Effects of high pressure and heat processing on the structure and rheological properties of food proteins

by

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A thesis Submitted to the University of Surrey in Partial Fulfilment of the Requirements for the Degree of Doctor of Philosophy in the Faculty of Science

School of Biomedical and Molecular Sciences
University of Surrey

December 2003

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ABSTRACT

This study evaluated the effect of heat and high pressure processing of whey protein isolate, β-lactoglobulin, egg albumen, ovalbumin and their binary mixtures. Rheological studies of whey proteins (15% w/w) in distilled water indicated that gels made by heating at 90°C for 30 min were stronger and more elastic compared with pressure-treated gels (400-600 MPa for 20 min). Gel strengths at higher pressure (650-800 MPa) were similar to heated samples. In contrast, egg albumen proteins (15% w/w) in distilled water showed no gelation below 500 MPa but increased in strength with increasing pressure, although values remained below those of heat-induced gels. Heat and pressure treatment of whey/egg albumen protein mixtures (10:5) produced gels stronger than expected indicating synergistic interactions.

Sucrose influenced gelation and interactions, with 20% sucrose being the optimal for egg albumen and whey proteins. Sucrose addition produced weaker pressure-treated gels compared with heated gels. Addition of 1% NaCl produced stronger whey protein gels compared with egg albumen but weaker mixed whey/egg albumen gels. Combined heat (50°C or 60°C) and pressure (600 MPa) produced weaker gels compared with heat treatment alone, but stronger gels than pressure treatment alone for both whey and egg albumen proteins and mixtures. Values increased with increasing temperature due to greater protein unfolding, as shown by Differential Scanning Calorimetry.

FT Raman spectroscopy indicated that both heat and high pressure affected α-helix, β-sheet structure, hydrophobic interactions and disulphide bonds. Heat caused greater changes in disulphide bonds and β-sheet structures but pressure produced greater changes in hydrophobic interactions.

Self deconvolution of the Amide I band showed quantitative changes in secondary structures. Random coil increased in high pressure treated (600 MPa, 30 min) β-lactoglobulin, whereas, for ovalbumin, β-turns doubled. The different mechanisms of gelation observed for heating and high pressure treated egg albumen, whey proteins and their mixtures can provide novel textures.
OBJECTIVES:

The aims of this study were to evaluate the effect of heat and high pressure processing on the structure and physical chemical properties and texture of selected food proteins.

The key objectives are outlined below:

I. To determine the structural and physicochemical properties of egg albumen, ovalbumin, whey protein isolate and β-lactoglobulin using small and large deformation rheology, FT Raman spectroscopy, differential scanning calorimetry and microscopy.

II. To compare the effect of high pressure, heat and combined high pressure/heat treatment on the structure and texture of egg albumen and whey proteins.

III. To relate the rheological behaviour of single and mixed proteins systems to the structures of gels formed under high pressure and heat treatment and determine the nature of protein-protein interactions.

IV. To investigate the effect of sodium chloride and sugar on the rheological properties and molecular interactions of unheated, heated and high pressure treated egg albumen and whey proteins.
ACKNOWLEDGEMENTS

Firstly, I would like to thank my two supervisors, Prof. Nazlin Howell (School of Biomedical and Molecular Sciences) and Prof Alf Adams (School of Electronics and Physical Sciences, Department of Physics) for their support and encouragement throughout the course of my work.

I am grateful to Dr. H. Herman (Chemistry) for his tireless support, for making Raman spectroscopy such a joy to understand and many helpful comments and discussions. I owe much gratitude to Nicola Walker (Chemistry) for her assistance with setting up Raman spectroscopy.

Particular thanks to all technical staff in the School of Biomedical and Molecular Sciences for their help in the laboratory.

Many thanks to Prof. D. Ledward and Aklile at the University of Reading for use of High pressure rig.

Finally I would like to thank my family and friends in particular my dearest brother Tendai M. Ngarize for his support, Tselo Dobeli (Switzerland), Khosi Manaka, Dr Lwazi Mdyesha and Susan Nyandoro for their friendship and support.

A studentship from the EPSRC is greatly acknowledged.
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Plate 4.3  TEM of egg albumen protein gel (15% w/w) heated at 90°C for 30 min (x 22 000).
Plate 4.4  TEM of whey protein gel (15% w/w) heated at 90°C for 30 min (x 22 000).
Plate 4.5  TEM of mixed protein gel 7.5:7.5 (15% w/w) high pressure treated (500 MPa for 30 min) (x 22 000).
Plate 4.6  TEM of mixed protein gel 7.5:7.5 whey/egg albumen (15% w/w) heated at 90°C for 30 min (x 22 000).

XV
1. INTRODUCTION

1.1 Protein structure and physicochemical properties

1.1.1 Amino acids

Proteins are biopolymers that consist of 50-300 amino acid residues. Essentially there are twenty naturally occurring amino acids. Amino acids are amphoteric compounds containing both the acidic group (-COOH) and a basic group (-NH$_2$). The common amino acids have the following general structure:

```
R
\[ \text{H} - \text{C} - \text{COOH (carboxylic group)} \]
\[ \text{NH}_2 \]

(side chain)

\[ \text{NH}_2 \]

(amic acid group)
```

Figure 1.1 Structure of an amino acid

All amino acids have a central carbon atom to which a hydrogen atom, R-group (side chain), an amino group (NH$_2$) and a carboxylic group (COOH) is attached. The central carbon atom is designated the $\alpha$-carbon atom and is a chiral centre; the side chains are commonly designated $\beta$, $\delta$, $\epsilon$, $\sigma$. All amino acids found in proteins encoded by the genome, have a L-configuration at this chiral centre (Berg and Tymoczko, 2002). The R-group, often referred to as the side chain, dictates the characterisation of the amino acid and confers important properties to a protein such as the ability to bind ligands and catalyse biochemical reactions.

Large aliphatic side chains like those found in leucine and isoleucine, or aromatic side chains such as those in phenylalanine and tryptophan are hydrophobic. Conversely, the moieties containing hydroxyl, carboxyl and amino groups are hydrophilic for example, serine, aspartic and lysine (Berg and Tymoczko, 2002). A sulphur atom is present in the side groups of the two amino acids cysteine (S-H) and methionine (S-CH$_3$). The SH group of cysteine is highly reactive and is capable of forming disulphide links which participate not only in the
stabilisation of the secondary structure of proteins, but also in the interactions with other proteins which contain free cysteine groups. A detailed structure of proteins and molecular properties is discussed by Creighton (1993).

1.1.2 The Peptide bond

Amino acids in proteins are joined together by the peptide bond which is the most important single feature of protein structure. The peptide bond occurs when the amino group attached to the alpha carbon of one amino acid, is joined to the carbonyl group attached to the alpha carbon of a second amino acid with the removal of water. The inability of a peptide bond to rotate constraints the conformation of the peptide bond backbone and accounts for the bond's planarity. In most proteins, the peptide bond nearly always has the \textit{trans} configuration, because the steric clashes between groups attached to \(\alpha\)-carbon atoms hinder the formation of the \textit{cis} form (Berg and Tymoczko, 2002).

The ionisation state of an amino acid depends on the pH of its environment; amino acids in solution at neutral pH exist predominantly as dipolar ions (also called \textit{zwitterions}). In the dipolar form, the amino group is protonated (-\(\text{NH}_3^+\)) and the carboxyl group is deprotonated (-\(\text{COO}^-\)). In acid solution, the carboxyl group is un-ionised and the amino group ionised, whereas in alkaline solution the carboxyl group is ionised and the amino group un-ionised. The pH at which the net charge is zero is known as the isoelectric point (pI) and depends on the R group of the amino acid. The ionic behaviour of a protein can be summarized as follows, if it is in solution at a pH higher than its isoelectric point, it will carry a net negative charge, while at a pH lower than its pI value it will be positively charged.

\[
\text{Protein}^{+ve} \rightarrow \text{Protein}^{+ve/-ve} \rightarrow \text{Protein}^{-ve}
\]

\[
\text{Acid} \quad \text{pI} \quad \text{Alkaline}
\]

Figure 1.2 The ionization behaviour of protein

Consequently, pH has a marked effect on the interactions of proteins since it affects the charge carried by amino acid residues (Morris, 1991; Howell, 1992; Comfort, 1995).
Chapter 1. General Introduction

1.1.3 Primary Structure

The primary structure of a protein constitutes the first level of protein structure. It is the covalently linear linked sequence of amino acids in the polypeptide chain. Each protein has a unique sequence of amino acids which includes the location of disulfides bonds, if present (Berg and Tymoczko, 2002).

1.1.4 Secondary Structure

Protein secondary structure is the regular arrangement or orientation of the main-chain atoms without regard to the conformation of side chains or relationships with other segments of the polypeptide chain (Zubay et al., 1995). Depending on where the amino acids are positioned in relation to one another, the polypeptide chain will fold in various ways to produce the three dimensional structure. The three common secondary structures in proteins include α-helices, β-pleated sheets, β-turns and random coil. There are two important aspects which characterise the secondary structure of proteins, (a) the way the protein is folded and (b) the nature of the linkage and bonds which stabilise them. The α-helix is a rod-like structure stabilised by hydrogen bonds between the N-H and CO groups of the main chain. Each residue is related to the next one by a rise in 1.5 Angstrom with 3.6 amino acid residues per turn of helix.

There are two sterically favourable arrangements for the β-sheets, the parallel β-pleated sheet and the anti-parallel β-pleated sheet, based on the relative directions of the two interacting β-strands (Phillips et al., 1994). β-pleated sheets are formed when a polypeptide chain abruptly reverses its direction and is stabilized by hydrogen bonds between the carboxyl groups of a residue on one sheet to an NH group of a residue on the anti-parallel β-strand. β-Sheets are almost fully extended and the distance between adjacent amino acids along a β-strand is approximately 3.5 Angstrom. In addition, β-turns are formed when a characteristic, extended polypeptide chain changes direction and involves a minimum number of residues (Creighton 1993). That which cannot be classified as possessing a periodic structure is usually grouped into a category called random coil or “other”. This designation is not absolute as no portion of a protein three-dimensional structures is truly random or coiled.
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Figure. 1.3 The four different hierarchies of proteins including α-helix and β-pleated sheet adapted from Zubay et al., (1995)(overleaf).

Packaging of the buried helices against other secondary structural elements in the buried core of a protein can lead to distortion since the side chains are on the surface of helices. In particular, proline residues are not compatible with the α-helix conformation (Creighton 1993, Berg and Tymoczko 2002). This is because proline residues induce distortion and the direction of the helix and proline cannot form a regular α-helix due to steric hindrance arising from its cyclic side chains which block the main chain NH group.

1.1.5 Tertiary Structure

Tertiary structure or the three dimensional structure of proteins refers to the spatial arrangement of all atoms in a single polypeptide chain or covalently linked chains (Zubay et al., 1995). It constitutes the native folded form most commonly occurring conformation at room temperature and displays biological activity. The folded structures of most small proteins are roughly spherical and compact with irregular surfaces. The structures of most proteins that have more than 200 residues appear to consist of 2 –3 or more structural units referred to as domains, these domains interact to varying extent (Creighton 1993).

