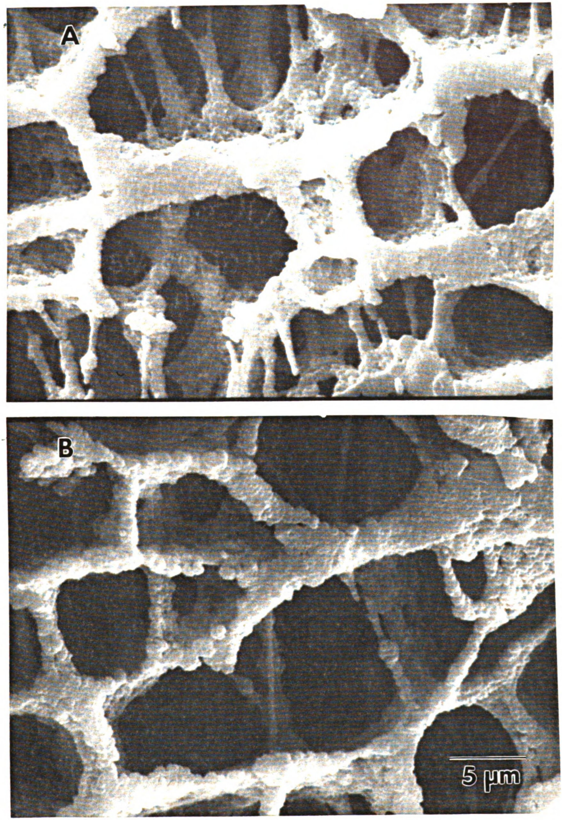
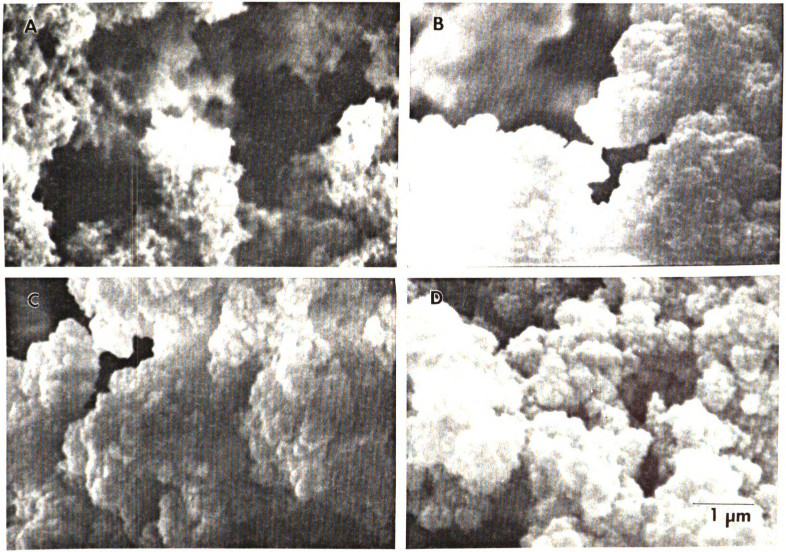

A
B
5 µm

**Fig. 14-Comparison of scanning electron microscopy chemical fixation techniques of 8% (w/v) S-ovalbumin gel at pH 7.0 in 0.5M NaCl heated at 80°C for 30 min A. gluteraldehyde B. osmium tetroxide C. osmium-thiocarbohydrazide-osmium D. osmium-tannic acid-uranyl acetate.**


