STRUCTURAL ANALYSIS OF PROTEINS IN
THERMALLY INDUCED OVALBUMIN
AND EGG WHITE GELS

by

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We accept this thesis as conforming
to the required standard

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Abstract

Hen egg white proteins have been extensively utilized as ingredients in food processing because of their high nutritious value and unique functional properties. The ability to form a gel upon heating makes them key ingredients in many food products. Three factors including pH, ionic strength and protein concentration play very important roles in determining the structure and thus the properties of egg white gels.

In this project, the relationship between the structure and functional properties of pure ovalbumin and egg white before and after gelation under different pH and ionic strength conditions were studied. Egg white is a complicated system containing many different kinds of proteins, among which ovalbumin is a major component. Therefore, the structure and function relationships of thermally induced pure ovalbumin gels were studied first as a representative of egg white gelation.

Raman spectroscopy was the major technique used in this project to study the protein structure changes of ovalbumin and egg white after thermal gelation. Other structure and functional properties of ovalbumin and egg white proteins before and after heat-gelation were also studied, including gel strength, turbidity and solubility, as well as the charges of protein molecules, interchange between SH groups and SS bonds and protein hydrophobicity difference before and after heating.

Depending on the conditions of the heating medium, ovalbumin and whole egg white formed transparent solutions, transparent gels, opaque gels or turbid gels after thermal treatment. Generally, the transparent solution and transparent gels were only formed by heating the protein solutions at pH distant away from the isoelectric point and very low salt concentration or even without salt. As the salt concentration increased, the turbidity of
the gels increased as well and eventually the opaque gels were formed. The turbid gels were formed by heating ovalbumin or egg white solutions at pH close to their isoelectric point regardless of the ionic strength. The strength of the heat-set ovalbumin and egg white transparent and opaque gels was much higher than that of the turbid gels.

The results of Raman spectroscopy showed that conformational changes of ovalbumin and whole egg white proteins upon thermal gelation involved the formation of stable intermolecular β-sheet structure. In other words, the heat-denatured ovalbumin and egg white proteins did not take on a random-coiled structure but rather retained a considerable amount of secondary structure, with the exposure of some hydrophobic regions to the solvent environment.

The solubility results of gels indicated that thermally induced ovalbumin and egg white gels were mainly formed by noncovalent crosslinks such as hydrophobic and electrostatic interaction or hydrogen bonds. Formation of thermally induced disulfide bonds was also involved during gel formation, especially in egg white gels.

The charges measured as the zeta potentials showed that the charges of ovalbumin and egg white protein solutions were high at pH 3.5 or 7.5 and low at pH 5.5. The sulfhydryl groups and disulfide bonds determined by Ellman’s or NTSB reagent showed the decrease of sulfhydryl groups and increase in disulfide bonds during heating, indicating the sulfhydryl/disulfide interchange or the oxidation of the sulfhydryl groups. The investigation of hydrophobicity by ANS fluorescence probe demonstrated a large increase of hydrophobicity of ovalbumin and egg white proteins after heating. This result suggested that heating caused the exposure of some hydrophobic regions to the solvent environment and the hydrophobic interactions were the main reason for gelation.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ANS</td>
<td>1-anilinonaphthalene-8-sulfonate</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>CPA</td>
<td>cis-parinaric acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
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<td>hr</td>
<td>hour</td>
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<tr>
<td>I.R.</td>
<td>infrared</td>
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<td>kDa</td>
<td>kilodalton</td>
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<td>l</td>
<td>liter</td>
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<tr>
<td>M</td>
<td>molar (moles per liter)</td>
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<td>mg</td>
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<td>nm</td>
<td>nanometer</td>
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<tr>
<td>NTSB</td>
<td>2-nitro-5-thiosulfobenzoate</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>PB</td>
<td>Phosphate buffer</td>
</tr>
<tr>
<td>PSC</td>
<td>phenyl-Sepharose chromatography</td>
</tr>
<tr>
<td>QSAR</td>
<td>Quantitative structure-activity relationship</td>
</tr>
<tr>
<td>RFI</td>
<td>relative fluorescence intensity</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RSAP</td>
<td>Raman spectral analysis package</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>sec</td>
<td>second</td>
</tr>
<tr>
<td>SH</td>
<td>sulfhydryl</td>
</tr>
<tr>
<td>SS</td>
<td>disulfide</td>
</tr>
<tr>
<td>U.V.</td>
<td>ultra violet</td>
</tr>
<tr>
<td>V</td>
<td>volt</td>
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<td>v/v</td>
<td>volume/volume</td>
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<td>w/v</td>
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<tr>
<td>ZP</td>
<td>zeta potential</td>
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Proteins are linear macromolecules, which possess relatively rigid and specific three-dimensional structure. The three-dimensional structure of proteins is determined by the structure of the 20 different amino acids and by the order in which they are linked together into a polypeptide chain (Creighton, 1992). Proteins are probably the most important components in food systems. In many foods, sensory qualities depend on the functional properties of proteins. Such properties determine the interaction among protein molecules and between proteins and other food elements. Recent research has shown that the molecular structure is crucial for the functional properties of proteins, thus the study of the relationship between the protein functional properties and its structure is an important goal of protein chemists.

The manufacture of processed foods often requires gelled ingredients to provide a desirable texture. There are a variety of gels with different molecular structures in food industry. Among them, gels from polysaccharides and gelatin, which are commonly used polymer compounds in food applications, have a transparent appearance and elastic texture. These properties of polysaccharides and gelatin gels are attributed to the formation of fine networks of fibrous chains with point contacts. However, food proteins are another group of polymer compounds that also possess good gelling properties.

Globular proteins such as egg white proteins are also known to possess good gelling properties upon heating. Gels formed by globular proteins are not as transparent as polysaccharide or gelatin gels. However, by manipulating the pH, ionic strength, protein concentration of the medium and the heating procedure, it is possible to produce
transparent as well as turbid gels from globular proteins. It was found by electron microscopy that transparent gels from globular proteins have a common property with polysaccharide and gelatin gels in terms of a three-dimensional network of linear polymers.

Egg white represents approximately 60% of the total egg weight and proteins form the major component of egg white solids. Egg white proteins are extensively utilized as valuable food ingredients not only because they are highly nutritious but also because they possess excellent functional properties, such as gelation, foaming and emulsification. Being able to form a gel upon heating make egg white excellent gelling agents and stabilizers in food processing. Many attempts have been made to improve the gelling properties of egg white protein and broaden their use in foods. Physicochemical treatment serves as an effective and attractive method for improvement of gel properties. The structure and properties of heat-induced ovalbumin gel are affected by the temperature and duration of the applied heat. Three factors including pH, ionic strength and protein concentrations play very important roles in determining gel structure and properties. By controlling these factors, a transparent gel, transparent solution, turbid gel or turbid solution can be obtained.

Various spectroscopic methods, including circular dichroism (CD), infrared, and Raman spectroscopy are often employed for analyzing protein structure. Raman spectroscopy is a branch of vibrational spectroscopy, which involves the inelastic scattering of radiation in the visible, UV or near-IR regions (Li-Chan, 1996). Proteins have been used extensively as examples of the application of the Raman spectroscopy to biomolecules (Carey, 1982). The observed Raman spectrum of a protein can provide useful information including the quantitation of polypeptide secondary structure such as α-
helix, β-sheet and unordered structure, characterization of cysteine-SH groups and
cystine-SS bonds, and the exposure of aromatic amino acid side chains to environments,
especially tyrosine and tryptophan (Carey, 1982). CD spectroscopy is a variation of
electronic absorption spectroscopy that uses circularly polarized light rather than normal
isotropic light. The greatest use of CD spectroscopy for proteins is for assessing the
distribution of secondary structure within a polypeptide backbone (Copeland, 1994). The	hree major secondary structures associated with proteins (α-helix, β-sheet and random
coil) give rise to very distinct signatures in the far-UV CD region (190-250 nm).

In order to obtain more detailed knowledge about Raman spectroscopy, a
preliminary experiment was performed in this project to compare Raman spectroscopy
with CD spectroscopy, which is the most commonly used technique to study protein
secondary structure, in determining the secondary structure of some model
homopolypeptides such as poly-lysine etc.

In this project, transparent, translucent and opaque ovalbumin and egg white gels
were prepared by fine adjustment of pH and ionic strength of the heating media. The
relationship between the protein structure and functional properties of these gels were
studied by using Raman spectroscopy and other physicochemical methods. By
understanding the structural differences of different kind of gels formed by egg white
proteins, it is possible to modify the physicochemical treatment of egg white proteins and
broaden their applications in food processing.

The following experiments were carried out for the above purposes: (1)
Comparison of the secondary structures of homopolypeptides using Raman spectroscopy
and circular dichroism (CD), (2) analysis of the effects of pH and heating on ovalbumin
structure using Raman spectroscopy, (3) Characterization of the properties of thermally induced ovalbumin gel, including the changes of protein structure upon gelation, the gel turbidity and strength, together with the charge, hydrophobicity, solubility and the SH versus the SS content of the gel, and (4) investigation of the thermally induced gelation of egg white from hen eggs including the changes of protein structure upon gelation, the gel turbidity and strength, together with the charge, hydrophobicity, solubility and the SH versus the SS content of the gel.
2.1. SPECTROSCOPIC METHODS FOR THE STUDY OF PROTEIN STRUCTURE

2.1.1. Raman spectroscopy

The Raman effect was first discovered by the Indian scientists C.V. Raman and K.S. Krishnan in 1928, who noted that some of the light scattered by a liquid changed in wavelength. J.T. Edsall was the pioneer in the use of Raman spectra for conformational information about biomolecules in the late 1930s, and he obtained the first Raman spectrum of lysozyme in 1958 using mercury lamp excitation and photographic spectroscopy (Lord, 1977). The laser as a Raman excitation source has produced an exponential growth in all kinds of Raman spectroscopy since the 1960s, including that of biomolecules. Since then, both Raman and resonance Raman spectroscopy have shown increasing importance for solving biochemical problems, and developments in optics, lasers and electronics have provided and will continue to promote advances in experimental Raman spectroscopy (Li-Chan et al., 1994).

The distinct advantages of Raman spectroscopy over other spectroscopic methods are its applicability to studying samples with high concentration as well as samples in various physical states, e.g. solids, gels, powders, crystals etc. (Painter, 1984). This advantage makes it especially useful for analysis of food that normally involve highly concentrated and diverse food components.
2.1.1.1. Basic concept of Raman spectroscopy

Raman spectroscopy is one type of vibrational spectroscopy, which involves the inelastic scattering of radiation in the visible, UV or near-IR regions (Li-Chan, 1996). The term scattering means that a beam of monochromatic radiation is randomly directed in all directions from the substance it strikes. It consists of two categories: elastic (Rayleigh) and inelastic (Raman) scattering (Fig. 1).

In the case of elastic (Rayleigh) scattering, the frequency of the scattered radiation is the same as the exciting radiation; there is no energy exchange between the molecules and the photons of the light beam.

![Diagram of Rayleigh and Raman scattering](image)

Figure 1: Schematic presentation of Rayleigh scattering and Raman scattering including stokes and anti-stokes lines (Adapted from Li-Chan et al. 1994).
According to Carey (1982), the physical origin of Raman scattering lies in inelastic collision between the molecules and the photons of the light beam. In this inelastic collision, there is an exchange of energy between the photons and the molecules with a consequent change in energy, and hence the frequency of the photon changes. Since total energy is conserved during the scattering process, the energy gained or lost by the photon must equal to the energy change within the molecule. Thus, by measuring the energy gained or lost by the photon one can probe changes in the energy of the molecule (Carey, 1982). In Raman spectroscopy, vibrational transitions of the molecules are measured in terms of the shift in frequency or wavenumber (cm$^{-1}$), $\nu_i$, from the incident wavenumber, $\nu_0$, of the photons of the light beam, resulting in Stokes ($\nu_0 - \nu_i$) lines and anti-Stokes ($\nu_0 + \nu_i$) lines. Because the intensity and frequency of the molecular vibration are sensitive to chemical changes and to the environment around the atoms, the Raman spectrum can be used as a monitor of molecular chemistry (Li-Chan et al., 1994).

2.1.1.2. Raman spectroscopy of proteins

Proteins have been used extensively as examples of the application of Raman spectroscopy to biomolecules (Carey, 1982). The observed Raman spectrum of a protein consists of contributions from various amino acid side chain vibrations as well as vibrations originating from the polypeptide backbone. Thus Raman spectroscopy can provide structural information about a protein that includes the quantification of polypeptide secondary structure (i.e., $\alpha$-helix, $\beta$-sheet etc. content), characterization of cysteine-SH groups and cystine-SS bonds, and the exposure to environments of aromatic amino acid side chains, especially tyrosine and tryptophan (Carey, 1982). There are some
excellent reviews (Carey, 1982; Tu, 1986; Susi and Byler, 1988; Li-Chan et al., 1994) about the assignment of various amino side chain vibration bands and conformationally sensitive bands (i.e., amide I and amide III) in protein Raman spectra.

The peptide backbone vibration. The amide I region near 1650 cm\(^{-1}\) results primarily from in-plane peptide C=O stretching vibrations (80\%) with some contributions from in-plane N-H bending vibrations. The amide III region near 1200-1300 cm\(^{-1}\) results mainly from C-N stretching and N-H in-plane bending vibrations (Tu, 1986). These two regions, especially the amide I band, have been used not only for qualitative but also quantitative estimation of the secondary structure of the polypeptide backbone.