The unifying principle of the tertiary structure is that the protein folds in a manner that allows the interior to consist of entirely non-polar residues such as leucine and phenylalanine and charged residues such as aspartate, glutamate, lysine and arginine to be on the surface of the protein molecule. Thus the polypeptide molecule folds such that the hydrophobic side chains are buried inside and polar charged groups are on the surface. Many α-helices and β-strands are amphipathic, i.e. possess both a hydrophobic core pointing into the protein interior where the hydrophobic effects are dominant and a polar surface which points in to the solution (Berg and Tymoczko 2002).
1.1.6 Quaternary Structure

Protein quaternary structures are produced by the association of separate folded polypeptides (subunits) into an aggregate multimeric structure under specified conditions of pH and temperature without regard to the internal geometry of subunits (Phillips et al., 1994). Thus, when proteins contain more than one polypeptide chain the quaternary structure relates to the spatial arrangement of such subunits. These subunits may be identical polypeptide chains or chemically distant species (Zubay et al., 1995, Berg and Tymoczko 2002). An example of a protein aggregation unit is human haemoglobin. The subunits in the quaternary structure must be in non-covalent association. Haemoglobin contains 4 polypeptide chains (α₂ β₂) held non-covalently in a specific conformation required for its biological function.

1.2 Forces stabilising protein structure

1.2.1 Introduction

Several forces, covalent and non-covalent, determine protein structure. These forces are also involved in interactions between proteins and have been subject to many reviews (Stryer, 1988; Howell, 1992; Koning and Viser, 1992; Creighton 1993; Privalov, 1999). The following section examines these forces in relation to food protein interactions.

1.2.2 Covalent Bonds

Covalent bonds include the peptide bond of the primary structure between amino acids in a polypeptide chain. It has a potential energy of 100 kcal/mol and is therefore not broken during denaturation, which relates to changes in the secondary and tertiary structures only (Howell, 1992). However, hydrolysis with a strong acid/alkali or proteolytic enzymes may break the peptide bond. The covalent bond between two cysteine residues is a disulphide (S-S) bridge. Disulphide bonds are involved in the stabilisation of secondary structure in many proteins for example, β-lactoglobulin (McKenzie, 1967; Creighton, 1993) and in the interactions of proteins. Disulphide bonds are therefore affected by denaturation during heat gelation and foaming (Comfort, 1995). These bonds are strong and have a heat of formation of 50 kcal/mol. Reports indicate that high pressure processing of food has little effect on covalent bonds, resulting in minimal changes in food colour (Ledward 2000, Heremans, 2002); at higher pressures of >300 MPa, sulphhydryl groups may oxidise to S-S bonds in the presence of oxygen (Galazka and...
Ledward, 1998). However, the effect of high pressure on disulphide bonds has not been investigated fully as yet.

### 1.2.3 Non-covalent interactions

#### 1.2.3.1 Electrostatic forces

Electrostatic interactions occur between two charged particles and can form salt bridges that stabilise secondary and tertiary structures. Protein molecules are zwitterions which contain both negative and positive charges depending on their isoelectric point and pH of the environment. Electrostatic interactions are repulsive forces for like charges and attractive for opposite signs of the charges; these forces occur over large distances and vary inversely with distance (Howell, 1992).

#### 1.2.3.2 Van Der Waal forces

Van der Waals forces are weak attractive interaction forces between polar and non-polar molecules. There are two principle van der Waals forces. Attractive forces exist between non-polar entities such as atoms and molecules, attractive van der Waals forces involve interactions among induced dipoles, that arise from fluctuations in the electron charge density of neighbouring non-bonded atoms. Repulsive interactions occur when non-covalently bonded atoms or molecules come very close together. An electron-electron repulsion arises when the electron charge clouds start to interpenetrate and this repulsive forces ensure that two different molecules cannot occupy same space at the same time. Van der Waal forces favour close packing in folded protein structures (Howell, 1992; Zubay et al., 1995).

#### 1.2.3.3 Hydrogen Bonds

Hydrogen bonds are a type of electrostatic interaction force between polar molecules. In hydrogen bonding there is a sharing of an hydrogen atom between an acidic group OH, NH (proton donor) and a basic group (C=O), a proton acceptor. The position of such reactive groups in the secondary structure of a protein influences the interaction between them. This is because water is a dipole and can form hydrogen bonds. Water is therefore known to compete for the reactive groups of proteins and reduce interactions between them. Hydrogen bonds in the interior of proteins can induce more significant enthalpy and entropy changes when compared with those
formed in an aqueous medium, these bonds play an important role in the stabilisation of proteins in the form of both β-sheets and α-helices (Koning and Visser, 1992).

1.2.3.4 Hydrophobic Interactions

In a polar aqueous environment the absence of hydrogen bonding between water and non-polar solute results in what is referred to as hydrophobic interactions. The amino acids tryptophan, leucine and phenylalanine have hydrophobic side groups and entropic effects strongly favour the internal location of these residues, where they are free from interacting with polar water molecules. Electrostatic and hydrogen bonds and van der Waals forces between protein molecules are not favourable energetically in aqueous solutions because of the tendency of polypeptide chains to interact with water. In order to compensate, water molecules interact strongly with each other. This in turn results in strong attractive forces between the non-polar groups causing them to aggregate in water.

The magnitude of hydrophobic interactions can be estimated by measuring the hydrophobicity of proteins for example by using a fluorescent probe such as cis-parinaric acid which is thought to bind aliphatic groups (Kato and Nakai, 1980; Howell, 1992). The native folded state of a globular protein reflects a delicate balance between opposing energetic contributions of large magnitude. Whereas entropic factors favour the packaging of the hydrophobic side chains into the interior regions of globular proteins, enthalpic factors favour placing hydrophilic side chains on the surface of the protein where they interact with water. An increasing temperature favours hydrophobic interaction until very high temperatures are reached when these interactions are disrupted due to increasing kinetic energy of the system (Koning and Visser, 1992).

1.3 Protein denaturation

1.3.1 Definition

Protein denaturation is a phenomenon that involves transformation of a well ordered, folded native structure to an unfolded state under non-physiological conditions, when forces stabilising protein structure are interrupted. Mulvihill and Donovan (1987) defined protein denaturation as “alteration in the original native structure without hydrolysis of primary covalent bonds”, while Sikorski (2001) described protein denaturation as a process involving
unfolding and at least an alteration of the native structure. Implicit in these definitions is the fact that the denaturation process affects the very forces that stabilise proteins. It follows that when proteins are denatured, their properties are completely altered due to the exposure of the functional groups of amino acids previously embedded inside the native structure. The exposed groups largely include hydrophobic residues, which align themselves towards the centre to minimise their interactions with water. As a result, the solubility of a protein is decreased by denaturation as hydrophobic groups are exposed. These newly exposed groups are free to interact with other sites within the protein or other proteins resulting in intermolecular association. Thus, those forces involved in stabilising the native structure are involved in the secondary interactions which may take place following denaturation. These secondary associations can be controlled to produce various phenomena such as gelation and foaming (Comfort 1995). Protein precipitation occurs most readily at the isoelectric point, when it is least stable. Proteins can be denatured by a variety of means which can be classified into two major groups: (a) chemical factors including, pH, chaotropic agents (urea, guanidine hydrochloride) and salts, and (b) physical factors including, temperature, pressure and shear (Sikorski, 2001).

1.3.2 Heat denaturation of proteins

During thermal denaturation, increasing temperature progressively disorders both protein and water structures. Koning and Viser (1992) reported that forces involved in the interactions between proteins are set against the kinetic energy of the molecule. This kinetic energy is driven by the thermal motion of the atoms and it is this thermal energy which allows the molecule to undergo conformational changes, particularly during heating. Thermal denaturation of proteins therefore affects both volume and entropy changes due to increasing kinetic energy of the system. As heating continues, some of the cooperative hydrogen bonds that stabilize the helical secondary structure will begin to break and facilitate unfolding. At this stage, the properties of the protein are altered owing to the exposure of the largely hydrophobic amino acids previously embedded inside the native structure (Ma and Harwalkar, 1990).

The nature of these interactions depends on heating conditions, structure and concentration of protein. For example, heating of β-lactoglobulin solutions in the range of 40 -60°C promotes
exposure of previously buried reactive protein groups and partial unfolding of protein (Kella and Kinsella, 1988). Further, heating of β-lactoglobulin solutions above 70-80°C is characterised by denaturation of the protein molecules and sulfhydryl-disulphide interchange reactions with other proteins that contain a free sulfhydryl group (Phillips et al., 1994, Howell, 1992). Thus heat denaturation involves the breaking and remaking of covalent disulphide bond structures.

### 1.3.3 Pressure denaturation of proteins

High pressure breaks down cell structure and also acts as a tool for obtaining thermodynamic and structural information and conformational properties of proteins. Unlike, other denaturants such as heat or chemicals, pressure acts on interatomic distances of molecular and macromolecular assemblies. The covalent bonds of the primary structure of proteins and low molecular weight biomolecules such as peptides, lipids and polysaccharides are not affected by pressures of 100-1500 MPa because of the insignificant compressibility of the covalent bond (Morild, 1981, Heremans and Smeller, 1998). In general, high pressure stabilises hydrogen bonds, so that secondary structures such as the α-helix and β-sheets which are stabilised by hydrogen bonds are not significantly affected by high pressure (Heremans, 1995, 2002; Galazka and Ledward, 1998). However, pressures above 400 MPa may cause changes in secondary structure, leading to irreversible denaturation depending on the rate of compression and extent of secondary structure rearrangement (Balny and Masson, 1993). The main targets of high pressure are hydrophobic and electrostatic interactions which play an essential role in the formation of tertiary and quaternary structure of proteins as well as dictating protein-protein interactions (Balny and Masson, 1993, Heremans 1995, 2002). Examples of changes in tertiary structure can be seen at pressures above 200 MPa; pressures below 150 MPa usually dissociate oligomeric proteins, while pressures above 150-200 MPa lead to unfolding of the protein and reassociation of subunits from the dissociated oligomer (Balny and Masson, 1993).

High pressure is known to place a constraint on volume of a system such that with increasing pressure a positive volume change (ΔV) causes the equilibrium to shift towards bond rupture. In contrast, a negative volume change (ΔV) causes bond formation (Galakza and Ledward, 1998). The volume of native proteins is the sum of three effects: the compositional or constitutive volume of atoms, the volume of internal cavities (conformational volume) and
solvation volume (Heremans et al., 1997). It follows, that the native conformation of a protein is a delicate balance of ionic, hydrophobic and van der Waals interactions and hydrogen bonds within the polypeptide chain and the solvent. The application of pressure leads to volume changes in the system that alters the balance of the intramolecular and solvent-protein interaction (Janicke, 1991; Heremans, 2002), which can lead to unfolding (denaturation) of the protein. Galazka and Ledward (1998) pointed out that this partial unfolding of the protein is a complex phenomenon which is mainly due to the splitting of intramolecular salt bridges or weakening of hydrophobic interactions, which tend to increase the area accessible to the solvent.