A
B
C
D
1 µm

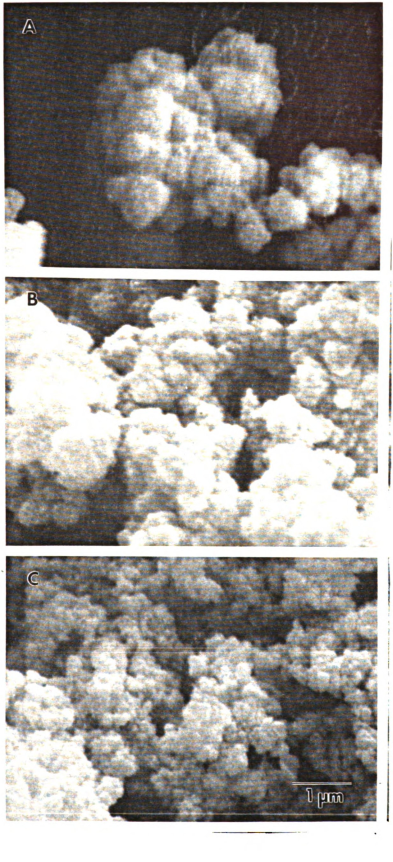
surface definition of the grape-like clusters (Fig. 14 b, d and e). The OTU prepared S-ovalbumin gel did not crumble as easily compared to other fixation methods (Fig. 14 e) therefore, it was the method of choice. Woodward and Cotterill (1985) reported 50% shrinkage of gluteraldehyde fixed egg white gels during CPD with pores size ranging from 0.1 to 0.2 um in diameter. Egg white gels fixed in OTU had pore size ranging from 0.1 to 0.8 um. Wollweber et al. (1981) reported treatment of peritoneal cells in OTU caused 5% shrinkage. Woodward and Cotterill (1985) attributed the enhanced structural stability of ovalbumin gel to the cross-linking of tannic acid with $OsO_4$ and uranyl ions.

## Effect of pH on Microstructure

Surface morphology of 8% ovalbumin in 0.5M NaCl at pH 3.0, 7.0 and 9.0 were compared (Fig. 15 a, b and c, respectively). Large irregular void areas, up to 6 um across were observed in ovalbumin gels at pH 3.0. The pI of ovalbumin is 4.5 therefore, at pH 3.0 electrostatic repulsion of the protein molecules resulted in large voids between clusters. Ovalbumin aggregates at pH 3.0 and 7.0 were approximately 0.5 um across while gel clusters at pH 9.0 were approximately 0.25 um indiameter. The large void areas exhibited in the S-ovalbumin gel at pH 3.0 were not

Fig. 15-Microstructure of 8% (w/v) S-ovalbumin gel in 0.5M NaCl heated at 80°C for 30 min prepared using the osmium-tannic acid-uranyl acetate fixation procedure A. pH 3.0 B. pH 7.0 C. pH 9.0.

in
he osmiu-
pH 3.0



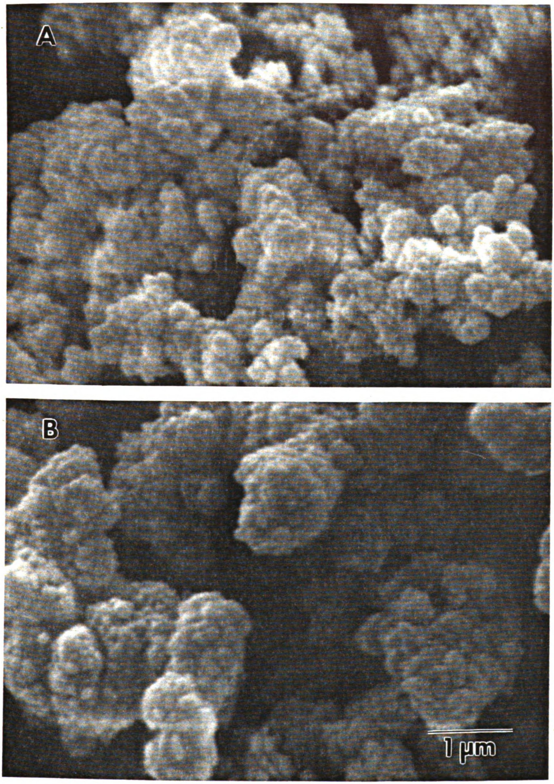
found at pH 7.0 and 9.0. At pH 7.0 and 9.0 S-ovalbumin molecules were tightly clustered together leaving small irregular voids. The decrease in large void areas exhibited at pH 7.0 and 9.0 were due to the balance of attractive and repulsive forces. The storage modulus (G') at pH 3.0 was larger than at pH 7.0 or 9.0 (Herald, 1991). Indicating the area between aggregates at pH 3.0 allow for more elastic behavior than exhibited at pH 7.0 or 9.0. Woodward and Cotterill (1986) a fine structured gel at pH 9.0 in 1.0M NaCl with numerous globules 0.1 to 1.2 um in size that were interconnected by fine protein strands and small and evenly distributed pores throughout the gel matrix. Harte (1989) described different sized grape-like clusters and void areas as non-homogeneous, in 5% ovalbumin gels at pH 6.0 and 7.0. Heertje and van Kleef (1986) suggested ovalbumin gelation at pH 5.0 occurred because of the presence of large aggregates that formed networks. Montejano et al. (1984) reported spherical particles in micrographs of low pH gels might be caused by random aggregation that resulted in non-homogenous structure.