In the amide I region, proteins containing predominantly \(\alpha\)-helical and \(\beta\)-sheet structures generally show bands centered around 1645-1657 cm\(^{-1}\) and 1665-1680 cm\(^{-1}\), respectively, while proteins with high content of random coil structure exhibit amide I band around 1660 cm\(^{-1}\) (Li-Chan et al., 1994).

In the amide III region, proteins usually show \(\beta\)-sheet structure bands around 1238-1245 cm\(^{-1}\), while random coil structure bands appear near 1250 cm\(^{-1}\). The intensity of \(\alpha\)-helix bands is generally weak, and these bands are located in a broad region from 1260-1300 cm\(^{-1}\). Thus the lack of strong peaks in the amide III region is characteristic of high \(\alpha\)-helix content (Li-Chan et al., 1994).

Various methods have been proposed for quantitation of secondary structural content of proteins from their Raman spectra, especially the amide I and amide III bands. Among them, the method developed by Williams (1986), which involves the analysis of protein amide I and amide III bands as linear combinations of the corresponding bands in spectra of proteins in a reference set whose secondary structures are already known, is one
of the most commonly used. This method was the theoretical basis of Raman Spectral analysis Package developed by Przybycien and Bailey (1989). RSAP is a set of computer programs for the analysis of amide I band of protein Raman spectra, which is sensitive to protein secondary structure. The relative amounts of α-helix, parallel and antiparallel β-sheet and random coil are assigned by deconvoluting experimental spectra in terms of contributions from reference spectra of proteins of known secondary structures.

*Amino acid side-chain vibration.* The Raman vibrational bands are generated by the specific chemical groups of various amino acid side-chains, including the disulfide bonds of cystine, the phenolic OH group of tyrosine, the carboxyl groups of aspartic and glutamic acids, as well as the aromatic rings and aliphatic chains of different amino acids. The Raman bands of disulfide bond stretching appear in the region of 500-550 cm\(^{-1}\), and proteins most frequently show this band near 510 cm\(^{-1}\). However, additional bands can also be seen at 525 and 540 cm\(^{-1}\) depending on the conformation of the C-C-S-S-C-C group. The tyrosine residue vibrations show several Raman bands; among them, the most useful pair for monitoring the microenvironment around tyrosine residues is located at 830 and 850 cm\(^{-1}\) region. The intensity ratio \(I_{850}/I_{830}\) depends on whether the tyrosine residue is exposed (i.e., its phenolic OH group can interact with solvent as a simultaneous acceptor and donor and form moderate to weak hydrogen bonds) or buried (i.e., the phenolic OH group acts as a hydrogen bond donor to the carboxylate ion of aspartate or glutamate residue). The intensity ratio \(I_{850}/I_{830}\) will be high (0.9-1.45) for exposed tyrosine residues and lower (0.7-1.0) for buried tyrosines (Li-Chan et al., 1994). There are also other useful bands for other aromatic amino acids. For example, the strong band near 1006 cm\(^{-1}\) attributed to the breathing vibration of the phenylalanine monosubstituted ring has been
used extensively as a useful internal standard (Harada et al., 1982). C-H stretching and
deformation bands of aliphatic amino acids were reported to appear in the 2800-3000 and
1400-1500 cm\(^{-1}\) regions, respectively. However, structural implications of these bands
have not been extensively studied and reported (Tu, 1986).

2.1.2. Circular dichroism (CD) spectroscopy

2.1.2.1. Basic principle of CD spectroscopy

CD spectroscopy is a variation of electronic absorption spectroscopy that uses
circularly polarized light rather than normal isotropic light. The term circularly polarized
light means that the electric vector of the light is constant in magnitude but the direction of
the electric vector is modulated, such that the resultant wave propagates as a helix instead
of a fixed axis (Johnson Jr., 1985). The helical propagation of such a wave can be
clockwise (right circularly polarized) or counterclockwise (left circularly polarized)
(Copeland, 1994).

CD of a molecule is defined as the difference between the extinction coefficients
for the left and right circular polarized light (Johnson Jr., 1988). Since each type of light
obeys Beer's law, the difference is given as \(\Delta A = A_L - A_R = \Delta \varepsilon c\), which means CD bands
can be either positive or negative depending on which type of light is absorbed more
strongly. This difference in extinction coefficient between the left and right circularly
polarized light depends on the chirality or optical activity of the sample.

CD is zero unless the sample molecule is asymmetric since the two types of
circularly polarized light only interact differently with asymmetric molecules. Most
biological molecules contain asymmetric carbon atoms; thus they are suitable to be studied
by this technique. CD is a commonly used type of absorption spectroscopy because it is particularly sensitive to configuration and conformation and can be applied to molecules in solution (Johnson Jr., 1985).

2.1.2.2. Application of CD in protein structure analysis

In proteins the major optically active groups are the amide bonds of the peptide backbone and the aromatic side chains (Greenfield, 1996). The greatest use of CD spectroscopy for proteins is for assessing the distribution of secondary structure within a polypeptide backbone; however, it has been suggested that CD spectroscopy can be of some use in determining some elements of tertiary structure as well (Copeland, 1994).

In the far-UV CD region (190-250 nm), the electronic transitions from amide bonds of peptides show optical activity (Fig. 2). As a consequence, the three major secondary structures associated with proteins (α-helix, β-sheet and random coil) give rise to very distinct signatures in the far-UV CD. This correlation was first demonstrated by Doty and coworkers using homopolypeptides such as poly-L-lysine (Holzwarth and Doty, 1965). Poly-L-lysine with α-helix structure displays a CD spectrum showing the double negative features at wavelength of 208 and 222 nm, while the CD spectra of β-sheet and random coil structure show a single negative feature at the wavelength of 215 nm and 198 nm, respectively. For most globular proteins, all three major secondary forms are present, and their CD spectra appear as a linear combination of the three characteristic spectra of poly-L-lysine which have been reported by some researchers (Holzwarth and Doty, 1965; Greenfield and Fasman, 1969; Copeland, 1994).
Figure 1: The classical CD spectra for poly-L-lysine in α-helix (a); β-sheet (b) and random coil (c) structure (Adapted from Copeland, 1994).

2.1.3. Homopolypeptides as model systems in Raman and CD spectroscopy

Synthetic homopolypeptides have been used as model compounds to study secondary structure by spectroscopic techniques such as Raman and CD spectroscopy. Parker (1983) reported the conformation sensitive Raman lines of different polypeptides. The Raman spectra of β-sheet and α-helix structure in homopolypeptides are considerably different; thus there is little difficulty in differentiating between these two conformations. It was reported that poly-L-alanine, poly-γ-benzyl-L-glutamate and poly-L-leucine had
typical α-helix structure while poly-L-valine, poly-L-serine and polyglycine had characteristic β-sheet structure (Koenig and Sutton, 1971).

Among synthetic homopolypeptides, poly-L-lysine has been used as a model compound for protein structure determination because Raman frequency and intensity changes which accompany conformational changes can be observed in aqueous solutions without compensation for the effects of differing side chains or solvents. Poly-L-lysine exists as random coil at low pH, as the α-helix at high pH and low temperature, and as the antiparallel β-sheet conformation at high pH and higher temperature (Lippert et al., 1976). The Raman spectra of poly-L-lysine in all three conformations were reported by Yu et al. (1973) and Painter and Koenig (1976a) in both H₂O and D₂O solutions.

Poly-L-lysine has also been used as a model compound for CD spectroscopic estimates of secondary structure content in proteins for the same reason. The CD spectra of poly-L-lysine in different secondary structure were first demonstrated by Doty and coworkers (Holzwarth and Doty, 1956). Greenfield and Fasman (1969) initiated the first method for analyzing the CD spectrum of a protein for secondary structure by fitting the spectrum to a combination of reference CD spectra for pure secondary structures, which were the CD spectra for poly-L-lysine in the α-helical, β-sheet and random coil forms.

2.2. RELATIONSHIP BETWEEN PROTEIN STRUCTURE AND ACTIVITY

2.2.1. Quantitative structure-activity relationship (QSAR)

Quantitative structure-activity relationship (QSAR) techniques use molecular structure and physical property data to make predictions about activity and reactivity of
compounds (Nakai et al., 1991). Charge, hydrophobicity and steric parameters were originally proposed as the three major classes of descriptors for QSAR investigation for smaller compounds (Nakai and Li-Chan, 1993). Although the techniques were originally developed for investigation of smaller compounds, they can also be applied to elucidating function of proteins in food systems such as solubility, gelation etc. But because of the complexity of determining absolute values of these parameters for proteins, empirical measurements based on physicochemical properties have been used. For example, the hydrophobicity is measured by using the aromatic fluorescence probe ANS and aliphatic probe cis-parinaric acid CPA, or by hydrophobic interaction chromatography on phenyl-Sepharose (PSC). Net charge is measured as zeta potential (ZP), and steric parameters as SH/SS content. However, these methods can only be applied to sample solutions or dispersions, which means only samples with relatively low concentrations can be used for such measurements.

It is generally accepted that hydrophobicity, electronic and steric effects contribute not only to the stabilization of the structure of native protein and to the mechanism of protein folding, but also to the functional properties of proteins that give the food its characteristic texture and form. These functional properties include solubility, gellability or coagulability, viscosity, emulsifying and foaming properties (Li-Chan and Nakai, 1991a).

2.2.2. Protein hydrophobicity in food systems

The term ‘lyophobic’ is used to describe a solute that has little or no affinity for the solvent medium in which it is placed. When the medium is composed of water or an aqueous solution, ‘hydrophobic’ is used as the more specific term (Nakai et al., 1996).
The forces that hold the nonpolar regions of the molecules together in aqueous solution are called hydrophobic interactions. The strength of these interactions is due to not only the tendency to reduce the entropically unfavorable contact between nonpolar groups with water but also to the tendency to form enthalpically favorable noncovalent associations including those interactions broadly classified as Van der Waals forces. Such as the net effect of attractive London interactions, repulsive electron cloud overlap and inducible dipole orientation and induction effects (Li-Chan and Nakai, 1991a).

The most popular definition for the hydrophobicity of a protein is based on the sum or average hydrophobicity of its constituent amino acids expressed as the free energy of transfer from water to ethanol, which is computed from the solubility difference of the amino acids in the two solvents. However this approach does not provide information on the hydrophobicity at the surface of the protein molecule, which is actually more important to protein function.

The quantitation of protein hydrophobicity, especially surface hydrophobicity or effective hydrophobicity, can be an essential step for accurate prediction of protein functionality (Nakai, 1983). However, measurement of surface hydrophobicity is controversial, as no standard method for quantification of this characteristic has been established. Probe spectrofluorometry serves as one of the most commonly used methods for determination of protein hydrophobicity.

Fluorescence probes can be defined as small molecules which undergo a changes in one or more of their fluorescence properties as a result of noncovalent interaction with a protein or other macromolecule (Brand and Gohlke, 1972). The fluorescence probe method can provide an indication of the availability of hydrophobic regions in protein
molecules even though it does not yield quantitative information on the distribution of individual side chains (Kronman and Robbins, 1970). Compounds for which quantum yields of fluorescence and wavelength of maximum emission depend on the polarity of their environment have been used as probes to assess the hydrophobic or non-polar nature of proteins (Nakai et al., 1996).

There are three major types of hydrophobic probes: anionic probes, cationic probes and neutral probes (Haugland, 1992). Among them, 1-anilinonaphthalene-8-sulfonate (ANS), an anionic probes of the aromatic sulfonic acid class, is most frequently used. ANS has an extremely low quantum yield of fluorescence in water. However, the quantum yield of the probe shows a dramatic increase when bound to membranes or relatively hydrophobic cavities in many proteins (Stryer, 1965). On the other hand, Penzer (1972) reported that the fluorescence emission of ANS was enhanced and blue shifted in concentrated aqueous MgCl₂ solution and suggested that molecular rigidity rather than solvent polarity is the dominant factor influencing the energy and quantum yields of ANS.

2.2.3. Influence of electrostatic interactions on protein function

Protein molecules bear a net charge except at their isoelectric point, being negatively or positively charged at pH values significantly higher or lower than their isoelectric point. Consequently, there is an appreciable electrostatic repulsion opposing self-aggregation (McClements and Keogh, 1995).

Two factors that influence the electrostatic interactions of proteins are the pH and the ionic strength (i.e., the salt concentration) of the protein solution. Changes in pH alter the charges of the proteins and thus influence their electrostatic interactions. The primary
The effect of salt on protein functionality is the electrostatic shielding effect, which is usually achieved at relatively low ionic strength and is only dependent on the ionic strength of the medium, not on the nature of the ion. Salts also exert ion-specific effects in hydrophobic interaction and affect the stability of proteins at higher concentration. This effect is believed to be through the modification of water structure, which subsequently cause perturbations at the protein-water interface. This effect of salts in the structure of water depends mainly on the nature of the salt rather than the ionic strength (Wang and Damodaran, 1991).

Electrostatic interactions can have an important effect on the functionality of food proteins. One example is the gelation of globular proteins. It has been shown that gels from globular proteins are clear and present a fine-stranded microstructure when obtained in conditions of strong electrostatic repulsion, but they are opaque and exhibit a coarse particulate microstructure in conditions of weak electrostatic repulsion (Renard and Lefebvre, 1992).