The values of volume change (\(\Delta V\)) for the native to denatured protein are generally negative (Isaacs, 1981). At higher pressures the volume change values become more negative because the compressibility of the denatured protein is higher than the native protein. The conformation of the pressure-denatured protein is more compact than that of chemically or thermally-denatured protein (Weber, 1986). The negative volume change is caused by the disruption of hydrophobic interactions and ionic bonds in the intersubunit region. The subunits formed from dissociation by pressure may undergo further conformational changes. The conformation of a pressure-denatured protein is more compact than that of chemically or thermally denatured protein (Mozhaev et al., 1994).

Thermodynamic studies have established that proteins in solution are known to be stable within a narrow range of temperature (Morris, 1991). It is now known that proteins can be denatured by both heat and pressure, giving rise to the unique elliptic phase diagrams of native-denatured protein equilibrium in the pressure-temperature plane. Galazka and Ledward (1998) using the elliptic phase diagram of proteins (Figure 1.4), proposed that at room temperature, temperature stabilises the protein against pressure denaturation, whereas at high temperatures, pressure stabilises the protein against temperature denaturation. Consequently, proteins denature at a lower pressure at room temperature than at a higher temperature. It is seen that, the difference between pressure and heat can be interpreted by the known effects of pressure and heat on the forces stabilising protein structure. At ambient pressure, heating to approximately 60°C increases the strength of hydrophobic interactions; since these are the most sensitive to pressure, such a protein will become more resistant to further pressure denaturation. Reports by Takahashi (1992) indicated that the stability of hydrophobic interactions with increasing temperature explains why high pressure is more effective in
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killing certain micro-organisms at chill and sub-zero temperatures rather than ambient temperature. Above 60°C increasing temperature weakens hydrophobic interactions and therefore at these temperatures the protein becomes more sensitive as the temperature is increased (Galakza and Ledward, 1998).

Figure 1.4. Phase diagram for native/denatured protein (adapted from Ledward, 2000). The contour line is the equilibrium concentrations of the native and denatured forms of the protein \( \Delta G \) is the free energy of denaturation, \( \Delta V \) is volume change and \( \Delta H \) is change in enthalpy.

The mechanism of pressure denaturation involves rupture of hydrophobic bonds and subsequent hydrogen bond breakage while temperature ruptures hydrogen bonds before hydrophobic interactions (Galakza and Ledward 1998). It follows that pressure and temperature act antagonistically with respect to protein unfolding. Ledward (2000) described the weak linkages that are most labile on heating, i.e. hydrogen bonds are stabilised or marginally affected by pressure, whilst bonds most labile under pressure (electrostatic and hydrophobic interactions) are far less temperature sensitive. However, in the presence of oxygen, both increasing temperature and pressure encourage disulphide bond formation and/or interchange.

The pressure/temperature dependence of many weak linkages differ so markedly that on pressure release, the ‘reformed’ structures often differ to that of the native structure, i.e. conformational drift. Therefore, the amount of \( \alpha \)-helix, \( \beta \)-sheet and random coil vary as do
properties such as surface hydrophobicity and charge. These ‘renatured’ pressure treated proteins yield very different structures. Therefore, they exhibit marked differences in fundamental behaviour to the native protein or to its heat denatured product (Ledward, 2000).

1.4 High pressure processing in foods

1.4.1. Historical background

High pressure processing (HPP) is of great interest to biological and food constituents primarily because it permits microbial inactivation at low or moderate temperatures. Hite (1899) reported the effects of high pressure processing on food micro-organisms bysubjecting milk to pressures of 650 MPa and obtained a reduction in viable numbers of microbes. However, lack of information about the effects of pressure on biochemical reactions and structure of biopolymers, meant that high pressure was thought to have no commercial application in the food industry and research work in this area was abandoned. HPP was then limited to materials science industries which process or use ceramics, carbon graphite, diamonds and plastics.

Many years later, the pioneering work of Bridgeman (1914) observed the coagulation of egg albumen by high pressure, demonstrating that apart from its ability to kill certain microorganisms, high pressure treatment could alter protein functionality. Thus, although high pressure has been so far commercialised as a preservation technique, it has great potential as a tool for manipulating the texture of foods.

It was not until the late 1980s that a major breakthrough was made. In Japan, Hayashi et al., (1989) successfully demonstrated the application of high pressure in food processing. There are several types of pressure-treated foods on the Japanese market mainly fruit jams and fruit juices. Examples of commercially pressure treated foods in Europe and United States include orange juice by UtriFruit, France; acidified avocado puree (guacamole) by Avomex Company in US (Texas/Mexico); and sliced ham by Espuna Company, Spain (Tewari et al., 1999). Today, there are a number of potential applications of high pressure processing apart from sterilisation of acid foods, which require that heat processing be applied together with pressure treatment in order to deactivate bacterial spores. In the food industry, heat treatment is the most common preservation or processing technology especially to denature proteins.
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and subsequently gel proteins. The use of high pressure induced gelation has recently received interest, because these foods retain nutritional and sensory properties and gels with unique textures are produced (Knorr, 1993). Some meats can be tenderised by application of pressure at 1000 MPa and 40-70°C. Pressure treatment increases the sensitivity to proteases, of many food proteins including haemoglobin, myofibrillar proteins and β-lactoglobulin. Such applications can lead to colour reduction in meat and increased protein digestibility. Other food constituents such as starch can be gelatinised, liquid triacylglycerols are reversibly crystallised and the melting point of ice is lowered (Heremans and Smeller, 1998; Tedford et al., 1998; Grant et al., 2000).

Commercial exploitation of high pressure technology depends on more detailed and schematic knowledge of (a) factors affecting microbial inactivation and resistance to high pressure and (b) the effect of high pressure on the physical and chemical changes in food systems which may alter component interactions. Since, an objective of this study was to compare effects of pressure with heat treatment on protein structure and functional properties, pressure effects will be examined alongside the effects of heat.

1.4.2 Basic principles of high pressure processing

Pressure like temperature, is a fundamental thermodynamic property which dictates the state of substances and governs their behaviour. High pressure processing of food is based on two general scientific principles (Cheftel, 1992, 1995):

(a) Le-Chatelier principle states that any reaction which is accompanied by a decrease in volume is enhanced by pressure, that is, when a constraint is placed on a system, the system will change in order to minimise the constraint. High pressure places a constraint on volume and encourages reactions to take place allowing the overall volume of the food to decrease. This is followed by changes in the balance of intermolecular forces and the hydration of molecules in solution. Therefore, biopolymers may unfold their structure and as a consequence, their functional behaviour can change; for example there is a possible loss of enzyme activity or cell death due to increasing permeability of microbial membranes.

(b) The isostatic principle states that the applied pressure is transmitted in a uniform and instantaneous manner throughout the whole sample, independent of size and geometry. Thus,
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unlike thermal treatment during pressure treatment, the whole sample is treated homogeneously, whereby no part is subjected to extreme treatment.

Another related concept is the principle of microscopic ordering which postulates that at constant temperature, an increase in pressure increases the degree of ordering of molecules of a substance, that is, entropy will decrease. On the basis of this principle one would expect the physical effects of pressure and temperature to be antagonistic in molecular terms (Heremans, 1982; Suzuki, 1992; Ledward, 2000).

For an elementary equilibrium $A \leftrightarrow B$ the following general expression holds:

$$\Delta G = -RT \ln k = \Delta E + P \cdot \Delta V - T \Delta S$$

**equation (1)**

Where $\Delta G$, $\Delta E$, $\Delta V$, $\Delta S$ are changes in free energy, internal energy, volume and entropy; $k$ is the equilibrium constant governing the process, $T$ is the temperature $P$ is the pressure and $R$ is the gas constant (Heremans, 1982)

Pressure, unlike temperature which may change both the internal energy and the volume of a system, only affects the volume of the system under study (Heremans 1982; Silva and Weber, 1993; Mozhaev et al., 1996).

1.4.3 High pressure equipment

A typical high-pressure system consists of a high pressure vessel and its closure, a pressure generation system, a temperature control device and material handling. Once loaded and closed, the vessel is filled with a pressure-transferring medium, which is simply water mixed with a small percentage of soluble oil for pump lubrication. After all air is removed from the vessel by using a low pressure, fast fill and drain pump in combination with automatic de-aerating valve, high pressure can be generated.

The advantage of using water as a medium is that the decrease in water volume under pressure is slight compared with gases (Morild, 1981; Gould 2001). Practically most foods have a compressibility (water compressibility is around 15% at 600 MPa) very close to that of the pressurisation fluid. However, if any air is present foods cells become pulp, but food vacuoles and intercellular air is very compressible (Johnston, 1997). There is a transient
temperature rise during pressurisation of about 11 degrees, due to the friction caused by high fluid velocity. The temperature reaches a maximum when the desired pressure is obtained; it then decreases back to the ambient temperature (Farr, 1990). Holding times vary and depend on the type of food and the processing temperature.

In practice, there are two main pressurisation methods: the pressure is applied either directly by forcing the fluid into the treatment chamber (direct approach) or indirectly by forcing a piston into a liquid-filled vessel containing the material to be treated, usually pre-packaged (Figure 5a,b below). The Stansted Food Lab high pressure rig was used in this study and is based on a plunger system which follows the direct approach. The pressure medium in the vessel is directly pressurised by a piston, driven at its large-diameter end by a low-pressure pump. This method allows very fast compression but is restricted to a small laboratory or pilot plant system due to the limitations of dynamic seal between the piston and internal vessel surface (Thakur and Nelson, 1998). In the indirect method, the pressurisation fluid is pumped from the pressure medium tank to the pressure vessel by means of an intensifier. The intensifier increases the pressure in the vessel by compressing the fluid and ultimately the product is pressurised (Ledward et al., 1994).

Figure 1.5. (a) Direct pressure generation method (b) Indirect pressure generation method (adapted from Ledward et al., 1994).
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1.4.4 Pressure Units

Pressure is defined as force per unit area (Suzuki 1992). Units of pressure commonly used in
the literature are the atmosphere (atm), the bar (b) and Kilobar (kbar), kilograms per square
centimetre (Kg/cm²) and pounds per square inch (lb/in², psi). The equivalence of units is
given below:

\[ 1 \text{ bar} = 10^6 \text{ dynes/cm}^2 = 0.9869 \text{ atm} \]
\[ = 1.0197 \text{ kg/cm}^2 \]
\[ = 14.504 \text{ lb/in}^2 \]

Since work on high pressure involves a number of disciplines, different measures of pressure
are used throughout the world as shown in table 1.1 below. High pressure applications in
food processing currently use pressures of between 100 and 800 MPa but can go up to 1000
MPa (1 MPa = 9.869 atm), (Ledward et al., 1994; Grant et al., 2000).