Arntfield et al. (1990b) used light microscopy to relate 10% ovalbumin gel microstructure to G'. The researcher observed that at pH 5.0 and 6.0 crosslinking was absent, therefore there was an increase in protein-solvent interaction. At pH 3.0 there was evidence of strand like structures that were not well cross-linked. Arntfield et al. (1990 b) suggested that an increase in G' has been attributed to increased crosslinking with the network.

## Effect of Salt Type on Microstructure

The ovalbumin gel prepared in NaI was more compact and had smaller grape-like clusters (0.5 um) compared to the gel prepared in $Na_2SO_4$ (1.0 um) (Fig. 16 a and b, respectively). The ovalbumin gel prepared with NaCl contained different size clusters ranging from 0.5 to 1.0 um across (Fig. 14 c). The G' at 80°C for 15 min NaI was larger than for NaCl and $Na_2SO_4$ suggesting the hydrophobic interaction was decreased causing an increased cross-linking. Woodward and Cotterill (1986) compared micrographs of egg white gels with and without salt at pH 9.0. Gels prepared in 1.0M NaCl contained aggregated proteins, resulting in large particles that were clustered tightly together, leaving irregular voids. Gels prepared in a salt free environment were coarser and less aggregated than gels prepared in 1.0M NaCl. Hegg et al. (1979) reported thermal aggregation of 4.4% ovalbumin in a salt free environment only took place around the pI indicating the number of net charges on ovalbumin determines aggregation. Harte (1989) observed round spherical aggregates in ovalbumin gels containing 3.0% NaCl supporting the hypothesis of electrostatic interaction. Using a phosphate buffer gel system at pH 6.0, Harte (1989)

**Fig. 16-Microstructure of 8% (w/v) S-ovalbumin gel at pH 7.0 heated at 80°C for 30 min prepared using the osmium-tannic acid-uranyl acetate fixation procedure A. 0.5M NaI B. 0.5M $Na_2SO_4$.**


A
B
1 µm

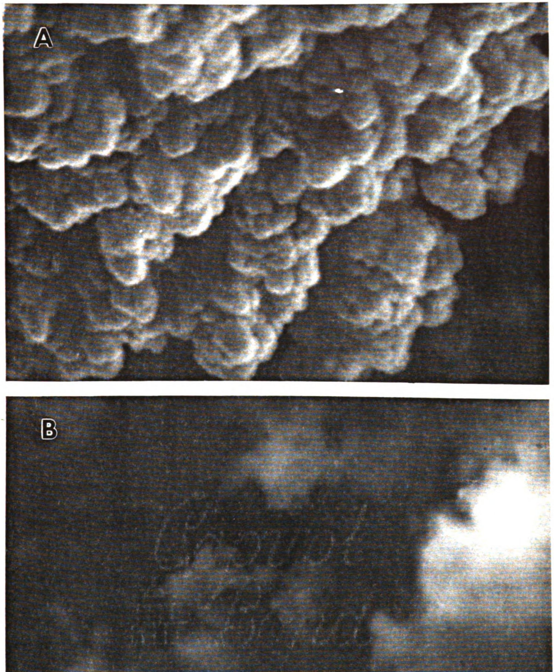
observered no difference between ultrastructure of 5.0% ovalbumin in 3.0% NaCl and without salt using OTO, CPD procedure. Hegg et. al. (1979) and Hatt et al. (1986) reported the addition of 170 mM NaCl shielded repulsive charges, thus promoting protein aggregation.