2.2.4. The sulfhydryl and disulfide groups of protein and their effects on functionality

Sulfhydryl (SH) and disulfide (SS) groups play important roles in the functionality of many food proteins. The SH and SS interchange reactions have been suggested as the principal mechanism for formation of β-lactoglobulin-κ-casein complexes when milk is heated (Noh and Richardson, 1989). Sulfhydryl and disulfide interchange also occurs between egg white proteins during heating (Mine et al., 1990)
Sulfhydryl groups of proteins have often been analyzed by using Ellman's reagent, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (Ellman, 1958). This reagent reacts quantitatively with the SH group and gives rise to a characteristic chromophoric product, 5-thiobis-(2-nitrobenzoic acid) (TNB). To quantify number of free SH groups in proteins, the reaction is generally run in denaturant solutions of high concentration to unfold the protein. However, some of the sulphydryl groups, which are buried in the interior of proteins, may not be able to exposure by some denaturants. Diez et al. (1964) reported that native ovalbumin showed no SH groups after two hours of reaction with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) but showed an average of 3.8 SH groups within five minutes in the presence of 0.48% SDS. Takeda et al. (1992) investigated the SH groups of ovalbumin in different denaturing reagents and found that most of the four SH groups of ovalbumin reacted with DTNB following exposure to 5 M guanidine? but only some reacted following exposure to 9M urea. Ludwig et al. (1989) also studied the influence of propane-1-ol on the determination of thiol and disulfide groups of ovalbumin, bovine serum albumin and several other proteins. It was found that propan-1-ol alone or together with urea was a good denaturant and made the SH and SS groups more accessible for detection in all cases.

Many methods have been proposed to determine the disulfide groups in proteins. Among them, the method reported by Thannhauser et al. (1987), which involves the use of the reagent 2-nitro-5-thiosulfobenzoate (NTSB), has significant advantages in terms of sensitivity and quantitative capability. The disulfide bonds of proteins are cleaved quantitatively by excess sodium sulfite at pH 9.5 and room temperature. Guanidine thiocyanate (2 M) is added to the protein solutions to denature them and thereby make the
disulfide bonds accessible. The reaction with sulfite leads to thiosulfonate and a free sulfhydryl group. The concentration of the latter is determined by reaction with NTSB in the presence of excess sodium sulfite (Thannhauser et al., 1984). The limitation of this method is that the chromophoric product formed in the reaction disappears rapidly in the presence of light. Thus, for the quantification of disulfide bonds using the NTSB method, the assay should be carried out in the dark (Damodaran, 1985).

2.3. THE RELATIONSHIP BETWEEN STRUCTURE AND GELATION PROPERTY OF EGG WHITE PROTEINS

2.3.1. Egg white proteins

Egg white or albumen represents approximately 60% of the total egg weight. It forms an important protection layer as well as the food supply for the incubation of embryo. As a protection layer, it provides both a physical (i.e., high viscosity and maintaining the yolk in a central location of the egg) and a chemical (i.e., high pH and presence of antimicrobial proteins) defense against microbial infection (Board and Tranter, 1990). Water is the major component of albumen (84-89%), while protein forms the major component of albumen solids (9.7 to 10.6%). The lipid, carbohydrate (both free and in the form of glycoprotein) and ash content of albumen are 0.03%, 0.4-0.9% and 0.5-0.6%, respectively (Powrie and Nakai, 1985). Egg albumen occurs structurally in four layers: outer thin white, thick white, inner thin white and chalaziferous layer, in approximate ratios of 23:57:17:3. Egg albumen contains more than 15 proteins, and it may be regarded as a protein system consisting of ovomucin fibers in an aqueous solution of numerous globular proteins (Li-Chan and Nakai, 1989a).
Ovalbumin is the most abundant egg white protein, constituting over half of the egg white proteins by weight. It is one of the most well studied proteins because it is easily purified and crystallized in abundant quantities. Purified ovalbumin is made up of three components, A₁, A₂ and A₃, which contain two, one and no phosphate groups per molecule, respectively. The proportion of these fractions are about 85%, 12% and 3% for A₁, A₂ and A₃, respectively (Froning, 1988). Ovalbumin is a monomeric phosphoglycoprotein comprised of 385 residues, and having a molecular weight of 44.5 kDa. The isoelectric point of ovabumin is 4.5. It is reported to have four sulfhydryl groups and one disulfide bond (Powrie and Nakai, 1986). However, the reports on the precise number of thiol vs. disulfide groups have been conflicting probably due to sulfhydryl-disulfide interchange (Li-Chan and Nakai, 1989a). The complete amino acid sequence of hen ovalbumin has been reported by Nisbet et al. (1981). The N-terminal of ovalbumin is blocked by an acetyl group. The fragment including the N-terminal region (1-20) is relatively hydrophobic and contains one Cys residue. The fragment of the C-terminal (365-385) is also hydrophobic containing two Cys residues. The single carbohydrate moiety (composed of a core of two N-acetylglucosamine and four mannose units, with variable numbers of additional residues of the same sugars) is attached to an asparagine residue, with Asn-X-Thr as the recognition site for glycosylation (Conchie and Strachan, 1978). Two phosphoryl groups are attached to serine residues and the recognition site for phosphorylation is Ser-X-Glu (Henderson et al., 1981). Phosphoserine groups of ovalbumin have a high degree of mobility and appear to be on the surface of the protein, an observation that has been found true for other phosphorylated proteins (Holt and Sawyer, 1988). The crystal structure of ovalbumin was reported by Stein et al. in 1991.
Doi et al. (1987) using CD spectroscopy observed 49% α-helix, 13% β-sheet and 24% random coil structure in native ovalbumin. No biological function has yet been ascribed to ovalbumin. However, in terms of functional properties, heat-induced denaturation and gelation represents one of its most important properties. Others include foaming (surface-induced denaturation) as well as changes which occur by proteolytic action and upon storage of shell eggs (Li-Chan and Nakai, 1989a).

Ovotransferrin, which is also called conalbumin, constitutes 12% of the egg white proteins with a molecular weight of 77.7 kDa and isoelectric point of about 6. It is one of the anti-bacterial agents in egg white because of its strong iron binding property (Li-Chan and Nakai, 1989a). Essentially, it belongs to the transferrin protein family, which is a group of homologous iron-binding glycoproteins that are widely distributed in various biological fluids (Williams et al., 1982). The complete amino acid sequence of hen ovotransferrin contains 686 amino acid residues with 15 disulfide bridges that assist in maintaining the molecule in a bilobal structure. The iron complex of ovotransferrin is much more stable to thermal denaturation and to physical and chemical treatment than is the iron-free protein. The differences in stability and other properties of the iron-saturated complex versus iron-free apoprotein have important effect on the functionality of egg white as a food ingredient.

Ovomucoid is a heat-resistant glycoprotein (containing about 25 % carbohydrate) with a molecular weight of 28.0 kDa and an isoelectric point of 4.1. It accounts for approximately 10% of the proteins of egg white from various avian species. Hen ovomucoid has nine disulfide bonds and no sulfhydryl groups. Its amino-acid sequence includes 186 residues and has been reported by Kato et al. (1987). Ovomucoid is best
known for its trypsin inhibitory activity. It is formed of three domains, each of which is homologous to pancreatic secretary trypsin inhibitor and has a putative reactive site for the inhibition of serine protease (Mine, 1995).

Ovomucin is a sulfated glycoprotein with a highly viscous and gel-like nature that comprises between 1.5 and 3% of the total egg white solids. It has been reported to play an important role in maintaining the physical structure of egg white. The molecular weight of soluble ovomucin in mild nondenaturing and nonreducing conditions is between 5000 and 8000 kDa. There are two types of ovomucin complexes in hen egg white: an insoluble ovomucin complex formed from whole thick egg white and a soluble complex formed from both thick and thin egg white (Li-Chan and Nakai, 1989a).

Lysozyme is the most extensively studied egg white protein. It has the ability to lyse certain bacteria by hydrolyzing the β-1,4-linkage between muramic acid and N-acetylglicosamine of mucopolysaccharides in the bacterial cell wall (Li-Chan and Nakai, 1989a). Lysozyme occurs in hen egg white to the extent of 3.4% dry mass/weight (Powrie and Nakai, 1985). Hen egg white lysozyme, classified as a c-type lysozyme (c for chicken), is a relatively small, stable and very basic enzyme molecule, with a molecular weight of 14.3 kDa and a pI of 10.7 (Mine, 1995). It consists of 129 amino acids and has four disulfide bonds. For lysozyme the sum of acidic amino acid residues, Asp+Glu (7+2) is considerably small than that of basic residues, Lys+Arg (6+11), which leads to a high isoelectric point (McKenzie and White, 1991). Its basic nature has been applied in the development of procedures for isolation, and it has also been implicated with electrostatic interaction with negatively charged proteins in egg white. These interactions may have considerable practical importance on the properties of egg white in storage as well as
processing (Li-Chan and Nakai, 1989a). The heat induced gelation of mixtures of ovalbumin and lysozyme was studied by Arntfield and Bernatsky (1993). They concluded that mixed protein systems could produce stronger, less structured networks than pure proteins. Removal of lysozyme from egg albumin can adversely affect its gel strength and hence reduce its contribution to the texture properties of food products.

There are many other proteins present in egg white, including globulin, ovoinhibitor, ovogloboprotein, ovoflavoprotein, ovomacroprotein, cystatin and avidin (Powrie and Nakai, 1985). They also play a remarkably important role in biological and functional properties of egg white despite their small amount.

2.3.2. Gelation as a functional property of egg white proteins

2.3.2.1. Gelation properties of proteins

2.3.2.1.1. Introduction and definition

The manufacture of processed foods often requires ingredients that can provide a desirable texture and mouthfeel. Food proteins are responsible for the texture of many foods by the formation of gel matrices. To study the gelation mechanism, one has to define the term ‘gel’ first. However this has proven difficult because the ‘gel’ definition varies depending on the research focus. The following definitions have provided the three criteria of compositional, rheological and appearance values, which can be used to determine if a material is a gel (Foegeding et al., 1995):

i). A gel is a soft, solid or solid-like material of two or more components, one of which is a liquid that is present in substantial quantity.
ii). Solid-like gels are characterized by the absence of an equilibrium modulus, by a storage modulus, $G'(\omega)$, which exhibits a pronounced plateau extending to times at least of the order of seconds, and by a loss modulus, $G''(\omega)$, which is considerably smaller than the storage modulus in the plateau region.

iii). The material must be homogeneous to the naked eye or at low magnification in a microscope; a sponge is not a gel.

There are various food gels with different molecular structure. However, most food gels are composed of networks of fibrous molecules such as polysaccharides, collagen, myosin and actomyosin (Clark, 1992). Globular protein solutions, such as egg white and soybean proteins, also form gels upon heating. The gelation of globular proteins involves the creation of a three dimensional network at optimum ranges of pH, ionic strength, time and temperature of processing (Harte et al., 1992). The globular gels can be classified as fine stranded, mixed or particulate based on the protein microstructure network appearance (Doi, 1993).

2.3.2.1.2. Heat induced gelation of globular proteins

Thermal gelation of globular proteins is a two-stage process of activation and association (Foegeding, 1989). During activation, heating causes the denaturation and exposure of the functional groups (such as CO and NH of peptide bonds, side-chain groups and hydrophobic groups). Under appropriate circumstances, these exposed groups facilitate association in which they interact with each other to form a three-dimensional gel network (Wang and Damodaran, 1991). Both processes are connected with abrupt changes of structure. At the first stage it is the structure of the individual protein molecule
that is varied; at the second, the structure of the solution as a whole changes (Sochava and Belopolskaya, 1992).

Hydrogen and disulfide bonds, hydrophobic and electrostatic interactions that stabilize proteins in aqueous solutions also stabilize thermally induced globular protein gels. Manipulation of these forces by altering pH, ionic strength, gelling time and temperature produces transparent, opaque or turbid gels. Electron microscopy has been used by many researchers as a powerful technique to study the architecture of solid gels (Foegeding et al., 1995; Tani et al., 1995; Kitabatake and Doi, 1993, Nakamura et al., 1984; Clark et al., 1981b). This technique reveals that a transparent gel is composed of a network of linear, fine-stranded aggregates of heat denatured molecules and suggests that transparent gels of globular proteins have a common property with polysaccharide and gelatin gels in terms of a three-dimensional network of linear polymers (Tani et al., 1995). On the other hand, turbid gels are made up of heat-denatured molecules gathered into a random, particulate network. The opaque gels are the intermediate state and are classified as having a mixed protein network appearance, containing both fine-stranded and particulate structures (Foegeding et al., 1995). The conditions required by different proteins to form fine-stranded, mixed or particulate gels varies depending on the specific physicochemical properties of the protein. However, in general, as the pH approaches the isoelectric point, the microstructure of gels becomes more particulate. Similarly, increasing ionic strength increases the particulate nature of gels (Doi, 1993).

The relationship between the gel properties (i.e. rheological properties and microstructures of gels) and changes in secondary structure of globular proteins has been studied by many researchers. Lin and Koenig (1976) used Raman spectroscopy to predict
changes in secondary structures of bovine serum albumin during gelation. They showed that heating causes a shift from α-helix to β-sheet, and proposed a gelation model based on intermolecular hydrogen bonding between β-sheets. Clark et al. (1981a) used a similar approach to determine if changes in secondary structure account for differences in rheological properties and microstructures of gels. They varied solution conditions to produce clear, elastic gels as well as turbid coagulates and found that bovine serum albumin showed similar secondary structure transitions in all cases. Thus, they concluded that clear, elastic or opaque gels and turbid coagulates were formed by proteins with similar infrared and Raman spectra and that secondary structure transitions were not indicative of the gel type formed. Similar results were also shown for β-lactoglobulin gelation; nearly identical circular dichroism spectra are seen for β-lactoglobulin denatured under solution conditions that lead to transparent or opaque gels (Foegeding et al., 1992).