Table 1.1: Conversion units for high pressure

<table>
<thead>
<tr>
<th>Mpa</th>
<th>Kg/cm²</th>
<th>Bar</th>
<th>Atm</th>
<th>psi</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>1.020</td>
<td>1000</td>
<td>986</td>
<td>14,503</td>
</tr>
</tbody>
</table>

1.4.5 Effects of pressure on protein structure

It is now well established that pressure affects protein structure and functionality. This has
been subject to many reviews (Balny et al., 1989; Gross and Janicke, 1994; Heremans, 1995;
Mozhaev et al., 1996; Galakza and Ledward, 1998; Heremans, 2002). Studies carried out on
volume changes in proteins, have shown that the main targets of pressure are hydrophobic
and electrostatic interactions. This section will consider the effects of pressure on the forces
involved in stabilising protein structure.
1.4.5.1 Electrostatic interactions

Electrostatic interactions maintaining protein structure can be easily cleaved by pressure. In an aqueous system an electrically charged ion causes the surrounding water molecules to align themselves according to its coulombic field. The result is a more compact arrangement of water molecules and a volume decrease. In biological molecules or food systems the ionisation of acidic or basic groups involves volume reduction and therefore, ionisation will be enhanced by pressure. Since high pressure induces ionisation, it can affect the pH of aqueous solutions. The volume change as a result of pressurisation of weak acids is due to dissociation, leading to pH changes (Johnston, 1997). Formation of electrostatic bonds is accompanied by a positive volume change of 10-20 mL mol\(^{-1}\) and a negative volume change of -10 mL mol\(^{-1}\), for the hydration of a charged group (separation of ion pairs) (Ledward, 2000).

1.4.5.2 Hydrogen bonds

Hydrogen bonding, which stabilises the \(\alpha\)-helical and \(\beta\)-pleated sheet structures of proteins is considered to be almost insensitive to pressure and is accompanied by small negative volume changes (Van Eldik et al., 1989 and Ledward, 2000). This stabilisation is attributed to the shortening of inter-atomic distances in the hydrogen bonded atoms (Morild 1981). The fact that high pressure stabilises hydrogen bonds has important implications for the secondary structures in proteins such as \(\alpha\) helices and \(\beta\)-structures. However, pressures above 400 MPa may cause changes in secondary structure, leading to irreversible denaturation depending on the rate of compression and the extent of secondary structure re-arrangement. (Balny and Mason 1993).

1.4.5.3 Hydrophobic interactions

Proteins may contain large regions which are non-polar in nature. Hydrophobic interactions play an essential role in forming the tertiary and quaternary structures as well as largely dictating protein-protein interactions (Heremans, 1982; Howell, 1992). Formation of hydrophobic interactions are thought to proceed with positive volume change values and are
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therefore enhanced by pressure (Mozhaev et al., 1996; Ledward 2000). The existence of hydrophobic regions in a protein causes a structuring effect on the layer of water molecules immediately adjacent to them, consequently restricting rotational and translational freedom. This is an unstable state, therefore, to create stability in the protein molecule, the hydrophobic regions interact so that they are surrounded by water molecules forming a single hydration shell. The overall movement of molecules from an ordered to disordered state causes a small volume increase to the system, consequently, hydrophobic interactions are disrupted by pressure (Johnston, 1997). However, stacking of interactions between aromatic rings show negative volumes changes and are stabilised by pressure (Van Eldik et al., 1989).

1.4.5.4 Covalent bonds

Covalent bond formation, gives a negative volume change of $-10 \text{ mL mol}^{-1}$ and almost zero values for exchanges between covalent bonds (e.g., $A-B+C-D=A-C+B-D$) (Mozhaev et al., 1994). At higher pressures >300 MPa sulphydryl groups may oxidise to S-S bonds in the presence of oxygen (Ledward, 2000). Covalent bond exchanges proceed with minimal changes in volume and the appreciable effects of pressure upon chemical equilibria result from differential effects on products and reactants that are practically unrelated to the difference in volume owing to the exchanged covalent bonds. Under pressure, bond angles behave similarly to bond lengths, so that with the exception of special cases like cis-,trans or boat-chair isomerisation the covalent architecture may be considered as unchangeable within the range of pressure at which water remains liquid at ordinary temperature, approximately 12 kbar (Mozhaev et al., 1996). From these precedents it is clear that the most significant equilibrium displacements under pressure occur in those reactions that involve differences in the intermolecular forces of the solvated products and reactants.

1.4.6 Advantages and limitations of high pressure processing

At present heat treatment is the most common method of food processing and preservation. The use of heat treatment has revolutionised the food industry and has made long term preservation of food a possibility. Heat, however, also leads to quality deterioration in certain foods by producing undesirable changes in sensory and nutritional properties of food (Thakur
Chapter 1. General Introduction

and Nelson, 1998). Therefore, preservation technologies that provide long shelf life without detrimental effects on the quality attributes of foods are gaining popularity in today's markets.

The consumer demand for minimally processed food stems from fears associated with use of chemical/preservatives in the food supply and conflicting reports regarding the pros and cons of existing processing technologies as well as increased awareness of nutritional issues. Minimal processing methods involve processing procedures that cause minimal changes in the inherent fresh-like quality attributes, at the same time enhancing the shelf life of food from time of processing to the consumer (Bengtsson 1994).

When contrasted with other processing techniques high pressure processing offers several advantages because:

(a) high pressure is considered not to break covalent bonds, therefore, there is minimal damage to flavour, and colour, thus maintaining the natural qualities of products.

(b) high pressure processing can be applied at room temperature thus reducing the amount of thermal energy needed for food products during conventional processing. Low temperature can also help to retain nutritional quality and functionality of raw materials treated (Knorr 1995).

(c) high pressure is isostatic (uniform distribution throughout the food) and the food is preserved evenly without any particles escaping treatment and shape is not distorted.

(d) high pressure processing is not time/mass dependent, it acts instantaneously, thus reducing the processing time. Once the desired pressure is reached, it can be maintained without the need for further energy input.

(e) high pressure processing is independent of size and geometry of the food. For example size reduction is required in conventional thermal processing to improve heat and mass transfer and this is often accompanied by elevated loss of nutrients (Knorr, 1995).

Like many processing technologies high pressure processing has certain limitations;

(a) Food enzymes and bacterial spores are very resistant to pressure and require very high pressure for their inactivation. Particular among the most studied organisms, *Staphylococcus aureus* appears to have a high resistance to pressure (Patterson and Kilpatrick, 1998; Knorr, 1993 and Hoover, 1993). However, it appears that high pressure processing when combined
with other parameters such as alteration in pH is more effective at controlling spore forming bacteria (Martin et al., 2002).

(b) There are also reports that HPP has detrimental effects on some food components: High pressure processing induces the induction phase strongly and accelerates the formation of primary oxidation products (hydroperoxides and peroxides) in fatty acids (Tauscher, 1995; Tauscher and Butz 1998).

(c) Residual enzyme activity and dissolved oxygen results in enzymatic and oxidative degradation of high pressure processed strawberry jam at room temperature, (Kimura et al., 1994).

(d) Most high pressure processed foods need low temperature storage and distribution to retain their sensory qualities.

(e) High pressure processing is a very expensive process and manufacturers have to consider the benefits before investing in such technology.

1.5 Functional properties of proteins

1.5.1 Introduction

Proteins are capable of performing a variety of functions in foods. The food industry routinely exploits a variety of textural functional properties produced when a protein is exposed to different processing conditions. According to Damodaran, (1996) and Howell (1996) the functional properties routinely exploited are gelling, foaming and emulsification. Since, these functional properties are influenced by protein solubility characteristics, this section will focus on protein-water interactions, that is, solubility, viscosity, gelation and emulsification properties (Table 1.2). These properties also directly relate to gelation properties investigated in this study.
Table 1.2. Functional roles of food proteins in food systems

Adapted from Damodaran (1996).

<table>
<thead>
<tr>
<th>FUNCTION</th>
<th>MECHANISM</th>
<th>FOOD SYSTEM</th>
<th>PROTEIN SOURCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solubility/Viscosity</td>
<td>hydrophilicity</td>
<td>Beverages</td>
<td>Whey Protein</td>
</tr>
<tr>
<td></td>
<td>water binding</td>
<td>Soups, Gravies</td>
<td>Soya protein</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Salad dressings</td>
<td></td>
</tr>
<tr>
<td>Water Binding</td>
<td>H-bonding</td>
<td>Meat, Sausages</td>
<td>Myofibrillar,</td>
</tr>
<tr>
<td></td>
<td>Ion hydration</td>
<td>Cakes and bread</td>
<td>Egg and soya proteins</td>
</tr>
<tr>
<td>Gelation</td>
<td>Water entrapment</td>
<td>Meat gels, cakes</td>
<td>Muscle protein,</td>
</tr>
<tr>
<td></td>
<td>immobilisation</td>
<td>Baked goods</td>
<td>egg and milk proteins</td>
</tr>
<tr>
<td></td>
<td>network formation</td>
<td>Cheese</td>
<td></td>
</tr>
<tr>
<td>Emulsification</td>
<td>Adsorption at</td>
<td>Sausages</td>
<td>Muscle</td>
</tr>
<tr>
<td></td>
<td>interfaces</td>
<td>Bolognas, soups</td>
<td>egg and caseinate</td>
</tr>
<tr>
<td></td>
<td>film formation</td>
<td>Cakes and dressings.</td>
<td>proteins</td>
</tr>
</tbody>
</table>

1.5.2 Solubility

Solubility of a protein is one the functional properties that is affected by the extent of interaction between water and protein and is considered to be a prerequisite for the performance of a protein in several food applications. Damodaran (1996) suggested that in order to obtain optimum functionalities in foods that require gelation, emulsification and foaming properties a highly soluble protein is desirable. Commercial proteins vary in their solubility characteristics as a result of partial denaturation of proteins during processing.

The solubility of a protein under a given set of environmental conditions is the thermodynamic manifestation of the equilibrium between protein-protein and protein-solvent interactions. It is related to the net free energy arising from the interactions of hydrophilic residues of the protein with the surrounding aqueous solvent. According to Cherry (1980) the solubility characteristics
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under various conditions provide a good index of the potential applications of proteins. The level of solubility of a protein is perhaps the most practical index of the extent of denaturation and is very dependent on prior treatment of the protein, extraction methods, ionic environments and the pH of the solvent.

1.5.3 Viscosity

Solubility of a protein is related to its viscosity. It follows that the solubility and viscosity are two experimentally measurable properties that can provide information about the functional behaviour as well as the physicochemical nature of proteins (Cherry, 1980). Many proteins upon hydration absorb water and swell thereby causing changes in their hydrodynamic properties that are reflected in thickening and concurrent increases in viscosity (Kinsella 1976). A knowledge of the flow properties and viscosity of protein dispersions are of practical significance in relation to the processing and design (i.e. pumping, feed rate, spray drying and heat exchange). Viscosity can be used as an index of structural changes in proteins and subsequently of the hydrodynamic/rheological properties of modified food proteins (Bourne, 2002).