Arntfield et al. (1990 c) reported that 10% ovablumin gel at pH 8.5 in 0.5M NaCl formed well cross-linked strands whereas ovalbumin gels at pH 8.5 in 0.5M $Na_2SO_4$ exhibited little evidence of cross-linking when observed using a light microscopy. The authors reported the relationship between the storage modulus and the position of a salt in the lytropoic series at a concentration of 0.5M provided evidence for the involvement of hydrophobic interactions as factors in determining the strength of heat-induced ovalbumin networks.

### Effect of Guanidine Hydrochloride on Microstructure

Ovalbumin gels prepared in 0.5M and 1.0M GuHCl (Fig. 17a) exhibited grape-like aggregates of approximately 1 um in diameter. The ovalbumin gel microstructure was composed of compact aggregates with irregular interstitial spaces. The 2.0M GuHCl ovalbumin gel was amorphous in structure (Fig. 17b) probably due to unfolding of ovalbumin molecules due to disruption of hydrogen bonds. The amorphous structure exhibited the ability of ovalbumin to

Fig. 17-Microstructure of 8% (w/v) S-ovalbumin gel at pH 7.0 in 0.5M NaCl heated at 80°C for 30 min prepared using the osmium-tannic acid-uranyl acetate fixation procedure
A. 1.0M guanidine hydrochloride B. 2.0M guanidine hydrochloride.


A
B
1 µm

cross-link and increase the G' of the ovalbumin gel compared to lower GuHCl concentrations (Herald, 1991). Amphorous microstructure due to GuHCl has not been reported before in literature. Heertje and van Kleef (1986) investigated the effects of urea at pH 10 (that was attributed to disruption of non-covalent bonds) and found an amphorous, homogeneous and non-cellular structure.

### Effect of β-Mercaptoethanol on Microstructure

S-ovalbumin gels prepared in 1.0% and 2.0% β-ME (Fig. 18a and b) were similar to each other in grape-like aggregate structure (approximately 1.2 um across) and irregular void area. The control had a denser microstructure than all β-ME containing treatments. The 3.0% ME (Fig. 18c) treatment was similar to 2.0M GuHCl in that an amorphous structure rather than the grape-like cluster structures were formed. Herald (1991) reported that as β-ME concentration increased G' for S-ovalbumin increased, however changes in surface morphology was exhibited only at 3.0% β-ME.

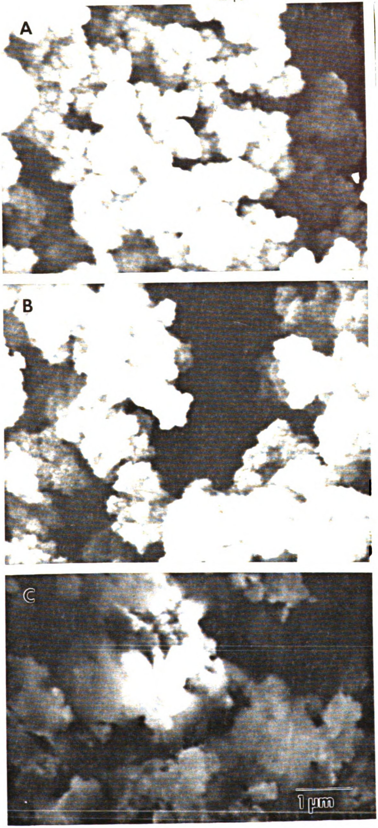
### Conclusion

Changes in S-ovalbumin gel pores sizes and strand diameter were related to gel structure. A smaller pore size and larger diameter strands were associated with a higher storage modulus and a lower thermal denaturation temperature.

Fig. 18-Microstructure of 8% (w/v) β-ovalbumin gel at pH 7.0 in 0.5M NaCl heated at 80°C for 30 min prepared using the osmium-tannic acid-uranyl acetate fixation procedure A. 1.0% β-mercaptoethanol B. 2.0% β-mercaptoethanol C. 3.0% β-mercaptoethanol

B. 0.5M $Na_2SO_4$.

pH 7.0
g the
. 1.0%
0% β-



# VII. SUMMARY AND CONCLUSIONS

## Study I.

This study used DSC and dynamic rheological testing to characterize ovalbumin gelation as a function of pH and salt type. At pH 3.0, thermal denaturation and structure develoment temperatures were reduced compared to pH 7.0 or pH 9.0. Below the pI the thermal denaturation temperature declined due to electrostatic replusion of the ovalbumin molecules causing unfolding at a lower temperature. At pH 3.0 there might be some aggregation, therefore decreasing the temperature of structure development. Denaturation enthalpy determined by DSC may be influenced by the high concentration of ovalbumin (8.0%) used. As a decrease in enthalpy may be the result of S-ovalbumin aggregation.