A new type of protein conformation state, molten globule state, has been demonstrated for many globular proteins. It is a partially folded conformation that can be distinguished from either the native or the fully denatured forms. ‘Globule’ refers to the native compactness and ‘molten’ refers to the increased enthalpy and the entropy on transition from the native structure to the new state (Mine, 1995). The molten globule state of a protein is often characterized as having minor changes in secondary structure relative to the native form and an increase in surface hydrophobicity (Bychkova et al., 1992). Therefore, hydrophobic-driven aggregation of globular protein in a molten globular structure can not be predicted by changes in secondary structure.
2.3.2.2. Gelation of egg white protein

Egg white proteins have been widely used in the food industry because of their unique functional properties. The ability to form a gel upon heating makes egg white a key ingredient in many food products because of the contribution to structure, binding and texture properties.

2.3.2.2.1. Gelation of ovalbumin

Since ovalbumin is the main protein component of egg white, the gelation together with other functional properties of egg white are related to the properties of ovalbumin (Kitabatake et al., 1989). Thermal gelation of ovalbumin has been studied extensively as a representative of egg white gelation.

Following initial denaturation and unfolding, thermal gelation of ovalbumin involves primarily the development of turbidity due to the formation of spherical aggregates via hydrophobic interactions, then the stiffening of the pre-formed aggregates through sulphhydryl-disulphide reactions and finally a sudden, large increase in elasticity upon cooling that results from the rapid formation of multiple hydrogen bonds (Mine, 1995).

Conformational changes accompanying the thermal treatment of ovalbumin have been studied using Raman (Painter and Koenig, 1976b) and circular dichroism (Mine et al., 1990) spectroscopy. The spectral changes indicate the formation of extensive regions of antiparallel β-sheet between ovalbumin molecules. The formation of β-sheet structure increased with increasing ovalbumin and salt concentrations as a result of enhanced intermolecular interaction. Thus it was concluded that heat-induced ovalbumin gels were formed from the partially unfolded molecules through the crosslinking of intermolecular β-sheet structures as a result of the exposure of hydrophobic residues (Mine, 1995).
Ovalbumin can form transparent gel, transparent solution, turbid gel and turbid solution depending on the protein concentration, pH and ionic strength of the heating medium. Hatta et al. (1986) noticed the importance of pH and ionic strength on the turbidity and hardness of heat-induced ovalbumin gels. It was reported that the transparent gel was formed at pH values distant from the isoelectric point and low salt concentration and that the gel hardness was maximal under conditions that yielded a transparent or slightly turbid gel. A transparent gel can also be prepared with high concentration of salt if the ovalbumin solution was heated in advance under salt free conditions. This process is known as two-step heating method (Kitabatake et al., 1988). The gels and coagula produced in this process could be solubilized by 1% SDS but not by 6 M urea nor by 50 mM 2-mercaptoethanol. It was concluded that the compensation between hydrophobic interactions and electrostatic repulsive forces influenced gel turbidity and hardness (Hatta et al., 1986).

It was reported by Kitabatake and Doi (1993) that at low ionic strength and neutral pH, ovalbumin formed soluble oligomers at high temperatures, but it did not gel. These authors examined the conformational changes related to this treatment by CD. The far-UV CD spectrum indicated that only small changes in secondary structure occurred on heating relative to those induced by a high concentration of guanidine hydrochloride. In contrast, the near-UV CD spectrum and differential UV absorption spectrum indicated disorganization of the conformation around the aromatic amino acid residues of the ovalbumin molecules on heating. Thus these authors concluded that ovalbumin took on a molten globule-like state at high temperatures.

Kitabatake et al. (1989) also determined some of the rheological properties of
ovalbumin gels prepared by one-step and two-step heating methods. They found that transparent gels formed by one- or two step heating were firm and elastic and had high water-holding capacity, while turbid gels formed by one-step heating were soft and less elastic and had low water-holding capacity. More recently, the turbidity of heated ovalbumin as well as egg white was measured by a microplate system, which provided a quicker and more accurate method to examine the turbidity (Kitabatake and Kinekawa, 1995).

Tani et al. (1995) studied the microstructure of transparent gels from ovalbumin and two other proteins (hen egg white lysozyme and bovine serum albumin) by using high-resolution scanning electron microscopy. It was clearly found that the transparent gel matrices from each of the three proteins were composed of a common structural unit in which heat-denatured molecules formed fine networks of linear aggregates. Tani et al. also studied the molecular conformation of these linear aggregates produced after heat treatment in the absence of salt by CD spectra, intrinsic tryptophan fluorescence and adsorption of the dye 1-anilinonaphthalene-8-sulfonate (ANS). All three heat-denatured proteins remained partially folded with some hydrophobic regions becoming exposed to the solvent environment, consistent with formation of conformation as the "molten globule" state.

2.3.2.2.2. Gelation of egg white

Egg white is a protein mixture. Gelation properties of egg white are similar to those of ovalbumin and involve formation of extensive regions of antiparallel β-sheet between protein molecules during gelation upon heating (Painter and Koenig, 1976b). At
pH values distant from the isoelectric point and low salt concentration, egg white can also form transparent gels. However, the pH and salt conditions for transparent gel formation of egg white is much narrower than those for pure ovalbumin (Doi and Kitabatake, 1994). The salt concentration range for forming the transparent gel can be widened by a preheating of egg white under salt free condition. Arntfield and Bernatsky (1993) found that mixed protein systems could produce stronger, less structured networks than can pure proteins. Thus egg white gel has greater strength than does an ovalbumin gel formed under the same condition, and the egg white gel can provide a greater contribution to the textural properties of food products.
Chapter 3. Materials and Methods

3.1. RAMAN SPECTROSCOPY

3.1.1. Spectra measurement

The Raman spectrum of each sample was measured with a Jasco Model NR-1100 laser Raman spectrophotometer (Japan Spectronic Co., Tokyo, Japan) with excitation from the 488-nm line of a Spectra-Physics Model 168B argon laser (Nonaka et al., 1993). The Raman scattering of samples in a transverse/transverse arrangement was measured either at room temperature or with the RT-IC temperature controller under the following conditions: laser power, 200 mW; slit height, 4 mm; spectra resolution, 5.0 cm\(^{-1}\) at 19000 cm\(^{-1}\); sample speed, 120 cm\(^{-1}\)/min with data collected every cm\(^{-1}\), 6 scans per sample. All data were collected at least in duplicates, and the results are reported as the average of these replicates with coefficient of variation of \(\leq 15\%\).

3.1.2. Spectra analysis

Spectra smoothing, baseline correction and normalization against the vibration of the phenylalanine at \(\sim 1007\) cm\(^{-1}\) (Li-Chan et al., 1994) were performed with LabCalc (Galactic Industries Corp., Salem, NY) and Square Tools software (Spectrum Square Associates, Ithaca, NY) with an IBM compatible personal computer. The secondary structure composition of the sample based on the Raman spectra in the amide I region was estimated using the Raman spectral analysis package (RSAP) of Przybycien and Bailey (1989).
3.2. INVESTIGATION OF SECONDARY STRUCTURE USING HOMOPOLYPEPTIDES

Homopolypeptides used for the study were purchased from Sigma Chemical Company (Table 1).

Table 1. List of polypeptides purchased from Sigma Chemical Company

<table>
<thead>
<tr>
<th>Polypeptide</th>
<th>Molecular Weight (Dalton)</th>
<th>Catalogue number</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-L-alanine</td>
<td>10,000-25,000</td>
<td>P-5517</td>
</tr>
<tr>
<td>P-D-lysine (Hydrobromide)</td>
<td>105,800</td>
<td>P-0899</td>
</tr>
<tr>
<td>P-L-lysine (Hydrobromide)</td>
<td>95,100</td>
<td>P-1274</td>
</tr>
<tr>
<td>P-L-lysine (Hydrobromide)</td>
<td>375,800</td>
<td>P-1524</td>
</tr>
<tr>
<td>P-L-valine</td>
<td>5,000-10,000</td>
<td>P-3908</td>
</tr>
</tbody>
</table>

3.2.1. Raman spectroscopy

The solid polypeptide samples were used directly to collect the Raman spectra. The poly-l-lysine solutions (10% w/v, pH 5.6) were prepared by dissolving in distilled deionized water, and also at pH 10.3 (adjusted with 0.1 M NaOH). The Raman spectra of poly-lysine at pH 5.6 and pH 10.3 were measured and compared. In addition, spectral data of polypeptide samples (pH 10.3) were collected at 4°C, 25°C or 55°C using the JASCO RT-IC temperature controller and analyzed as described in section 3.1.

3.2.2. Circular Dichroism

The poly-lysine solutions used to collect the circular dichroism (CD) spectra were prepared under the same pH and temperature conditions but at a lower concentration
(0.02% w/v) than those used for Raman spectroscopy, because CD spectroscopy is only applicable to low sample concentration. CD spectra of poly-lysine at 4°C, 25°C or 55°C were measured with a Jasco model J720 spectropolarimeter (Japan Spectronic Co., Tokyo, Japan), acquired with an IBM compatible computer, and plotted with Microsoft Excel.

3.3. INVESTIGATION OF OVALBUMIN

3.3.1. The effect of pH and heating on ovalbumin structure

Ovalbumin (Sigma, Product number A5503; Grade V, crystallized and lyophilized; essentially salt free) was used to make a 20% w/v solution in distilled deionized water. Sodium azide (0.02%) was added to prevent possible microbial contamination during experiments and storage. The solution was divided into four aliquots, which were then adjusted to pH 6, 7, 8 and 9, respectively, using 1M NaOH or 1M HCl. The solutions at pH 6 and 9 were sealed in capillary tubes, and subjected to five minutes heating at 60, 70, 80, 90, and 100°C. The samples were then cooled immediately by immersion in an ice water bath. The Raman spectral data collections of this study were performed by Ms. Grace Lee.

3.3.2. Properties of ovalbumin gel

The same grade of ovalbumin was also used to prepare approximately 10% (w/v) solution containing 0.02% sodium azide. The pH 3.5, 5.5 and was chosen as the testing pH for ovalbumin (Doi and Kitabatake, 1994). After adjusting the pH of the solution to 3.5,
5.5 and 7.5 by 1 M NaOH or HCl, the exact concentrations of the protein solutions were
determined from the absorbance values at 280 nm based on the extinction coefficient of
albumin (Tani et al., 1985): $E_{280\text{nm}}^{1\%} = 7.12$. The solutions of different pH were separated
into 9 aliquots and 5 M NaCl solution was added to give a final salt concentration of 0,
10, 20, 30, 40, 50, 60, 70, or 80 mM. For example, to 1 ml sample solution, 16 μl of 5 M
NaCl solution was added to make the final salt concentration 80 mM. The solutions were
then used in the following experiments before and after being heated at 80°C for one hour.
All following experiments were performed twice, and the results are reported as the
average of the duplicates.

3.3.2.1. Raman spectroscopy

Initially, each of the ovalbumin solutions was sealed in a capillary, and the Raman
spectrum of the solution was collected. The capillaries were then heated at 80°C in a water
bath for one hour and cooled in an ice water bath for 30 minutes. After setting in the cold
room (~5°C) overnight, the Raman spectrum of the ovalbumin gel in the capillary was
measured. The spectra were then analysed as a function of pH, salt concentration and
heating condition to obtain information about protein structure changes.

3.3.2.2. Gel turbidity

Turbidity of ovalbumin solutions at various pH and salt concentrations was
determined with a microplate system before and after heating each sample at 80°C
(Kitabatake and Kinekawa, 1995). To the wells in 3 rows (row A, C and E) of the 96 well
microplate (Nunc, tissue culture plate), 0.25 ml of each sample solution was added. The
plate was covered and sealed with parafilm and plastic cling wrap, heated in an 80°C water bath for 1 hour and then cooled in an ice water bath for 30 minutes. After the plate was set in the cold room for 24 hours, another 0.25 ml of each sample was added to the wells in 3 other rows (row B, D and F) to compare the samples before and after heating. The absorbance of the plate was measured with a microplate reader (Labsystems iEMS Reader MF, Helsinki, Finland), and the turbidity was expressed in terms of the absorbance at 595 nm.

3.3.2.3. Gel strength

The strength of the ovalbumin gels formed at pH 7.5 with salt concentration of 20, 40, 60 and 80 mM and at pH 3.5 and 5.5 with salt concentration of 0 and 80 mM was measured. The gel strength was expressed as the peak force (Newton) multiplied by the distance (mm) to the peak, obtained from force deformation curves measured by compression with the Texture Analyser (model TAXT2, Stable Micro Systems, England) (Distance format, strain; pre-test and post-test speed, 10.0 mm/sec; test speed, 0.1 mm/sec; strain, 50.0 %; trigger type, auto; trigger force, 0.05 kg). The gels were formed in a plastic tube with a diameter of 15 mm and a height of approximately 10 mm. The diameter of the compression plate was 50 mm, and the compression went to 50 % of the gel height.