1.5.4 Emulsification

An emulsion is a two phase liquid system in which one liquid is dispersed as droplets in the other liquid. Food emulsions can be divided into two types: systems in which an oil is dispersed in an aqueous continuous phase or oil in water emulsions and systems in which water is dispersed in an oil continuous phase or water in oil emulsions (Damodaran, 1996). As differences in interfacial tensions between water and oil are quite high, emulsions are thermodynamically unstable and phase separation occurs over time. Stability of emulsions can be improved by the addition of amphiphilic surface active molecules that adsorb at the oil-water interface and reduce the interfacial tension. Proteins being amphiphilic are well suited to act as macromolecular surfactants. Factors affecting emulsifying properties include the amount of protein adsorbed, rate of adsorption at the oil-water interface, extent of conformational rearrangement at the interface, and on ability to form a continuous, cohesive and viscous film via covalent (disulphide bonds) and non-covalent interactions (Damodaran, 1996).
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The most common types of food emulsions are mayonnaise or milk (oil in water emulsion) and butter and margarine (water in oil emulsions). The inherent properties of proteins such as molecular conformations, denaturation, aggregation, pH and solubility affect their performance in model and commercial system. Emulsion capacity profiles of proteins closely resemble protein solubility curves and thus factors that influence solubility properties or treatments used to modify protein character also influence emulsifying properties (Cherry 1980).

1.5.5 Gelation

Gelation is an important functional property as it allows food proteins to be fabricated into novel and structured products for example, egg white and comminuted meat products (Kinsella, 1976; Barbut and Findlay, 1990; Clark, 1998). Manufacturing of certain food products and development of new products requires ingredients which gel to provide a structural matrix for holding water and other ingredients while providing desirable texture and mouthfeel (Howell and Lawrie, 1984; Phillips et al., 1994). In these products proteins are thought to contribute in different ways to the solid or elastic properties of food by formation of an orderly, three-dimensional network of associated or aggregated protein molecules that are capable of entrapping large amounts of water within the matrix (Hermansson, 1978). Indeed, Kinsella (1976) reiterates this concept by asserting that the ability of protein gels to act as a matrix for holding water, lipids, sugars, flavours and other ingredients is useful in food applications and in new product development. Gelation is described in more detail below as it is the main functional property investigated in this study.

1.5.5.1 Definition

There is no universal definition of what constitutes a protein gel. Gelation is considered a rheological property and its definition and that of its product, a gel is subject to definitions based on the observer and the techniques used to measure it, for example, gels may be described by their capacity to immobilize a liquid, their macromolecular structure, and by their textural and or rheological properties (Kinsella 1976, Clark and Lee-Tuffnell 1986, Howell, 1992, 1996; Ziegler and Foegeding 1990, Clark, 1998). There is general consensus that a protein gel consists of a three-dimensional matrices or networks, this is reflected in definitions by (Kinsella, 1976; Howell and Lawrie, 1984) that a protein gel is generally considered as being composed of three-dimensional matrices or networks of intertwined, partially associated, polypeptides in which water is entrapped. (Hermansson, 1979) adds that
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proteins form gels by polymerizing into an ordered, three-dimensional network which transforms viscous liquids into a viscoelastic matrix. Cherry (1980) defines gelation as a protein aggregation phenomenon in which polymer-polymer and solvent-polymer interactions and attractive and repulsive forces are so balanced that a well-ordered tertiary network or matrix is formed and this matrix is capable of immobilising or trapping extremely large amounts of water.

Common to all these definitions is the fact that gels consist of structured networks in which the solid component is protein dispersed in a liquid component. Indeed, early work by Ferry (1948) on protein gelation indicated that polymer gels are differentiated from other systems in which small proportions of solid are dispersed in relatively large proportions of liquid by the property of mechanical rigidity or ability to support shearing stress at rest. Implicit in this proposition is the fact that rigidity may be accompanied by viscous retardation which delays response to stress or it may be associated with slow deformation under stress, i.e. gels which consist mostly of fluid behave as a solid and yet retain many properties characteristic of a liquid component (for example, compressibility). Mulvihill and Kinsella (1987) commented that gels have this remarkable ability to behave like a solid while still retaining the many properties characteristic of the fluid component.

1.5.5.2 Mechanism of gelation

The formation of a gel occurs under conditions which disrupt the native protein structure. It follows that denaturation of protein is a prerequisite for protein gelation. (Hermansson 1978, Calrk, 1998) reported that conditions which favour more complete denaturation prior to aggregation produce networks with a lower opacity (greater homogeneity) and higher elasticity than if aggregation and denaturation occur simultaneously or if aggregation precedes denaturation (Howell, 1995). Denaturation makes new interactions possible, if protein-protein and protein-solvent interactions lead to the formation of a three-dimensional network capable of entraining solvent molecules, a gel is likely to form. Comfort and Howell (2002) pointed out that the ability of denatured proteins to associate and form gels depends on their properties and the environmental conditions to which they are exposed. The environmental conditions, include protein concentration, other proteins and non-protein components, pH, ionic and or reducing agents and heat treatment (Cherry, 1980; Howell and
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Lawrie, 1984). The presence of different amino acids in the polypeptide chain with differently charged, hydrophobic or hydrophilic side groups has dramatic effects on the gelling behaviour of a protein (Murphy and Howell 1990, Paulsson and Dejmek 1990). Huang et al (1990) studied the gelling behaviours of A and B β-lactoglobulin isoforms which differ only by one or two amino acids and reported that their gelling behaviours differ significantly. It is well known that there is a well defined critical concentration \( (C_0) \) below which no continuous network is formed, when the protein concentration is increased above this critical level the gelling time is reduced and gel strength is increased (Mulvihill and Kinsella, 1987).

The most commonly accepted view on protein gelation is that of Ferry (1948). He proposed that gelation is a two step process involving an initial change in conformation, unfolding of the native protein, thereby exposing reactive groups followed by an association and or aggregation of the long unfolded polypeptide chains via covalent and non-covalent forces. For successful network formation and stabilization a critical balance of attractive and repulsive forces must be present.

Native protein \( \Leftrightarrow \) Denatured protein \( \Leftrightarrow \) network formation

step 1

step 2

Step 1 is the initial denaturation step and is usually induced by heat and is reversible. Step 2 indicates formation of a network which may also be induced by heating or cooling. This last step was once thought to be irreversible, it is now known to be reversible for some protein gels, for example whey proteins. Kinsella et al., (1994) reported whey proteins as being capable of forming thermoplastic (reversible) gels. A thermoplastic gel as the name implies, melts and flows upon heating (Rector et al., 1989).

There was no real departure from these early views of Ferry (1948) until Barbu and Joly (1953) and (Kratchovil et al., 1961; 1962) proposed that aggregation of partially-unfolded globular proteins took place when repulsion was large, whereas random aggregates or clumping occurred when repulsion was small. Tombs (1974) distinguished between ordered and random aggregation and suggested that protein gelation could be considered in terms of statistical theories developed by Flory (1942) for synthetic polymers. However, Bezrukov (1979) reported that statistical aggregation was unlikely to occur because protein globules
have a surface mosaic structure with sites differing in charge, density, degree of hydrophobicity and presence of disulphide bonds. Howell and Lawrie (1985) pointed out that protein gelation at least that of globular proteins, supports the theory that partially-unfolded globular proteins associate linearly at low heating temperatures and randomly to form aggregates at high temperature or at the pI of the protein. Provided the temperature, pH and concentration are maintained accurately, the gelation pattern is reproducible (Howell and Lawrie, 1985).

1.5.5.3 Protein-protein interactions

Protein-protein interactions occur in many food systems, including in mixtures of ingredients used in food processing and manufacture (Howell, 1995, Howell and Lawrie, 1984). In order to achieve a certain desired functional property it may be necessary to mix two or more proteins as well as a wealth of other ingredients. There are a number of reasons for investigating protein-protein interactions including acquiring knowledge of structure-function relationships, optimising product constituents (thereby improving quality, nutrition and safety), cost reductions and new product development (Howell, 1995).

Proteins may interact with one each other in a number of ways to affect the properties of the gel formed and the overall texture of the food. After mixing, proteins may be qualitatively considered incompatible or compatible depending on whether the two immiscible phases are formed (Manson and Sperling, 1979). There are a variety of ways in which proteins can interact which will affect the properties of a multi-component gel. Composite or multi-composite gels are produced from mixtures of two or more gelling agents or single gelling agent and non-gelling agents. A second protein capable of gel formation may act as a non-gelling component if it is present in a mixture at a concentration below its critical concentration for gel formation (Ziegler and Foegeding 1990; Comfort and Howell, 2002). The second protein component may behave like a filler, interspersed throughout the primary gel network. The gel may be single phase with the filler remaining soluble or two phase where there exists an incompatibility (Howell, 1996). The single phase was labelled by Tolstoguzov (2002) as type 1 and phase separated as type 2 filled gel. The two proteins may co-polymerise to form a single heterogeneous network, an example of this type of polymerisation occurs in BSA-ovalbumin gels (Clark et al., 1982; Howell and Lawrie, 1985).
Howell (1995) highlighted various interactions of proteins with other proteins which affect food texture (a) **synergistic interactions** and compatible gel formation and stable forms: synergistic interactions relate to those interactions that result in an effect which is greater than that produced by each protein in isolation; the effect manifests itself in greater viscosity, cohesiveness or gel strength values and the structure indicates compatible gel networks. A practical example of this phenomenon is the interaction of plasma proteins and egg albumen proteins in a cake type model system (Howell and Lawrie, 1985).

(b) **Aggregation** through predominantly electrostatic interactions: Howell *et al.*, (1995) reported that electrostatic interactions between positively charged (pI 10.1) lysozyme with bovine serum albumin and α-lactoglobulin (pI 5.0) enhanced foaming properties.

(c) **Phase separation:** In phase separation, two phases exists where thermodynamic incompatibility causes phase separation to occur with filler existing as dispersed particles of liquid or as secondary gel network. An example of a phase separated system is a mixture of soya and whey proteins. These proteins do not associate due to physical and chemical differences, i.e. soya isolate proteins are larger (MW, 140,000-190,000 for 7S globulins and 300,000-400,000 for the 11S globulins) compared with whey proteins (MW, 14,000 for α-lactalbumin and 18,000 for β-lactoglobulin. It is likely that due to molecular incompatibility and steric hindrance soya proteins can inhibit the gel formation of globular proteins like whey or plasma proteins, however, when a small amount of the second protein is added to the larger concentration of the first protein, the mixture can result in high gel strength of the resultant gels (Comfort and Howell 2002).

1.6 Composition and properties of egg albumen proteins

1.6.1 Introduction

The major constituents of egg albumen and their physicochemical properties and biological or functionally important characteristics are outlined in Table 1.3. Protein is a major component of egg albumen comprising of 9.7%-10.6% (w/w). Carbohydrates account for 0.5-0.6% of egg albumen and exist either in a combined or free form. Glucose at 0.5% comprises 98% of the total free carbohydrates. The amount of lipids 0.01% in egg albumen is negligible compared with the amount present in egg yolk (Mine 1995). The composition and properties
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of egg albumen components have been extensively reviewed (Vadehra and Nath, 1973; Mine, 1995; Nakamura and Doi, 2000).