Inclusion of 0.5M NaI to 8% ovalbumin at pH 7.0 decreased thermal denaturation and structure development temperatures. The decrease in thermal stability suggest a reduction in water structure and an increase in exposed hydrophobic residues that were able to cross-link.

The results suggest that pH and salt type are a significant factor in the thermal behavior of ovalbumin as measured by DSC and dynamic rheological testing. By manipulating pH and salt type a desired textural characteristics can be obtained under specific conditions of processing.

This study used DSC and dynamic rheological testing to characterize thermal behavior of ovalbumin as a function of GuHCl and ß-ME concentration. Increasing concentrations of either denaturant decreased the thermal stability of 8% (w/v) S-ovalbumin at pH 7.0 in 0.5M NaCl by either disrupting hydrogen bonds or reducing the disulfide bond. Using denaturants helped to provide information on structural changes occurring during thermal processing and their influence on the storage modulus or denaturation temperature.

**Study III.**

This study used second derivative FTIR spectra to monitor changes in S-ovalbumin solution as a function of pD and temperature. Three bands and 2 shoulders representing ß-sheet, $3_{10}$-helix, α-helix and turn were observed at 30°C. Type III turn and intermolecular hydrogen bonding increased at the expense of both helicies and ß-sheet.

Conformational changes in S-ovalbumin such as a decrease in $3_{10}$-helix and an increase in type III turn related to denaturation temperatures and temperatures of structure formation as measured by DSC and dynamic rheological testing, respectively.

Using FT-IR to monitor secondary structure has provided an addition tool to the already established DSC and dynamic rheological testing methods. Research can now be done to relate specific conformations to functinal attributes that will aid food processor during manufacturing.

**Study IV.**

This study showed that LTSEM micrographs of S-ovalbumin gels exhibited honey comb structures compared to grape-like cluster shown for the chemical fixation procedures. LTSEM preparation and viewing of specimens was faster than the chemical fixation methods.

A trend was observed when comparing the LTSEM method to the different S-ovalbumin gel treatments at 80°C. When the S-ovalbumin gel microstructure exhibited thick strands and small pore sizes the G' was greater as compared to thin strands and large pore sizes. The thin strands and large pore sizes were weaker due to the more heat stable environment provide by pH 9.0 or $Na_2SO_4$. The OTU method was not as conclusive in relating S-ovalbumin microstructure to G' due to similar grape like cluster sizes and void areas.

Microscopy contributed physical meaning to ovalbumin gelation. However, more work must be done in order to understand the effects of fixation procedures on gel microstructure.

# VIII. RECOMMENDATION FOR FUTURE RESEARCH

1. Study the influence of prosthetic groups on ovalbumin secondary structure. Monitor interaction between the prosthetic group and polypeptide chain such as:
   - a. isolate each ovalbumin variant ($A_1$, $A_2$ and $A_3$) and compare secondary structure.
   - b. remove mannose groups one at a time and compare secondary structure.
2. Use modeling and FT-IR to determine if further differences between ovalbumin and S-ovalbumin can be discerned.
3. Compare structure-function relationship between ovalbumin and other members of the serpin family.
4. Use N-ethlylmaleimide (NEM) to block S-S formation during heating and monitor conformational change. Determine if partial or total loss of S-S formation has any significant influence on functionality (solubility or texture) and relate to changes in secondary structure.
5. Study the effects of protein concentration on activation energy and reaction order and note if differences can be observed between aggregation versus denaturation.
6. Study the effect of frozen storage of ovalbumin on structure function relationship. Does low temperature

change ovalbumin conformation that correlates to specific protein functions within food systems such as texture or solubility.

7. Elucidate the presence of a $3_{10}$-helix or ß-sheet at 1638 $cm^{-1}$ by determining the number of psi, phi and omega angels of the 3-dimensional ovalbumin structure.

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