3.3.2.4. Solubility of the gel

The solubility of the gel was determined as described by Li-Chan and Nakai (1989b). Ovalbumin gels (0.1g) at various pH and salt concentration were weighed into
centrifuge tubes, and 1 ml of one of the following buffers containing 0.02 % sodium azide was added as solvents: 1) 0.1 M sodium phosphate buffer (PB), pH 7.5; 2) PB containing 1% (w/v) SDS; 3) PB containing 8M urea; 4) PB containing 1% β-mercaptoethanol; 5) PB containing 1 M NaCl; 6) PB containing 1% SDS and 1% β-mercaptoethanol; 7) PB containing 1M NaCl and 1% β-mercaptoethanol; 8) PB containing 8 M urea and 1% β-mercaptoethanol. After sitting with occasional agitation at 22°C for 22 hours, the samples were centrifuged at 3000xg for 20 minutes. The protein concentration in the supernatant fluid was determined from the absorbance at 280 nm. The percentage of solubilization was calculated as (% protein in supernatant)/(% protein before centrifugation) x 100%.

3.3.3. Other physicochemical properties

For the following experiments, the same ovalbumin (Sigma) was dissolved in 0.01 M citric-phosphate buffer at pH 3.5, 5.5 and 7.5, respectively. All the following experiments were performed twice, and the results are reported as the average of the duplicates.

3.3.3.1. Electric parameter

The net charge of the protein was expressed as the zeta potential. The zeta potential of the ovalbumin solutions (4 mg/ml) at pH 3.5, 5.5 and 7.5 were measured with a Model 501 Laser Zee Meter (Pen Kem Inc., Bedford Hills, NY) before and after the solutions were heated at 80°C for one hour. A mixture of 5 ml of ovalbumin solution and 0.15 ml of 3,3'-dimethylbiphenyl (Aldrich Chemical Co.) were emulsified with the Polytron homogenizer at 2200 rpm for 20 seconds. The emulsion was then diluted with a
50-fold volume of the same citric phosphate buffer. The zeta meter chamber was filled with 25 ml of the protein colloid emulsion using a syringe according to the manufacturer's recommendation. The observed zeta potential (in mv) was corrected for the temperature according to the equation: \( ZP(\text{corrected}) = ZP(\text{measured}) \times (1-0.02T) \) where \( T \) is the temperature at the time of measurement.

3.3.3.2. SH groups and SS bonds

The steric parameter of ovalbumin solutions was investigated as a function of pH by determining the SH/SS content before and after the solutions (4 mg/ml) were heated at 80°C. The SH group determination was performed by a modification of the method described by Hardham (1981) and Beveridge et al. (1974) in a colorimetric reaction with Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid, MW=396.35, 4 mg/ml in Tris-Gly). To an 0.8 ml of the sample solution (4 mg/ml), 2M guanidine thiocyanate in Tris-Gly-EDTA buffer (pH 8.0) was added to a final volume of 4 ml. The solutions were then incubated at room temperature for 30 min prior to addition of 50 \( \mu \)l Ellman's reagent. After standing for another 30 min at room temperature following addition of Ellman's reagent, the absorbance was measured at 412 nm, and SH content was determined from the extinction coefficient (13600 M\(^{-1}\) cm\(^{-1}\); Beveridge et al., 1974).

The content of SS groups was investigated using the method of Thannhauser et al. (1984). The NTSB (2-nitro-5-thiosulfobenzoate) stock solution was synthesized as follows: 100 mg of Ellman's reagent was dissolved in 10 ml of 1 M \( \text{Na}_2\text{SO}_3 \) and the pH of the solution was adjusted to 7.5 to produce in a bright red color. While heating in a 38°C water bath, the solution was bubbled with oxygen until a pale yellow color appeared (~ 2
hrs). The stock solution was stored at -18°C for future use. The NTSB assay solution was prepared by diluting the stock solution 1 to 100 with a freshly made solution containing 2 M guanidine thiocyanate, 50 mM glycine, 100 mM sodium sulfite and 3 mM EDTA (pH 9.5). To measure the disulfide bond content, 100 µl of ovalbumin solution (15 mg/ml) was mixed with 1.5 ml of NTSB assay solution, and the mixture was incubated in the dark for 25 min. The absorbance at 412 nm was recorded against a blank of 1.5 ml of NTSB assay solution mixed with 100 µl water and total SH content was calculated from the extinction coefficient (13900 M⁻¹ cm⁻¹; Thannhauser et al., 1984).

3.3.3.3. Hydrophobicity

Hydrophobicity was investigated using the fluorescence probe ANS (Hayakawa and Nakai, 1985). The fluorescence of ANS was measured on spectrofluorophotometer (Shimadzu Model RF-540, Shimadzu Corp., Kyoto; Japan). To a series of tubes each containing 4 ml of 0.1 M phosphate buffer, 10, 20, 30, 40, and 50 µl of the ovalbumin stock solution (4 mg/ml) were added using a positive displacement pipette and each sample was mixed with a vortex mixer. The relative fluorescence intensity of these diluted solutions was measured as the protein blanks, starting from the lowest to the highest protein concentration. After addition of 20 µl of 8 mM ANS stock solution, the same protein dilution solutions were incubated at room temperature for 15 min, and the relative fluorescence intensity (RFI) of each solution was measured in the same sequence. The quartz fluorometer cell was rinsed with distilled water after each measurement. The net relative fluorescence intensity was calculated by subtracting the fluorescence intensity of the protein blank from that of the corresponding protein dilutions with ANS, and the
hydrophobicity was then calculated by linear regression (Minitab) as the initial slope of the net relative fluorescence intensity at each dilution versus the protein concentration at that dilution. The concentrations of the protein dilutions were measured from the absorbance at 280 nm. The RFI scale was standardized to 40 by measuring the RFI of 10 μl ANS in 10 ml methanol.

3.4. INVESTIGATION OF EGG WHITE

The eggs were purchased from a local store, and the egg white was tested within two days. The egg white sample was prepared as described by Kitabatake and Kinekawa (1995). The egg white was first dialyzed overnight against distilled water with 0.02% w/v sodium azide with three changes of the water. After dialysis, the egg white was centrifuged at 3,000g for 30 min at 4°C, and the supernatant fluid was used for the experiment within two days.

The pH of the egg white samples was adjusted to 3.5, 5.5 and 7.5 by adding 1 M NaOH or HCl, and all experiments were performed as described in objective 3.
Chapter 4. Results and Discussion

4.1. SECONDARY STRUCTURE DETERMINATION OF HOMOPOLYPEPTIDE USING RAMAN AND CD SPECTROSCOPY

Raman spectroscopy was the main technique used in all the studies throughout this thesis. For this reason, it was important to obtain detailed knowledge about this technique and how it relates to other spectroscopic methods. The following experiments were performed to compare Raman spectroscopy with CD spectroscopy, the most commonly used technique to study protein structure, in determining the secondary structure of some model homopolypeptides. The Raman spectra were also used to estimate quantitatively the secondary structure of poly-lysine by RSAP.

4.1.1. Results and discussion

The Raman spectra of poly-L-alanine and poly-L-valine in the solid state are shown in Fig. 3 and 4.

Poly-L-alanine in the solid state was reported as the simplest polypeptide that has an α-helical conformation. Previous work (Fanconi et al., 1971) demonstrated that there are 18 peptide residues in five turns and intrachain hydrogen bonds are found between each pair of every third neighbor. As shown in the spectrum of poly-L-alanine, the peak in the amide I region is at 1658 cm$^{-1}$, and there is no strong peak in the amide III region, which indicates the high α-helical content of this peptide structure. This character is also reflected in the skeletal vibration at 910 cm$^{-1}$, which is usually a strong band arising from
Figure 3. Raman spectrum of poly-L-alanine in the solid state.

Figure 4. Raman spectrum of poly-L-valine in the solid state.
the α-helical structure of synthetic polypeptides (Parker, 1983).

Poly-L-valine in the solid state was reported to have typical β-sheet structure in which the polypeptide chain is nearly fully extended and the hydrogen bonds are formed between sheets (Parker, 1983). The amide I peak for the poly-L-valine is at 1670 cm⁻¹, and there is a very intense and sharp band at 1232 cm⁻¹ in the amide III region and a skeletal vibration at 1018 cm⁻¹, all of which reflected the high content of β-sheet structure. These results for poly-L-alanine and poly-L-valine are coincident with those reported by Parker (1983).

Poly-L-lysine is often used as a model compound for estimation of protein secondary structure content. This peptide forms each of the three most common secondary structures as a function of solution conditions, i.e. random coil at low pH, α-helix at high pH and low temperature, and β-sheet conformation at high pH and high temperature (Lippert et al., 1976). The Raman and CD spectra of poly-L-lysine in the three pure secondary structures have been reported by many researchers (Greenfield and Fasman, 1969; Painter and Koenig, 1976a).

In this study, the Raman and CD spectra were collected for poly-L-lysine (MW=375,800), poly-L-lysine (MW=95,000) and poly-D-lysine (MW=105,000). Fig. 5, 7 and 9 show the Raman spectra collected at 4°C, 25°C and 55°C of these three samples (10% w/v, pH 10.3), respectively. The CD spectra of the same sample solutions at a lower concentration (0.2 % w/v) are shown in Fig. 6, 8 and 10.

Both Raman and CD spectra demonstrated the tendency of all three peptide samples to change from α-helix structure to β-sheet structure upon increasing temperature from 4°C to 55°C. The amide I region in Raman spectra shifted from ~1655 cm⁻¹ to higher wavenumber at ~1671 cm⁻¹, and the CD spectra indicate the change from typical α-helix
character to β-sheet character. This result showed that Raman and CD spectroscopy provided comparable qualitative information for the estimation of protein secondary structure. However, the concentration of the solutions used for Raman spectroscopy is much higher than that used for CD spectroscopy, which makes Raman spectroscopy more useful in analysis of food protein structure.

Figure 11 displays the secondary structure fraction estimated by RSAP of the Raman amide I band for the three preparations of poly-lysine. Each preparation exhibits decreasing α-helix and increasing β-sheet structure with increasing temperature. However, the amount of different secondary fraction is not identical in each scan. This behavior may result from the varying poly-lysine peptide chain lengths, which may affect folding of the polypeptide. The difference between D- and L-polymer may also affect the structure.

4.1.2. Summary and conclusion

Polypeptides with typical α-helix or β-sheet structure give rise to unique and distinguishing amide I and amide III bands in Raman spectra. The RSAP results of the solutions (10 % w/v) of three different samples of poly-lysine at pH 10.3 showed the conversion from typical α-helix to predominant β-sheet structure with increasing temperature. The same tendency was also observed in the CD spectra of these poly-lysine samples at much lower concentration (0.2 % w/v). These results demonstrates that Raman spectroscopy provides information about the secondary structure of polypeptides, that is comparable to information provided by CD spectroscopy. Moreover, the applicability of Raman spectroscopy to high protein concentration makes it more useful for analysis of food systems.
Figure 5. Raman spectra of 10% w/v poly-L-lysine (MW=375,800) at pH 10.3 and temperature of 4°C, 25°C and 55°C, respectively.

Figure 6. CD spectra of 0.02% w/v poly-L-lysine (MW=375,800) at pH 10.3 and temperature of 4°C, 25°C and 55°C, respectively.
Figure 7. Raman spectra of 10% w/v poly-L-lysine (MW=95,000) at pH 10.3 and temperature of 4°C, 25°C and 55°C, respectively.

Figure 8. CD spectra of 0.02% w/v poly-L-lysine (MW=95,000) at pH 10.3 and temperature of 4°C, 25°C and 55°C, respectively.
Figure 9. Raman spectra of 10% w/v poly-D-lysine (MW=105,800) at pH 10.3 and temperature of 4°C, 25°C and 55°C, respectively.

Figure 10. CD spectra of 0.02% w/v poly-D-lysine (MW=105,800) at pH 10.3 and temperature of 4°C, 25°C and 55°C, respectively.
Figure 11. Secondary structure fraction of poly-lysine at pH 10.3 and different temperature estimated by RSAP of the amide I band of the Raman spectra.
4.2. ANALYSIS OF THE EFFECTS OF pH AND HEATING ON OVALBUMIN STRUCTURE USING RAMAN SPECTROSCOPY

The main objective of this section was to use Raman spectroscopy in the structure determination of real protein samples, i.e. to study ovalbumin solutions during pH and temperature changes. From this work, the role of Raman spectra in protein structure determination, such as the characteristic band for protein secondary structure, the band for disulfide bond and aromatic amino acid side chain such as tyrosine, were more clearly demonstrated.

4.2.1. Results and discussion

The unheated ovalbumin (20% w/v) solutions at pH 6, 7, 8 and 9 were all clear. The ovalbumin solutions at pH 6 and 9 were subjected to heat treatment. Upon heating, the clear solutions at pH 6 turned turbid with increasing temperature, and eventually an opaque off-white gel was formed at and above 80°C. Upon heating the clear solution at pH 9, the samples turned into a transparent and slight yellowish gel at and above 80°C.

For all the samples subjected to various conditions of pH and temperature, Raman spectra were measured between 400-1800 cm\(^{-1}\) and between 2500-3400 cm\(^{-1}\). These regions of the spectrum were used to estimate protein secondary structure content and to study various side chain vibrations.

The Raman spectra of ovalbumin solutions at pH 6, 7, 8 and 9 are shown in Fig. 12, and tentative assignments of major bands of the spectrum at pH 6 are listed in Table 2.
The pH change from 6 to 9 at room temperature had no obvious effect on the Raman spectra.