Table 1.3: Composition and some physicochemical and functional characteristics of proteins in egg albumen (adapted from Nakai & Doi, 2000)

<table>
<thead>
<tr>
<th>Protein</th>
<th>%Total Protein</th>
<th>M.W.</th>
<th>I_p</th>
<th>CHO Moiety</th>
<th>Important Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovalbumin</td>
<td>54</td>
<td>45,000</td>
<td>4.7</td>
<td>Yes</td>
<td>Phosphoglycoprotein, foaming, Gelation, Immunogenic</td>
</tr>
<tr>
<td>Ovotransferrin</td>
<td>12</td>
<td>77,000</td>
<td>6.0</td>
<td>Yes</td>
<td>Binds iron, antimicrobial</td>
</tr>
<tr>
<td>Ovomucoid</td>
<td>11</td>
<td>28,000</td>
<td>4.1</td>
<td>Yes</td>
<td>Trypsin inhibitor</td>
</tr>
<tr>
<td>Ovomucin</td>
<td>1.5-3.5</td>
<td>0.23-8.3x10^6</td>
<td>4.5-5.0</td>
<td>Yes</td>
<td>Viscous, role in age-thinning, Viral hemagglutination inhibitor</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>3.4-3.5</td>
<td>14,300</td>
<td>10.7</td>
<td>No</td>
<td>Lysis of bacterial cell wall, Anti-microbial</td>
</tr>
<tr>
<td>G2 globulin</td>
<td>4.0(?)</td>
<td>49,000</td>
<td>5.5</td>
<td>Yes</td>
<td>Anti-microbial</td>
</tr>
<tr>
<td>G3 globulin</td>
<td>4.0(?)</td>
<td>49,000</td>
<td>5.8</td>
<td>Yes</td>
<td>?</td>
</tr>
<tr>
<td>Ovoinhibitor</td>
<td>0.1-1.5</td>
<td>49,000</td>
<td>5.1</td>
<td>Yes</td>
<td>Serine proteinase inhibitor, wide spectrum of inhibitory activity</td>
</tr>
<tr>
<td>Ovoglycoprotein</td>
<td>0.5-1.0</td>
<td>24,400</td>
<td>3.9</td>
<td>Yes</td>
<td>?</td>
</tr>
<tr>
<td>Ovoflavoprotein</td>
<td>0.8</td>
<td>32,000</td>
<td>4.0</td>
<td>Yes</td>
<td>Binds riboflavin</td>
</tr>
<tr>
<td>Ovomacroglobulin</td>
<td>0.5</td>
<td>0.76-0.90x10^6</td>
<td>4.5-4.7</td>
<td>Yes</td>
<td>Strongly antigenic, high Immunological cross reactivity</td>
</tr>
<tr>
<td>Cystatin</td>
<td>0.05</td>
<td>12,700</td>
<td>5.1</td>
<td>No</td>
<td>Thiol proteinase inhibitor</td>
</tr>
<tr>
<td>Avidin</td>
<td>0.05</td>
<td>68,300</td>
<td>10.0</td>
<td>Yes</td>
<td>Binds avidin, antimicrobial</td>
</tr>
</tbody>
</table>

(?) refers to unknown or uncertain data,
M.W = Molecular weight, I_p = Isoelectric Point, CHO= Carbohydrate moiety
1.6.2 Ovalbumin

Ovalbumin is the principal protein of chicken egg albumen which is used extensively in the food industry because of its ability to foam and to form gels upon heating. Ovalbumin constitutes approximately 54% of egg albumen. Ovalbumin is classified as a monomeric phosphoglycoprotein with a molecular weight of 45.5 kDa and an isoelectric point (pI) of 4.5. The molecule comprises 385 amino acids residues and its crystal structure has been reported at 1.95 Angstrom (Nisbet \textit{et al.}, 1981; Stein \textit{et al.}, 1991).

Purified ovalbumin is made up of 3 components differing only in the phosphorous content namely, A1, A2 and A3 which contain two, one and no phosphate groups (Mine, 1990). The relative proportion of A1, A2 and A3 components in the ovalbumin fraction is about 85:12:3. The two phosphate groups are attached to serine residues 68 and 344 (Nisbet \textit{et al.}, 1981). One carbohydrate moiety is attached to the polypeptide chain through asparagine residue 292 (Nisbet \textit{et al.}, 1981). The secondary structure of ovalbumin has been derived from circular dichroism (CD) spectroscopy and is reported to be made up of 40% $\alpha$ helix, 15% $\beta$-sheet, 15% $\beta$-turns and 28% unordered structure (Mine \textit{et al.}, 1990). However, some reports indicate that CD cannot identify turns or distinguish between $3_{10}$ and $\alpha$ helices (Susi and Byler, 1988; Prestrelski \textit{et al.}, 1991). Figure:1.6. below shows the secondary structure of ovalbumin.
Figure 1.6: Secondary structure of ovalbumin: strands of β sheet are represented by arrows (labelled, s) and α-helices by helical ribbons (labelled, h). sites of post translational modification are shown. (C) Carbohydrate side chain; (P) phosphoserine and (S-S) disulphide bond are shown (Nisbet et al., 1981).

During storage ovalbumin is converted to S-ovalbumin, a more heat stable protein. The denaturing temperatures of native and S-ovalbumin are 84.5°C and 92.5°C respectively (Stadelman and Cotterill, 1986). No difference in the amino acid composition of ovalbumin and S-ovalbumin have been demonstrated and that disulphide-sulphydryl interchange may be involved in ovalbumin-S-ovalbumin transition. Forthergill and Fothergill (1970), Konig and Painter (1976), Nisbet et al., (1981) and Stein et al., (1991) indicated that in addition to 4 sulphydryl groups, ovalbumin contains one disulphide group per molecule. Ovalbumin is easily denatured and coagulated by exposure to new surfaces but is resistant to thermal denaturation (Stadelman and Cotterill, 1986).

### 1.6.3 Gelation properties of egg albumen proteins in foods

The term functional properties refers to the attributes of egg proteins which make them a useful ingredient in food such as meringues, cakes and candy. Stadelman and Cotterill (1986) and Nakamura and Doi (2000) classified the major functional properties of egg proteins as gelation, foaming and emulsification. In addition, eggs serve as colouring and flavouring ingredients. The ability of a protein to coagulate upon heating is utilised when eggs are used as binding agents and when they are used in custards, cakes and pie fillings (Howell, 1978). Changes in egg albumen protein molecule resulting in loss of solubility and gel formation can be brought about by heat, salts, acids, alkali and reagents such as urea (Howell and Lawrie, 1985; Stadelman and Cotterill, 1986). Reports on pressure induced gelation showed that egg albumen protein does not form a gel when pressurised for 30 min below 400 MPa (Hayakawa, 1996, Smith et al., 2000).

The success of many cooked foods is dependent on coagulation of egg protein, especially irreversible heat coagulation of egg albumen proteins. It is this unique property of egg albumen protein which give rise to products such as angel cake and their characteristic textural qualities. In addition, thermal setting is required for cake
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batters, custard and puddings has been attributed in part to denaturation and coagulation of egg albumen proteins (Stadelman and Cotterill 1986).

The mechanism resulting in gelation of egg albumen proteins have been studied using spectroscopic methods (Painter and Koenig 1976, Kato and Takagi 1988 and Mine et al., 1990, Howell and Li-Chan 1996). It has been suggested that the formation of stable intermolecular β-sheet structures is the major change occurring during thermal denaturation and aggregation of egg albumen proteins including ovalbumin. Using Raman spectroscopy, Painter and Koenig (1976) indicated that thermal denaturation of ovalbumin molecules resulted in an intense amide III line at 1236 cm\(^{-1}\) (beta-sheet structure) and a shift in the amide 1 band from 1667 to 1672 cm\(^{-1}\). These changes indicate the formation of extensive regions of anti-parallel β-sheets between ovalbumin molecules. The formation of β-sheet in heat denatured ovalbumin was enhanced with increasing ovalbumin and salt concentration. More recently Smith et al., (2000) using Fourier transform infra-red spectroscopy analysed pressure treated ovalbumin and indicated that there was some change in the β-structure of the protein even after pressure treatment of 400 MPa. In the same study, circular dichroism spectra also revealed a small loss of ellipticity around 212 nm 400MPa, consistent with some change in β-secondary structure.

The heat-induced ovalbumin aggregates or gels were thought to be formed from the partially unfolded molecules through the crosslinking of intermolecular β-sheet structures as a result of exposure of hydrophobic residues (Kato and Takagi 1988). Previous reports by Howell and Lawrie (1985) demonstrated the mechanism of gelation by chemical modification showing that gelation via disulphide bonds was predominant in all plasma and egg albumen proteins whilst hydrophobic and hydrogen mainly bonds prevailed in plasma proteins gels.

Hatta et al., (1986) and Nakamura and Doi (2000) reported the importance of pH and ionic strength on turbidity and hardness of heat-induced ovalbumin gels. At pH values near the pI of ovalbumin or higher ionic strengths, the denatured proteins randomly aggregated by hydrophobic interactions. Thus pH far from the pI and low ionic strength, electrostatic repulsive forces hinder the formation of random aggregates and more ordered linear polymers are formed.
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1.6.4 Egg albumen proteins and high pressure processing

Thermal treatment of egg albumen proteins result in the modification or loss of most of the functional properties even after mild treatment (Hayakawa et al., 1996). In contrast, high pressure processing can be used to obtain stable products with minimal effects on flavour, colour and nutritional value or create products with a novel texture and taste (Lametti et al., 1998; Galazka et al., 2000).

As early as 1914, Bridgeman observed that egg albumen proteins coagulated at pressure of 500-600 MPa. Grant et al., (2000) verified that the mechanism of gelation observed by Bridgeman was due to denaturation under high pressure. Such pressure denaturation is now believed to be caused by rearrangement or destruction of non-covalent bonds such as hydrophobic interactions and ionic bonding of the tertiary structure of protein while covalent bonds are considered not to be affected (Okamoto et al., 1990, Cheftel, 1995; Mozhaev et al., 1996; Ledward 2000).