The Raman spectra of ovalbumin samples at pH 6 before and after heating to 60°C to 100°C are shown in Fig. 13. At higher temperature, the amide I band was sharper, and the peak shifted from around 1660 cm\(^{-1}\) to over 1670 cm\(^{-1}\), indicating the conversion from \(\alpha\)-helix to \(\beta\)-sheet structure. This conclusion was also supported by the secondary estimation of RSAP shown in Table 3 and was consistent with the data reported by Painter and Koenig (1976b). They found that upon thermal denaturation of ovalbumin samples, the amide I band shifted from 1667 to 1672 cm\(^{-1}\), indicating the formation of \(\beta\)-sheet structure in heat-denatured ovalbumin.

Table 2. Tentative assignment of some major bands in the Raman spectrum of the ovalbumin solution at pH 6 (Li-Chan et al., 1994).

<table>
<thead>
<tr>
<th>Wavenumber (cm(^{-1}))</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>526</td>
<td>S-S stretching</td>
</tr>
<tr>
<td>835, 856</td>
<td>Tyr</td>
</tr>
<tr>
<td>887</td>
<td>Trp</td>
</tr>
<tr>
<td>906</td>
<td>(\alpha)-helix</td>
</tr>
<tr>
<td>1007</td>
<td>Phe</td>
</tr>
<tr>
<td>1036</td>
<td>Phe</td>
</tr>
<tr>
<td>1210</td>
<td>Tyr, Phe</td>
</tr>
<tr>
<td>1231, 1251, 1283</td>
<td>Amide III</td>
</tr>
<tr>
<td>1409</td>
<td>C=O stretching of Asp, Glu COO(^{-})</td>
</tr>
<tr>
<td>1455</td>
<td>(\text{CH}_2) bending</td>
</tr>
<tr>
<td>1664</td>
<td>Amide I</td>
</tr>
</tbody>
</table>
Table 3. Changes in secondary structure content estimated by RSAP of the amide I band, disulfide bond SS stretching intensity at 525 cm\(^{-1}\) and tyrosine doublet (835/856 cm\(^{-1}\)) ratio for ovalbumin samples at pH 6 and 9.

<table>
<thead>
<tr>
<th></th>
<th>α-helix</th>
<th>β-sheet</th>
<th>Random coil</th>
<th>SS stretching</th>
<th>Tyrosine doublet ratio</th>
</tr>
</thead>
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<td>pH = 6</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Untreated</td>
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<td>0.47</td>
<td>0.26</td>
<td>0.32</td>
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</tr>
<tr>
<td>60°C</td>
<td>0.14</td>
<td>0.58</td>
<td>0.28</td>
<td>0.26</td>
<td>1.12</td>
</tr>
<tr>
<td>70°C</td>
<td>0.15</td>
<td>0.58</td>
<td>0.27</td>
<td>0.26</td>
<td>1.18</td>
</tr>
<tr>
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<td>0.66</td>
<td>0.27</td>
<td>0.41</td>
<td>1.45</td>
</tr>
<tr>
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<td>0.00</td>
<td>0.72</td>
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<td>100°C</td>
<td>0.00</td>
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<td>pH = 9</td>
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<td>100°C</td>
<td>0.12</td>
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<td>0.30</td>
<td>0.07</td>
<td>0.78</td>
</tr>
</tbody>
</table>

Conformational changes accompanying the thermal gelation of ovalbumin have been qualitatively studied by Raman spectroscopy (Painter and Koenig, 1976b). The secondary changes of egg white solution at low protein concentration (2 %) before and after thermal treatment were also determined quantitatively by CD spectroscopy (Mine et al., 1990). Both studies demonstrated the formation of extensive regions of antiparallel β-sheet structure after thermal treatment. Our results shown in Table 3 provide quantitative evidence of the formation of β-sheet structure in ovalbumin gels.

The intensity ratio of the tyrosine doublet (835/856 cm\(^{-1}\)) decreased from greater than 1 to less than 1 after being heated at above 80°C, suggesting a change from buried to more exposed form.
The intensity of SS stretching band at around 525 cm\(^{-1}\) reached its highest point at 80\(^\circ\)C and then decreased slightly at 90\(^\circ\)C and 100\(^\circ\)C, indicating the first step for ovalbumin gelation involved SS bond formation. During further heating, ovalbumin molecules became polymerized and the formation of intermolecular disulfide bonds may play a role in the formation of stable gels. On the other hand, the SS bonds formed during gelation and might be disrupted after heating at even higher temperature. The changes in conformation around some S-S bonds from gauche-gauche-trans to gauche-gauche-gauche and trans-gauche-trans forms were indicated by the split of the peak at 525 cm\(^{-1}\) into two peaks at 530 cm\(^{-1}\) and 510 cm\(^{-1}\) (Fig 13).

The Raman spectra of ovalbumin sample at pH 9 before and after being heated from 60\(^\circ\)C to 100\(^\circ\)C is shown in Fig. 14. Similar changes were observed by heating ovalbumin solution at pH 9 as that at pH 6. However, the vibration in the amide I region had lower intensity and the wavelength shift was less significant, only from 1660 cm\(^{-1}\) to less than 1670 cm\(^{-1}\). RSAP analysis of the amide I band suggested less \(\beta\)-sheet structure was formed at pH 9 than at pH 6 (Table 3). At high pH, ovalbumin are highly charged and this affects not only the intermolecular interaction between ovalbumin molecules but also the intramolecular interaction within the molecules, which may explain why less \(\beta\)-sheet structure was formed at higher pH.

The trend of the decreasing in the intensity ratio of tyrosine doublet (835cm\(^{-1}\)/856cm\(^{-1}\)) after heating at pH 9 also indicated the change from buried to more exposed form of the tyrosine residues.
Figure 12. Raman spectra of ovalbumin solution (20% w/v) at pH 6, 7, 8 and 9.
Figure 13. Raman spectra of 20% w/v ovalbumin samples at pH 6 before and after heating at 60°C, 70°C, 80°C, 90°C and 100°C.
Figure 14. Raman spectra of 20% w/v ovalbumin samples at pH 9 before and after heating at 60°C, 70°C, 80°C, 90°C and 100°C.
Also, the peak for the S-S bond disappeared at 90°C and 100°C, possibly implying either S-S bond cleavage and loss or “burying” of cystine residues at high pH and temperature.

4.2.2. Summary and conclusion

Raman spectroscopy can provide useful information about protein secondary structure and some information about tertiary structure, which represents the environment of the different amino acid side chain such as cysteine or tyrosine etc. It was suggested by the result that temperature changes in the range from 60-100°C had a greater effect on the structure of ovalbumin than pH variation in the pH 6 to 9 range. The formation of stable intermolecular β-sheet structure was the major change observed during the thermal denaturation and aggregation of ovalbumin molecules. Heat also caused the disruption of ovalbumin tertiary structure, as indicated by exposure of hydrophobic aromatic amino acid side chain such as tyrosine to the solvent and the formation of covalent bond such as disulfide bond between cysteine residues.

4.3. THE PROPERTIES OF THERMALLY INDUCED TRANSPARENT OR TURBID OVALBUMIN GELS

In this section, different kinds of ovalbumin gel (transparent, translucent and opaque) were prepared by adjusting the pH and salt concentration of the heating media. The structural and functional properties of these gels were then investigated with several physicochemical techniques. The objective of this study was to understand the structural basis for the various appearances and textures of thermally-induced ovalbumin gels.
4.3.1. Properties of ovalbumin gel

After heating at 80°C for one hour, ovalbumin samples (10% w/v) at pH 5.5 formed very weak, turbid gels regardless of the salt concentration. Opaque but relatively strong gels were observed at pH 3.5 although a slightly translucent gel formed for the sample in the absence of added salt. At pH 7.5, ovalbumin at zero salt concentration remained as a transparent solution after heating. With increasing salt concentration, the turbidity as well as the gel strength increased at this point.

4.3.1.1. Raman spectra analysis

The Raman spectra of ovalbumin solutions and gels formed by heating at pH 3.5, 5.5 and 7.5 without adding NaCl are shown in Fig. 15, 17 and 19, respectively. The state of ovalbumin samples at these conditions was translucent gel, opaque gel and transparent solution, respectively.

The Raman spectra of the ovalbumin samples with 80 mM NaCl at pH 3.5, 5.5 and 7.5 are also shown in Fig. 16, 18 and 20, respectively. At this condition, opaque gels were formed at pH 3.5 and 7.5 and turbid sponge like gel at pH 5.5.

The spectra of ovalbumin molecules showed that regardless of the pH and salt concentration, the amide I band shifted to higher wavenumber and the intensity of amide III region at around 1240 cm\(^{-1}\) increased after after heat-gelation. This observation suggested the formation of more \(\beta\)-sheet structure, which is consistent with previous reports that ovalbumin forms extensive regions of antiparallel \(\beta\)-sheet structure after thermal gelation (Painter and Koenig, 1976b; Mine et al., 1990).
Figure 15. Raman spectra of ovalbumin solution (10% w/v) and gel formed at pH 3.5 with 0 mM NaCl.

Figure 16. Raman spectra of ovalbumin solution (10% w/v) and gel formed at pH 3.5 with 80 mM NaCl.
Figure 17. Raman spectra of ovalbumin solution (10% w/v) and gel formed at pH 5.5 with 0 mM NaCl.

Figure 18. Raman spectra of ovalbumin solution (10% w/v) and gel formed at pH 5.5 with 80 mM NaCl.
Figure 19. Raman spectra of ovalbumin solution (10% w/v) and gel formed at pH 7.5 with 0 mM NaCl.

Figure 20. Raman spectra of ovalbumin solution (10% w/v) and gel formed at pH 7.5 with 80 mM NaCl.
Heating ovalbumin also produced a decrease in the intensity ratio of tyrosine doublet from 1.1 to 0.8, implying that heat treatment increased the exposure of tyrosine side chains. This conclusion was also supported by the change in the hydrophobicity as detected by fluorescence probe measurement (see section 4.3.2.3.). The exposure of aromatic groups increased the binding of hydrophobic fluorescent dye to produced increased ANS fluorescence intensities.

The SS stretching band around 525 cm\(^{-1}\) were barely visible before heating, probably because the disulfide bonds of ovalbumin in the native protein were buried and not readily to be detected. This band may also be difficult to detect in these spectra because the ovalbumin concentration was 10 % w/v instead of 20 % w/v, which was too low to produce a satisfactory signal to noise ratio for the SS stretching region. Nevertheless, this band at 525 cm\(^{-1}\) increased in intensity after heating, suggesting the formation of SS bonds.

The secondary structure composition determined by RSAP of the amide I band of ovalbumin samples before and after heating is shown in Fig. 21 and 22, which represent the fraction of \(\alpha\)-helix and \(\beta\)-sheet structure, respectively. As observed for the model peptides, the content of \(\beta\)-sheet structure increased and the \(\alpha\)-helix and random coil content decreased after heating. The effects of ionic strength on the secondary structure were small. The observation was that ovalbumin molecules retained high content of secondary structure after heat-denaturation, relative to denaturation induced by concentrated guanidine hydrochloride reported by Kitabatake and Doi (1993).
Figure 21. The α-helix structure of ovalbumin solutions (10% w/v) before and after being heated at different pH. The legends ‘a’ and ‘b’ mean after and before heating, respectively.
Figure 22. The β-helix structure of ovalbumin solutions (10% w/v) before and after being heated at different pH. The legends 'a' and 'b' mean after and before heating, respectively.
4.3.1.2. Turbidity

The absorbance of the ovalbumin samples at 595 nm was used as an indication of turbidity. The absorbance of samples heated at pH 7.5 increased gradually from 0.07 to 2.4 with increasing ionic strength (Fig. 23), which means a transparent gel was formed at low salt concentration, and the turbidity of the gel was increased with increasing salt concentration. At pH 5.5, the absorbance was always high, and the turbid gels were formed regardless of the salt concentration. At pH 3.5, only ovalbumin sample without adding salt formed slightly translucent gel. These results indicated that a transparent gel only formed at pH distant from the pI and low salt concentration. At this condition, the ovalbumin molecules are charged thus the electrostatic repulsive forces are high enough to compensate with the hydrophobic attractive forces between heat denatured ovalbumin molecules. Therefore molecules formed more uniform linkages instead of the large random aggregates which are usually the reason for the formation of turbid gels.

4.3.1.3. Gel strength

Figure 24 shows the typical deformation curves of ovalbumin gels at pH 7.5 with salt concentration of 20, 40, 60 and 80 mM, respectively. The gel strength, expressed as peak force (Newton) multiplied by the distance (mm) to the peak, varied with pH and ionic strength (Fig. 25).

The strength of the gel formed at pH 7.5 increased with the salt concentration and reached the highest value at 80 mM NaCl. The gels formed at pH 3.5, though they were opaque, had relatively high strength especially for the gel at high salt concentration. At pH above or below the isoelectric point, the protein was charged, and there were fewer
Figure 23. The turbidity of 10% w/v ovalbumin samples before and after heating expressed as absorbance at 595 nm.
Figure 24. Deformation curve of ovalbumin gels formed by heating at 80°C for 1 hr at pH 7.5 and salt concentration of 20, 40, 60 and 80 mM.

Figure 25. Gel strength of ovalbumin gels heating at different pH and salt concentration, 7.5/20 means being heated at pH 7.5 and 20 mM NaCl. There is no 7.5/0 data point since ovalbumin did not form a gel after being heated at this condition.
intermolecular protein-protein interaction crosslinks due to the electrostatic repulsion. At these conditions, the ovalbumin molecules in the gel connected with one another to form a strand-shaped structure arranged in a homogeneous network that produced a much stronger gel. On the other hand, the gel formed at pH 5.5 had a very poor gel strength, since large aggregates are formed in the gel through extensive protein-protein interaction. The presence of compact rigid protein aggregates also causes a less homogeneous gel network so that the resulting gel was much weaker, turbid and granular.

4.3.1.4. Solubility

The solubility of the ovalbumin gels in the eight different buffers is shown in Fig. 26. All gels were highly soluble in SDS solution and were slightly more soluble in SDS with mercaptoethanol, suggesting that hydrophobic interactions between the exposed aromatic and aliphatic amino acid groups are the primary forces for gelation.

Although the exchange of the sulfhydryl groups and the disulfide linkage may also play a role in the gel formation, this was not the primary force since 1% mercaptoethanol could only partially dissolve the gel. Moreover, hydrogen bonding between the β-sheet structures of the ovalbumin molecules may also contribute to gel formation because all of the gels tested showed moderately soluble in 8 M urea and in urea plus mercaptoethanol.

The gel formed at pH 7.5 was slightly more soluble than the gels formed at pH 5.5 and 3.5 in all of the buffers, probably because the gel matrix is more accessible to the solvent than the large aggregates present in the opaque or turbid gel.
Figure 26. Solubility of ovalbumin gels formed at 0 and 80 mM NaCl in different dissociating media. Bars represent the standard deviation of duplicate samples. There are no data points for pH 7.5 and 0 mM NaCl because only transparent solution was formed after heating at this condition.
With the exception of solubilizing media containing SDS, lower solubility was observed in all solubilizing buffers for gels formed in the presence of 80 mM NaCl than in the absence of salt. These results suggest that hydrophobic interactions are enhanced in the presence of salt.

4.3.2. Other physicochemical properties

4.3.2.1. Zeta potential

The zeta potential of the ovalbumin solutions before and after heating is shown in Fig. 27. Ovalbumin at pH 7.5 had highest negative charge among the three pH conditions compared. The electrostatic repulsive force of ovalbumin at this pH was sufficient to offset or counter the increase in hydrophobic attractive force between ovalbumin molecules during heat-denaturation. Thus, linear ovalbumin aggregates were formed, and transparent gels resulted from crosslinks of the linear aggregates. High net charge restricted thermal aggregation due to repulsive force, which explains why ovalbumin did not form a gel at low salt concentration at pH 7.5. Higher salt concentration could overcome the net charge of the protein to result in ovalbumin gel formation.

At pH 3.5 or with higher salt concentration at pH 7.5, the electrostatic repulsive force can only partially compensate for the hydrophobic attractive force, so heat-denatured ovalbumin formed a mixture of linear and large cluster aggregates. Under these conditions, such aggregates occur simultaneously in the gel network to result in formation of the translucent or opaque gels. At pH 5.5, the charge on ovalbumin is low
enough that intermolecular hydrophobic interactions are very high, and only large cluster of aggregates were formed, resulting in soft, turbid gels.

In fact, the result in Fig. 27 shows that the ovalbumin is more highly charged at pH 5.5 (≈ -6 to -12) than at pH 3.5 (≈+3 to +4). The reason why only turbid gels were formed at pH 5.5 while translucent and opaque gels were formed at pH 3.5 is not clear. It is also noticeable that the electrostatic charge of ovalbumin was slightly changed after heating. The linear regression trendline was drawn through the three charge point. The isoelectric point is estimated to be 4.4 before heating and 3.8 after heating from the intersection of the line and the x axis.

Figure 27. The Zeta potential of ovalbumin solution before and after heating.
4.3.2.2. SH/SS groups

To measure sulphydryl group content, the denaturing reagent 8 M urea was used initially but resulted in underestimation of thiol content. Evidently SH groups are inaccessible and are not exposed enough to be detected even by 8 M urea. It was also reported (Takeda et al., 1992) that urea is unable to expose all the SH groups of ovalbumin even at a concentration of 9 M. Therefore, 1.6 M guanidine thiocyanate was used throughout subsequent experiments for consistency with the conditions used in the measurement of the disulfide group.

The number of sulphydryl and disulfide groups of the ovalbumin samples measured in the presence of 1.6 M guanidine thiocyanate before and after heating is shown in Table 4. It can be seen that the sulphydryl group content was still underestimated, indicating that most sulphydryl residues are located in the interior of the protein. It was likely that there were four sulphydryl groups and one disulfide bond before heating. Three of the four sulphydryl groups were more reactive and can be detected in the presence of 1.6 M guanidine thiocyanate. After heating, SS bonds formed by SH oxidation or SH/SS interchange reaction. Only two sulphydryl groups were detected after heating, one was reactive while the other was buried. Two disulfide bonds were present after heating.

Although ovalbumin is reported to contain four SH groups and one SS bond, there have been conflicting reports, possibly owing to sulphydryl/disulfide interchange (Li-Chan and Nakai, 1989a). Three of the four sulphydryl groups have been shown to be more reactive (Diez et al., 1964), which is consistent with the results shown in Table 4.
Table 4. The sulphydryl group and disulfide bond content of ovalbumin samples before and after heating

<table>
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<th>salt concentration (mM)</th>
<th>SH groups (mol SH/mol protein)</th>
<th>total (mol SS+SH/mol protein)</th>
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<td>after heating</td>
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<tr>
<td>80</td>
<td>3.26</td>
<td>0.69</td>
</tr>
</tbody>
</table>
4.3.2.3. Hydrophobicity

The hydrophobicity of ovalbumin samples before and after heating at 80°C is shown in Fig. 28 and 29. The hydrophobicity of the ovalbumin samples before heating was very low, suggesting that most of the hydrophobic groups were buried in the native state. However the hydrophobicity increased significantly after heating, indicating the remarkable exposure of many of the hydrophobic groups upon heating. The same result was also obtained by others (Tani et al., 1995). These authors used the ANS probe to measure the surface hydrophobicity of heat-denatured globular proteins including ovalbumin and found that ovalbumin significantly increased ANS fluorescence after heat denaturation. The Raman spectra of the ovalbumin samples also supported this result. The decrease in the intensity ratio of the tyrosine doublet (835 cm⁻¹/856 cm⁻¹) from 1.1 to 0.8 suggested a change in tyrosine environment from buried to more exposed form. Comparing the samples after heating, the ovalbumin solution heated at pH 7.5 and 3.5 gives higher hydrophobicity reading than the samples heated at pH 5.5, suggesting that the hydrophobic groups are more exposed under conditions that promote the homogeneous gel formation. It appears that, the net charge on the protein causes the protein to become extended. At the isoelectric pH, this effect is minimized. This charge repulsion effect promotes denaturation and exposes more hydrophobic groups to solvent but with less aggregate formation.

4.3.3. Summary and conclusion

Transparent, opaque and turbid ovalbumin gels were obtained in this study. When pH is far from the isoelectric point of ovalbumin molecules and the ionic strength was low, the gels were transparent. The turbidity of the gel increased as the increasing of ionic strength.
Figure 28. Surface hydrophobicity of ovalbumin solutions before incubation at 80° as a function of pH and salt conditions. 1-anilinonaphthalene-8-sulfonate (ANS) was used as the fluorescent probe for hydrophobicity determination.

Figure 29. Surface hydrophobicity of ovalbumin solutions after incubation at 80° as a function of pH and salt conditions. 1-anilinonaphthalene-8-sulfonate (ANS) was used as the fluorescent probe for hydrophobicity determination.
Turbid gels were formed at pH near the isoelectric point and/or at high ionic strength. Transparent and opaque gels were harder and more elastic than the turbid gels, which were soft and had poor elasticity.

Raman spectroscopy clearly demonstrated that conformational changes accompanying the thermal gelation of ovalbumin involved the transformation of α-helix and random coil to antiparallel β-sheet structure regardless of the appearance of the gels.

The gels were fully solubilized by 1% SDS and only partially soluble in 8 M urea or 1% mercaptoethanol. The surface hydrophobicity of ovalbumin, measured by the fluorescence probe ANS, increased significantly after heat-denaturation. These results indicated that thermally induced ovalbumin were formed primarily by noncovalent crosslinks such as hydrophobic and electrostatic interactions or hydrogen bonds. Formation of thermally induced disulfide bonds was not a prerequisite for the formation of the gels. The zeta potential measurement suggested that ovalbumin possessed high net charge at pH conditions in which the transparent or translucent gels were formed. Thus the compensation between hydrophobic interactions and electrostatic repulsive forces influenced gel turbidity and hardness.

4.4. THE PROPERTIES OF THERMALLY INDUCED TRANSPARENT OR TURBID EGG WHITE GELS

The same approach used for studying ovalbumin gels in the previous section was applied to whole egg white, which is more applicable to real food systems. Transparent, translucent and opaque egg white gels were prepared by heating at various conditions of
pH and salt concentration. The structural and functional properties of these gels were then investigated with several physicochemical techniques to understand the structural basis for the variable appearance and texture of thermally-induced ovalbumin gels.

### 4.4.1. Properties of the gel

As for the purified ovalbumin, transparent gels were obtained from fresh egg white by heating at pH away from pI. A transparent gel was formed at pH 3.5 for egg white compared to pH 7.5 for ovalbumin, indicating the average isoelectric point is more basic for egg white than for ovalbumin. However, the transparent gel formed by egg white was slightly yellowish.

#### 4.4.1.1. Raman spectra analysis

The Raman spectra of egg white solutions at pH 3.5, 5.5 and 7.5 in the absence of NaCl before and after heating at 80°C are shown in Fig. 30-32. The egg white gels formed at pH 3.5, 5.5 and 7.5 after incubation at 80°C were transparent, turbid and opaque, respectively.

Figures 33-35 show the Raman spectra of the egg white solutions with 80 mM NaCl at pH 3.5, 5.5 and 7.5. As observed for ovalbumin gel, opaque egg white gels were formed at pH 3.5 and 7.5 while turbid gel formed at pH 5.5.

The Raman spectra of the egg white solutions at various conditions were similar to those of ovalbumin at the same conditions. This result is, perhaps, not surprising because ovalbumin is the most abundant protein in egg white and the other major protein, ovotransferrin, is a poor Raman scatterer (Li-Chan and Nakai, 1989a). However, the
signal to noise ratio in the Raman spectra of egg white solutions after heating was poor, probably because of the interference of other minor protein components in egg white.

The secondary structure composition derived by RSAP of the egg white samples before and after heating is shown in Table 5. The change in secondary structure produced by heating was similar to that of pure ovalbumin sample. There was an increase in β-sheet structure and a decrease in α-helix and random coil structure. This result agrees with Painter and Koenig’s conclusion (1976b) that the formation of stable intermolecular β-sheet structure is the major consequence of the thermal denaturation and aggregation of egg white.

![Raman spectra of egg white before and after heating](image)

**Figure 30.** Raman spectra of egg white before and after heating at pH 3.5 and 0 mM NaCl
Figure 31. Raman spectra of egg white before and after heating at pH 5.5 and 0 mM NaCl.

Figure 32. Raman spectra of egg white before and after heating at pH 7.5 and 0 mM NaCl.
Figure 33. Raman spectra of egg white before and after heating at pH 3.5 and 80 mM NaCl.

Figure 34. Raman spectra of egg white before and after heating at pH 5.5 and 80 mM NaCl.
Figure 35. Raman spectra of egg white before and after heating at pH 7.5 and 80 mM NaCl.

Table 5. The secondary structure fraction of egg white samples before and after heating estimated by RSAP.

<table>
<thead>
<tr>
<th>Salt concentration</th>
<th>α-helix</th>
<th>β-sheet</th>
<th>Random coil</th>
<th>α-helix</th>
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<td>0.21</td>
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</table>
4.4.1.2. Turbidity

The turbidity of egg white solutions and gels is shown in Fig. 36. Egg white protein solution at pH 5.5 exhibited high turbidity even before heating. Proteins have the lowest solubility at pH near their isoelectric point because of the high hydrophobic interaction, and pH 5.5 is very close to the isoelectric point of egg white proteins. As the salt concentration increased from 0 to 80 mM, turbidity decreased slightly due to increasing solubility of egg white proteins. This increase in solubility occurs because the potential salt-binding sites of the proteins are occupied at high salt concentration, and thereby reduce hydrophobic intermolecular interactions.

After thermal treatment, transparent gels were formed only at very low salt concentration at acidic pH (pH 3.5), because the average pI of egg white proteins is higher than that of pure ovalbumin. At acidic pH the protein is more highly charged, and intermolecular electrostatic repulsion is great enough to compensate the intermolecular hydrophobic interactions. The result is formation of a uniform protein gel network. In addition, the egg white solutions only formed transparent gels in a very narrow range of pH and ionic strength, because egg white is composed of heterogeneous proteins. Different proteins in egg white have different isoelectric points (e.g., the isoelectric points of ovalbumin and lysozyme are 4.5 and 10.7, respectively). Thus the pH control is more crucial to formation of a transparent egg white gel.

4.4.1.3. Gel strength

Figures 37 and 38 show the deformation curves of egg white gels formed without NaCl at pH 3.5, 5.5 and 7.5, and the strength of the different egg white gels, respectively.
Figure 36. Turbidity of egg white solutions before and after heating expressed as absorbance at 595 nm.
Figure 37. The deformation curve of egg white gels formed by heating at 0 mM NaCl and 80°C for one hour at pH 3.5, 5.5, 7.5, respectively.