Liquid egg white partially coagulated when treated at pressures >500 MPa and strong self-supporting gels were formed at pressures >600 MPa (Messens et al., 1997). Okamoto et al., (1990) compared application of heat treatment for 10 min at 100°C and pressure treatment at 100-700 MPa for 30 mins, in fresh egg albumen proteins and observed that the pressure treated gels had increased hardness and elastic modulus but remained significantly softer than gels obtained by heat treatment. The pressure treated gels kept their original colour and flavour and were glossy and soft in comparison with heat-induced gels. The glossy and transluscent appearance observed in pressure treated egg albumen proteins is thought to be due to the rearrangement of water molecules surrounding amino acids residues in the denatured state as opposed to the vigorous movement of molecules which leads to the breaking and formation of covalent bonds during heat denaturation. Heat treatment is accompanied by the destruction or formation of covalent bonds to produce off-flavours or toxic compounds. However, such unfavourable reactions are not observed in pressure treated egg albumen proteins. Hayashi et al., (1989) indicated that there was no amino acid change after pressure treatment at 400-1000 MPa, while, lysine and sulphur-containing amino acids were observed in egg white and yolk boiled for 5-10 minutes. Lysinoalanine residues are thought to be formed easily by boiling egg white
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for 5 minutes (Hayashi 1989). However, formation of Lysinoalanine under pressure has not been investigated.

1.7 Composition and properties of whey protein isolate proteins

There are two main sources of whey protein. Most fractionation procedures begin with the precipitation of casein from skim milk by the addition of acid in situ to produce acid whey. Sweet whey is produced when the casein fraction of milk is removed by the action of the digestive enzyme rennet/rennin on k-casein at the specific Phe$_{105}$-Meth$_{106}$ bond. The whey is separated from the casein curd and concentrated by ultrafiltration or evaporation to produce a number of commercial whey protein powders, Bipro whey isolate (99.9 protein w/w) was produced by ultrafiltration.

1.7.1 Composition and some physicochemical properties of whey proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular Weight (Da)</th>
<th>Isoelectric Point</th>
<th>Approximate percentage of total whey protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-lactoglobulin</td>
<td>18 363</td>
<td>5.2</td>
<td>5</td>
</tr>
<tr>
<td>α-lactalbumin</td>
<td>14 175</td>
<td>4.2-4.5</td>
<td>12</td>
</tr>
<tr>
<td>Immunoglobulins</td>
<td>(1.6-10) x 10$^5$</td>
<td>5.5-8.3</td>
<td>10</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>66 267</td>
<td>5.13</td>
<td>5</td>
</tr>
<tr>
<td>Proteose-peptone, minor proteins and caseins</td>
<td>4,100 - 40,800</td>
<td>3.3-3.7</td>
<td>23</td>
</tr>
</tbody>
</table>
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1.7. 2 β-Lactoglobulin

β-lactoglobulin is a globular protein with a monomer molecular weight of 18.4 kDa and accounts for 50% of bovine whey protein isolates and 12% of protein in bovine milk. The protein exists as dimer in solution above its isoelectric point, because of the electrostatic attractions between Asp\textsuperscript{130}-Glu\textsuperscript{134} of one monomer with corresponding lysyl residues of another monomer (Creamer \textit{et al.}, 1983). The native conformation is sensitive to heat and pH. The structure and aggregation of the protein are dependent on pH (Pittia \textit{et al.}, 1996).

Below pH 3.5 and above pH 7.0 the dimer dissociates into a slightly expanded monomer and above pH 7.5 the protein polymerises forming 147 kDa octomers (Morr and Ha, 1993). β-lactoglobulin is composed of 162 amino acids. Creamer \textit{et al.}, (1983) determined the primary sequence of amino acids figure 1.7.

Figure 1.7. The amino acid sequence of β-lactoglobulin B. The residues marked with an asterisk vary from species to species with the genetic variant (from Creamer \textit{et al.}, 1983)
The secondary structure of β-lactoglobulin has been calculated from circular dichroism data and contains approximately 17% α-helix, 54% β-structure and 25% random coil (Clark and Smith, 1989; Creamer et al., 1993). β-lactoglobulin exists as five different genetic variants. The two most common variants are known as A and B, they differ at position 63 and 118 where the aspartic acid and valine in the A variant are substituted by a glycine and alanine in the B variant (Eigel et al., 1984).

Native β-lactoglobulin possesses 2 disulphide bonds (cys 66-cys160 and cys106-cys119) and a free thiol group at amino acid 121 which facilitates the potential for inter and intra molecular S-S-interchange during the pH or heat treatment (Papiz et al 1986). Above 65°C β-lactoglobulin undergoes time/temperature dependent conformational transition (molecular expansion) that expose highly reactive nucleophilic (SH and ε-NH₂) groups in the hydrophobic regions (McKenzie 1971, Kella and Kinsella 1988).

1.7.3 Functional properties of whey proteins

Whey proteins possess outstanding physicochemical properties in their gelation and binding; they are widely used as functional ingredients in many formulations of bakery, dairy and sausage products (Kinsella and Whitehead, 1989; Morr and Ha, 1993). Commercial whey proteins vary appreciably in their functional properties depending on their origin and method used to isolate them. This variability may be attributed to the differences in the amount of protein, lactose, fat, and minerals; in the ratio of the major whey proteins, and to the differences in the degree of protein denaturation and aggregation caused by processing. (Huffman, 1996). The variability of whey protein presents a major challenge to food researchers studying the basis of functionality. Mulvihill and Donovan, (1987), Morr and Ha, (1993) and Clark, (1998) provide excellent reviews on the impact of processing conditions on functional attributes of whey proteins. Food manufacturers are increasingly designing new products and then seeking functional ingredients to meet the specifications for that particular product, rather than allowing the type of product to be limited by the properties of the traditional protein preparations. In the view of these developments the producers of whey proteins recognize that the requisite functional properties in the final preparation must meet a set of decisive criteria. Food applications of whey
proteins require a different set of functional properties. For example, in the bakery applications, whey powders should readily hydrate. The most studied functional properties of whey proteins include their emulsification and gelation properties (Phillips et al., 1994; Huffmann, 1996).

1.7.4 Gelation of whey proteins

One of the main functional applications of whey proteins is as a gelling agent. Whey proteins can form gels that range in properties from viscous fluid, soft, smooth paste or curds to stiff rubbery gels. These gels vary in hardness, cohesiveness, stickiness, colour and mouth feel. Whey protein concentrate gels also vary in visual appearances from elastic transparent to opalescent curdlike coagula, (Schmidt, 1978; Kinsella and Whitehead, 1989).

The mechanism of whey protein gelation is similar to other globular proteins with an initial denaturation step followed by interaction to form a gel matrix (Mulvihill and Kinsella, 1987). The gelling properties of whey proteins depend on a number of factors; the composition and processing conditions used to produce the whey proteins, protein concentration (Hillier and Cheeseman, 1979). Mulvihill and Kinsella (1987) indicated that a minimum of 8% protein was required for gelation. The presence of lipids and lactose impairs the gelation of whey proteins (Schmidt et al., 1978).

The gel structure of whey proteins is affected by salt concentration and type of salt; addition of sodium chloride results in coarse gels with large aggregates while calcium chloride restores the strength of whey protein gels (Comfort and Howell, 2002). Formation of bridges between Ca$^{2+}$ and specific residues of a polypeptide chain are known to be important in the stabilising gel network structure. This cation reduces the electrostatic repulsion and permits greater protein-protein interactions and network formation. However, once the level of ion concentration is exceeded, calcium bridging becomes overwhelming and the matrix collapses (Mulvihill and Kinsella, 1988; Matsudomi et al., 1991).

Whey proteins have excellent gelling characteristics particularly above pH 7.0. The higher gelation tendency in the region above pH 8 may reflect some disulphide crosslinks and matrix formation via thiol-disulphide interchange (Kinsella and
Whitehead, 1989). Although much information exists concerning factors affecting gel formation and properties of whey gels there is still limited data on the kinetics of gel formation and gelling properties of individual whey proteins (Dunkerley and Hayes, 1981; Kinsella Mulvihill, 1987).

### 1.7.5 Whey proteins and high pressure processing

High pressure processing offers new opportunities for processing dairy products. Pressure treatment can be performed at room temperature and it is expected that gels formed from whey proteins by high pressure may have different properties from those formed by heat treatment (Hayashi et al., 1992). β-lactoglobulin which accounts for half the amount of whey protein has been shown to aggregate at low protein concentration during high pressure processing due to the generation of intermolecular disulphide bridges through SH/S-S interchange reactions (Dumay et al., 1994 and Funtenberger et al., 1995). The formation of intermolecular S-S bonds have also been reported in heat induced gelation (Howell and Lawrie, 1985; Iammetti et al., 1995).

High pressure processing has been reported to produce whey protein or β-lactoglobulin gels which possess sponge-like structures and are susceptible to exudation (Van Camp and Huyghebaert, 1995, Dumay et al., 1998). Other workers reported that whey and β-lactoglobulin gels can be stabilised against syneresis by the addition of xanthan gum and that a more elastic protein network was formed by the addition of the polysaccharide (Zasypkin et al., 1996).

Some studies have investigated the gel strength of high pressure-induced whey protein concentrate gels as a function of pH and reported that the optimum pH for the formation of high pressure-induced gels with greatest firmness was pH 9, whilst, lowest hardness occurred in the acidic pH range (Van Camp and Huyghebaert, 1995; Kanno et al., 1997; Walkestrom and Hermansson, 1997). Microscopy studies revealed structural differences between pressure-induced (400 MPa for 30 min) and heat-induced (0.1 MPa for 30 min at 80°C) whey protein gels. Heat-induced gels were stronger and possessed a greater number of permanent crosslinks between polypeptide chains. In contrast, high pressure-induced gels produced gels with a more porous network and fewer intermolecular crosslinks (Van Camp and Huyghebaert, 1995; Dumay et al., 1998).
A number of food and pharmaceutical products that contain whey protein as functional ingredients also contain sugar. For this reason Kulmyrzaev et al., (2000) investigated the influence of sucrose (0-40%) on the thermal denaturation and functionality of whey proteins at pH 7. Sucrose increased the gelation temperature and rigidity of the final cooled gels. The proposed mechanism for the thermal stability of proteins is that sugars decrease the thermodynamic affinity of protein for the solvent molecules (Timasheff and Arakawa 1993, Pasegian et al., 1995).

1.8 Rheological properties of proteins
1.8.1 Principles of rheology

Many foods are formed by the gelation of proteins. Gelation transforms the material physically from a viscous sol or liquid into a solid which is elastic in its response to a physical force application. Rheology is defined as the study of flow and deformation of a material (Barnes et al., 1989; Bourne, 2002). Flow relates to liquids and deformation to solid material; deformation maybe irreversible (viscous) and reversible (elastic). The science of rheology can be applied to study the texture and mouthfeel of food during processing and manufacturing. The importance of texture in the overall acceptability of foods varies depending upon the type of food. Rheology covers the behaviour of elastic solids through to viscous liquids. Many foods exhibit the properties of both an elastic solid and viscous liquids and these materials are said to be “viscoelastic” (Whorlow, 1992). In order to deform a material or make it flow, a force must be applied which should change only the shape and not the volume of sample. Rheology is concerned with relationships between force per unit area acting upon and within the material (shear stress) and the amount of deformation induced by this stress (shear strain). Rheology is therefore important in determining the texture of food products and subsequent acceptability to the consumer. The rheological properties of foods are also used in the design of food manufacturing and process systems in order to ensure that suitable equipment is put in place to handle the food throughout production. In practice rheological measurements can be divided into those that induce small or large deformation, small deformation tests probe viscoelastic parameters; large deformation tests measure stress, strain and failure properties of a given material (Hermansson, 1994, Bourne, 2002)
1.8.2 Small deformation testing

1.8.2.1 Principles

Small deformation oscillatory technique is non-destructive and allows the simultaneous measurement of visco-elastic behaviour of a material, by subjecting the material to a sinusoidal variable stress and measuring the resultant strain (deformation of sample) via a geometry (Prentice, 1984; Hamann, 1991). The sample is placed between the two surfaces comprising a stationary part with the sample and a geometry applying stress by the rheometer. The phase-angle between the input and output signals is also measured. For an ideal elastic solid (Hookian solid) the stress and the strain waves would be in phase (δ=0) as the sample is able to store the applied energy, then return to the energy system when relaxed. In contrast, a Newtonian fluid will dissipate the applied stress energy and the phase difference will be exactly 90° out of phase with the strain (δ=90°). As foodstuffs are generally visco-elastic the difference in their behaviour lies between 0 and 90° (figure: 1.8).