Figure 38. The strength of egg white gel formed at different heating conditions, 3.5/ means being heated at pH 3.5 and 0 mM NaCl.
The average charges of egg white proteins were high at pH away from their average isoelectric point. When the proteins were highly charged, the intermolecular electrostatic repulsive forces restricted the attractive intermolecular forces. Thus, the heat-denatured protein formed intermolecular crosslinks to result in a homogeneous network and a much stronger gel. However, the gels formed at pH 7.5 also had low gel strength, probably because of the influence of the basic proteins in egg white such as lysozyme.

On the other hand, the gel formed at pH 5.5 had a very poor gel strength because large aggregates are formed in the gel by protein-protein interaction. The presence of compact rigid protein aggregates also causes a less homogeneous gel network and so the resulting gel was much weaker, turbid and granular.

4.4.1.4. Solubility

The solubility of egg white gel is shown in Fig. 39. All the gels were highly soluble in SDS plus mercaptoethanol (>90% solubility) regardless of pH and salt concentration. The solubility in SDS alone of the gels formed without salt were 83%, 62% and 68% at pH 3.5, 5.5 and 7.5, respectively. The gels formed at 80 mM salt were slightly less soluble than those without salt except at pH 5.5. At this pH the solubility was much lower (44%). Urea and mercaptoethanol only partially solubilized the gels. Opaque gels formed at high ionic strength and pH close to the isoelectric point were the most difficult to solubilize. These results suggested that hydrophobic interactions are the main force underlying gel formation and that although hydrogen and disulfide bonds are also formed during gel formation they are not the primary forces stabilizing the gel network. This was consistent with the suggestion by Clark (1992) that a protein gel network is usually formed.
Figure 39. Solubility of egg white gels in various dissociating media. Gels were formed by heating egg white as a function of pH and salt concentration at 80°C for 1 hr. Details of the dissociating media are given in the materials and methods section.
through non-covalent intermolecular cross-linkages such as hydrophobic interaction, hydrogen bonds or electrostatic interactions and less frequently by covalent interactions such as disulfide bonds.

The tendency of the solubility of egg white gels was similar to that of ovalbumin. However the solubility of egg white gels were generally lower than that of ovalbumin gels, probably because there were heterogeneous proteins of egg white, which might influence the solubility of the gels formed. There were also greater involvement of disulfide bonds in the egg white gels compared to ovalbumin gels especially at pH 5.5 and 7.5, because the addition of β-mercaptoethanol to SDS and urea obviously increased the solubility of egg white gels while there were no apparent influence of β-mercaptoethanol to the solubility of ovalbumin gels in SDS and urea.

4.4.2. Other physicochemical properties

4.4.2.1. Zeta potential

Figure 40 shows the zeta potential of the egg white solution before and after heating. The linear regression trendline was drawn through the three charge point. The weight average isoelectric point of egg white was estimated as 5.0 before heating and 4.3 after heating, slightly higher than pure ovalbumin solution. This explains the reason that transparent gel was formed at pH 3.5 for egg white and at pH 7.5 for pure ovalbumin. At pH 3.5, most of the egg white proteins were positively charged, the electrostatic repulsion
was high enough to compensate with the hydrophobic attractive interaction between the different protein molecules. The protein molecules can only form linear aggregates and these linear aggregates crosslinked with each other to form a gel network and the appearance of the gel was transparent.

A similar result as for the ovalbumin solutions was observed for egg white gels. In fact the charges at pH 3.5 ($\approx +5$ to $+10$) were less than the charges at pH 7.5 ($\approx -15$), however transparent gel was only formed at pH 3.5. The reason was not clear for the result, possibly because of the influence of basic protein presenting in egg white.

Figure 40: The Zeta potential of egg white solutions before and after heating.
4.4.2.2. SH/SS groups

The literature value of the average number of total available sulfhydryl (SH) groups and disulfide (SS) bonds in native egg white have been reported to be 50.0-51.7 µmol/g protein and 79.9-84.5 µmol/g protein, respectively (Mine et al., 1990). Table 6 shows the number of sulphhydryl and disulfide groups of the egg white samples before and after heating at 80°C. The data before heating is consistent with the literature value. However, there was a decrease in the number of SH groups and increase of the number of SS bonds for the heated samples. This indicates that heating causes egg white proteins to polymerize by sulfhydryl-disulfide exchange or the oxidation of sulfhydryl group to disulfide bond.

<table>
<thead>
<tr>
<th>Salt concentration</th>
<th>SH groups (µmol SH/g protein)</th>
<th>SS bonds (µmol SS/g protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>before heating</td>
<td>after heating</td>
</tr>
<tr>
<td>pH 3.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>50.6</td>
<td>21.8</td>
</tr>
<tr>
<td>20</td>
<td>49.8</td>
<td>18.7</td>
</tr>
<tr>
<td>80</td>
<td>49.9</td>
<td>16.7</td>
</tr>
<tr>
<td>pH 5.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>50.7</td>
<td>16.8</td>
</tr>
<tr>
<td>80</td>
<td>50.8</td>
<td>13.7</td>
</tr>
<tr>
<td>pH 7.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>51.0</td>
<td>19.8</td>
</tr>
<tr>
<td>80</td>
<td>49.3</td>
<td>14.9</td>
</tr>
</tbody>
</table>

4.4.2.3. Hydrophobicity

Table 7 shows the hydrophobicity of egg white protein measured by ANS fluorescence probe before and after heating. It can be seen that the hydrophobicity of egg
white proteins at pH 3.5, at which transparent gels were formed, was higher than at pH 5.5 and 7.5. At pH distant away from the isoelectric point, the net charge may cause the protein molecules to become extended and this effect allowed more hydrophobic groups to be exposed and thus higher hydrophobicity.

The exposed hydrophobic residues on the molecular surface remarkably increased with the heat denaturation of egg white protein. Similar as ovalbumin, higher hydrophobicity were generally obtained at the conditions which transparent gels are formed, i.e. at pH regions far from the isoelectric point of the main protein components of egg white. At these conditions, the net charge of the protein molecules caused the mutual repulsion of protein molecules to increase. In consequence, protein-protein interactions by hydrophobicity were reduced, this may induce protein molecules to connect with one another head to tail and form an uniformed network, allowing more hydrophobic group exposed to the surface.

Table 7. Surface hydrophobicity of egg white solution before and after heating measured by ANS probe.

<table>
<thead>
<tr>
<th></th>
<th>Before heating</th>
<th>After heating</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH=3.5</td>
<td>0</td>
<td>710</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>850</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>880</td>
</tr>
<tr>
<td>pH=5.5</td>
<td>0</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>290</td>
</tr>
<tr>
<td>pH=7.5</td>
<td>0</td>
<td>220</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>270</td>
</tr>
</tbody>
</table>

5150 5120 5050 4150 4120 5880 5420
4.4.3. Summary and conclusion

The conditions to form the transparent heat-induced egg white gel were generally comparable to those of ovalbumin.

Transparent, opaque and turbid egg white gels were also obtained by fine adjustment of the pH and ionic strength. When pH was 3.5, which was lower than the isoelectric point of most egg white protein molecules and no salt was added to the protein solution, the gels were transparent. The turbid gels were formed at pH around the isoelectric point and/or at high ionic strength. The transparent gels formed at pH 3.5 were harder and more elastic than the turbid gels formed at pH 5.5, which were soft and had poor elasticity.

The result from Raman spectroscopy clearly demonstrated that conformational changes accompanying the thermal gelation of egg white, regardless of the appearance of the gels, involved the transformation of α-helix and random coil to antiparallel β-sheet structure.

The results from gel solubility indicated that thermally induced egg white gels were mainly formed by noncovalent crosslinks such as hydrophobic and electrostatic interaction or hydrogen bonds. Formation of thermally induced disulfide bonds was also involved during the gel formation, especially at pH 5.5 and 7.5.

The surface hydrophobicity of egg white protein molecules, measured by fluorescence probe ANS, showed a significant increase after heat-denaturation of egg white proteins. The result of zeta potential suggested that egg white protein molecules contain high net charges at pH conditions in which the transparent or translucent gels were
formed. Thus the compensation between hydrophobic interactions and electrostatic repulsive forces influenced gel turbidity and hardness.
Chapter 5. General Conclusions

Hen egg white proteins have been extensively utilized as ingredients in food processing because of their unique functional properties, among which thermal gelation is one of the most important properties of egg white proteins. The thermal setting required for cake batters, custards and puddings has been attributed in part to the denaturation and coagulation of egg white proteins (Mine, 1995). Egg white proteins usually form opaque gel upon heating. However, by controlling the pH, ionic strength and heating procedure, it is possible to obtain desirable transparent gels from egg white protein solutions, which are more nutritious than the similar gels formed from polysaccharide and gelatin. This characteristic property has a high potential in the food processing industry for providing food gels with high nutritious value as well as desirable appearance and texture.

The main focus of this project was to study and compare the structure and functional properties, including the changes of protein structure upon gelation, the gel turbidity and strength, the charge, hydrophobicity, solubility and the SH versus the SS group of the gel, of thermally induced transparent or turbid gels formed from pure ovalbumin or hen egg white.

Depending on the conditions of the heating medium, ovalbumin and whole egg white formed transparent solutions, transparent gels, opaque gels and turbid gels after thermal-gelation. Generally, the transparent solution and transparent gels were only formed by heating the protein solutions at pH distant from the isoelectric point and very low salt concentration or no salt. As the salt concentration increased, the turbidity of the gels increased as well. Turbid gels are formed by heating ovalbumin or egg white solutions at
pH close to their isoelectric point and the opaque gels were formed at pH away from the isoelectric point and relatively high ionic strength (i.e. high salt concentration).

The rheological properties of the heat-set ovalbumin and egg white gels also strongly depend on the pH and ionic strength of the solvent with which the protein is mixed and heated. The ionic strength of the solvent had a less pronounced effect than the pH. The transparent and opaque gels formed by heating at pH distant from the isoelectric point of the proteins had higher strength, while the turbid gels formed at pH close to the isoelectric point had lower strength.

The results of Raman spectroscopy showed that conformational changes of ovalbumin and egg white proteins upon thermal gelation involved the formation of stable intermolecular β-sheet structure. In other words, the heat-denatured ovalbumin and egg white proteins did not take on a random-coiled structure but rather retained a considerable amount of secondary structure, with the exposure of some hydrophobic regions to the solvent environment. This was distinguished from either the native or the fully denatured forms and it could be suggested that ovalbumin and egg white proteins took on a molten globule like state at high temperature. Heat denaturation involved the transition of protein molecular conformation from the native state to a molten globule state, followed by the association of heat-denatured molecules either in a highly ordered manner in a transparent protein gel or in random aggregates in a turbid gel.

The gels were fully solubilized by 1% SDS and only partially soluble in 8 M urea or 1% mercaptoethanol. This indicates that thermally induced ovalbumin and egg white gels were mainly formed by noncovalent crosslinks such as hydrophobic and electrostatic
interaction or hydrogen bonds. Formation of thermally induced disulfide bonds was also involved during gel formation; however, it may not be the primary force.

Other experiments performed in this project including measurement of the net charges of ovalbumin and egg white protein solutions, determination of sulfhydryl groups and disulfide bonds and investigation of the surface hydrophobicity of the proteins before and after heating also supported the above conclusions.

The charges were measured as the zeta potentials of ovalbumin and egg white protein solutions. It was found that protein solutions had high zeta potentials (either positive or negative) at pH 3.5 or 7.5, in which condition the transparent or translucent gels were formed. At pH 5.5, which was the pH near the pI of protein solutions, the zeta potentials of the protein solutions were close to zero.

The sulfhydryl groups and disulfide bonds were determined by Ellman’s or NTSB reagent, respectively. The result showed the decrease of sulfhydryl groups and increase in disulfide bonds, indicating the sulfhydryl/disulfide interchange or the oxidation of the sulfhydryl groups to form disulfide bonds during heating.

The investigation of hydrophobicity by ANS fluorescence probe demonstrated a significant increase of hydrophobicity of ovalbumin and egg white proteins after heating. This result suggested that heating caused the exposure of some hydrophobic regions to the solvent environment and the hydrophobic interactions were the main force involved in gelation.

Application of thermal induced transparent egg white gel in food processing is one of the recent new techniques that are likely to be the highly promising approaches. However, transparent and firm gel was only obtained within relatively narrow ranges of
pH and ionic strength (Doi and Kitabatake, 1994). This would limit the practical application of the transparent gel as a food material. Preparation of a hard, transparent gel in wider ranges of pH and ionic strength is of most interest to food scientists. The limits can be overcome, as suggested by some of the researchers (Kitabatake and Doi, 1993), by the introduction of the two step heating method. In this method, a transparent egg white gel can be prepared when the salt free egg white solution is heated prior to the addition of high concentration of salt. The two-step method has been applied to some globular proteins including egg white proteins to form transparent gels over a range of salt concentration (Mine, 1995; Tani et al., 1993). This technique can offer a convenient way of producing transparent ovalbumin or egg white gels for practical uses as a new food ingredient.

Other than fresh egg white, spray dried egg white powders have also been used widely in the food processing industry. Studies have shown that heating egg white proteins in a dry state significantly improve their functional properties including gelling property. However, there is very limited study about the possibility of making transparent gels from dry egg white powder and how to make them, which would be a very interesting topic for the continuation of this study.
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