![Phase Lag Phase (δ)](image)

Figure 1.8: Dynamic response of a viscoelastic material

The measurements result in the evaluation of the two moduli, the first is storage modulus $G'$ (elastic component signifies that strain is recoverable in elastic behaviour) which is a measure of how well structured a sample is. The second is loss modulus $G''$ (viscous component, i.e. permanent deformation of flow) of the material, which represents the viscous dissipation of
energy and is related to dynamic viscosity (Gregory, 1994; Bourne, 2002). The loss modulus will be large if the sample is predominantly viscous. The viscous and elastic components contribute to the overall response and can be found by taking the ratio of the stress amplitude to the strain amplitude. This enables the calculation of the complex modulus $G^*$. $G^*$ can be calculated by dividing the applied stress by the measured strain. Best results are usually obtained at low frequencies because it is at low strain rates that molecular properties are elucidated (Hamann, 1991). This approach is useful for the discussion of the mechanical properties of the gel and to relate these to structure.

![Diagram of $G'$, $G''$, and $G^*$](image)

Figure 1.9: The relationship of $G'$, $G''$, and $G^*$

$G^*$ : Complex modulus = \[ \frac{\text{Shear stress}}{\text{Shear strain}} \]

$G' = G^* \cos(\delta)$ (storage modulus)

$G'' = G^* \sin(\delta)$ (loss modulus)
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1.8.3 Large deformation testing

1.8.3.1 Principle

The fracture properties of a gel are studied using compression testing techniques. Hence large deformation is a destructive technique which simulates the action of mastication (Prentice, 1984). Large deformation testing is used to study solid like samples. The parameters of interest are the Young's modulus of the gel, as given by the initial slope of the stress-strain graph, the force at fracture Fc (break strength) and deformation at fracture Δh (cohesiveness) (Comfort and Howell, 2002). The Young's modulus is the ratio of stress to strain when an elastic solid material is compressed or extended; it is a measure of stiffness and was developed by Thomas Young, an English physicist. It is described by the equation:

\[ E = \frac{\text{stress}}{\text{Strain}} = \frac{F/A}{\Delta L/L} \]

\[ \text{equation 2} \]

Where \( E \) is Young's modulus, \( F \) is the applied force perpendicular to the area defined by stress, \( A \) is the cross-sectional area of the test material, \( L \) is the change in length resulting from the application of force \( F \). The prefix “Young’s” is often omitted making this term “modulus of elasticity”. Theoretically, the Young’s modulus of elasticity is used to describe elastic materials. However, most foods are visco-elastic in nature, opponents of this term argue that it should be used where the mechanical behaviour of a food is well defined and suggest the term “modulus of deformability” (Bourne, 2002). According to Hamann (1991) information obtained from large deformation testing is directly related to sensory perceptions of food. Fracture, shear stress or force of fracture is correlated with sensory hardness and fracture shear strain or deformation at fracture with sensory cohesiveness.

1.9 Raman spectroscopy

1.9.1 Principle

Raman spectroscopy is a branch of vibrational spectroscopy, based on the premise that molecules possess specific vibrational modes that result from chemical bonds or chemical groupings in a molecule. When a beam of light impinges on a sample, photons are absorbed and scattered by the material. The vast majority of these photons have exactly the same wavelength as the incident photons and are known as Rayleigh scatter; however, a tiny portion (approximately 1 in \( 10^7 \)) of scattered radiation is shifted to a different wavelength.
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These wavelength-shifted photons are called Raman scatter, named after Sir-Raman. Most of the Raman scattered photons are shifted to longer wavelength (Stokes shift) but a small portion are shifted to shorter wavelength (anti-Stokes shift). Figure 1.10 shows a diagram of the molecular origin of Rayleigh scatter, Stokes Raman and anti-Stokes Raman scattering. In normal Raman scattering, these are two-photon processes passing through virtual electronic state, and resulting in the creation of a quantum of vibration in a particular chemical bond in the sample.

In essence, Raman spectroscopy is based on the shifts in wavelength or frequency of the exciting incident beam resulting from inelastic collisions with the sample molecules (Carey, 1982). Inelastic collisions occur when there is an energy exchange between photon and molecule. In classical terms the interaction can be viewed as a perturbation of the molecule’s electric field. In quantum mechanics the scattering is described as an excitation to a virtual state lower in energy than the real electronic transition. In both types of Raman scattering the molecular vibration goes to a different level. Stokes Raman occurs when the final energy level is higher than the initial level. Stokes scattering is more common than the anti-Stokes Raman scattering because molecular vibration mode at normal temperatures is most likely to be in its lowest energy state in accordance with Boltzmann distribution. Typically, only the Stokes region is used in spectroscopy rather than anti-Stokes as the former is more intense (Li-Chan, 1996).

Figure 1.10: Relationship between, Rayleigh scattering, Raman Stokes scattering and Raman Anti-Stokes scattering (Li-Chan, 1996).
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A Raman spectra is a plot of the intensity of Raman scattered radiation as a function of its frequency difference from incident radiation (usually in wavenumbers, cm\(^{-1}\)). The difference is called Raman shift. It should be noted that, because it is a difference value, the Raman shift is independent of the frequency of incident radiation. Since both the intensity and frequency of the molecular vibrations are sensitive to chemical changes and environment around the atoms, the Raman spectrum can be used as a monitor of molecular chemistry and can act as a useful tool to probe protein structure in solid and liquid food systems (Li-Chan et al., 1994). Bands in the Raman spectrum arising from amide I, amide II and skeletal modes of peptides are useful for characterizing backbone conformation, including the estimation of secondary structure fractions. Bands attributed to various stretching or bending vibrational modes of functional groups of amino acids residues can be used to monitor the environment around these side chains. Raman spectroscopy can also be used as a quality control aid; a method of adulteration detection and as general procedure for studying changes in food structure during food processing (Li-Chan, 1996).

1.9.2 Comparison of infrared and Raman spectroscopies

Raman spectroscopy is complementary to infrared absorption spectroscopy in that both are based on the 'discrete vibrational transitions that occur in the ground state of molecules which correspond to various stretching and bending deformation modes of individual chemical bonds' (Li-Chan, 1996). The complementarity of infrared and Raman spectroscopy results from the different selection rules which determine the appearance in the infrared and or Raman spectrum of a band corresponding to a given vibration of the molecule. If the vibration causes a change in the dipole moment, which happens when the vibration changes the symmetry of charge density distribution, it is active in the infrared spectrum. If the vibration produces a change of the molecular polarizability, it is active in the Raman spectrum (Baranska et al., 1987).

It is therefore possible to say if there is a centre of symmetry in the molecule, a vibration active in the infrared spectrum is inactive in the Raman spectra and vice versa, such that a change in the intrinsic dipole moment is needed for infrared and changes in polarizability of functional groups are essential for Raman spectra. For example polar groups such as C=O and
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O-H have strong IR stretching vibrations; non-polar groups such as C=C and S-S have Raman bands (Li-Chan, 1996).

Water is a polar molecule and therefore exhibits strong infrared absorption. Consequently, infrared spectroscopy can only be applied to analysis of dry/non aqueous samples and is therefore not a suitable method for analysis of foods which are aqueous in nature. In contrast Raman spectroscopy has weak scattering properties for water, so the technique can be applied to analyse food samples, solutions, gels, powders or precipitates. This also explains the dissolving of samples in D$_2$O (Deuterium Oxide) instead of water during Raman experiments.

1.9.3 Quantitative analysis of secondary structure

In Raman spectroscopy experiments, it is now possible to obtain quantitative data. The use of the Amide 1 band in the estimation of the secondary structure is based on the principle that each amide group in a protein has a Raman intense frequency that depends on conformation, on whether the protein is hydrogen bonded to water or another amide (Williams and Dunker, 1981) and on coupling effects between residues. The Amide 1 band is primarily due to the C=O stretching vibration of the amide group weakly coupled with the in-plane NH bending and CN stretching (Susi and Byler, 1988).

The position of these bands is sensitive to differences in the orientation of molecular subgroups or changes in interchain and intra-chain hydrogen bonding interactions of peptide groups (Krimm and Bandekar, 1986). The Amide 1 band of proteins is a complex composite which consists of a number of component bands in terms of $\alpha$-helices, $\beta$-sheets (parallel pleated sheet and anti-parallel pleated sheet), turns and random coil structures, which cannot be resolved by conventional spectroscopic techniques because their inherent widths are greater than the instrumental resolution (Byler and Susi, 1986). The resolution enhancement techniques such as Fourier Self Deconvolution, are used to reveal the individual peaks attributable to various secondary structures. In this study Fourier Self Deconvolution was applied to determine component bands and their positions. Changes in secondary structure of globular proteins are known to accompany heating for both ovalbumin and $\beta$-lactoglobulin (Clark et al., 1981; Kato and Tagaki, 1988; Mine et al., 1990; Kinsella and Donovan, 1987; Casal et al., 1988; Boye et al., 1996; Howell and Li-Chan, 1996). Pannick et al., (1999)
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reported changes in secondary structure of \( \beta \)-lactoglobulin, following high pressure treatment. Details of these investigations are discussed in 5.4.1.2.

1.9.4 Raman Equipment

The traditional spectrometers are based on the monochromatic grating or prism which disperses a collimated beam of infrared light onto a slit which effectively blocks all but a narrow range of frequencies from reaching the detector. By continuously changing the angle of the grating with respect to the incident light beam, a complete spectrum can be scanned, one spectral resolution at a time (Byler and Susi, 1986).

Recent modification of the technique in the form of Fourier Transform Raman spectroscopy means that, the FTIR instrument by contrast, is non-dispersive and makes use of the interferometer to encode data from the whole spectral range simultaneously. The interferometer is illuminated with a polychromatic source of light, the detector receives a signal (interferogram) which is a summation of all the interferences resulting from constructive or destructive interaction between each wavelength component and all others (Parker 1983). Once an interferogram is digitalised and stored by the computer a complex Fourier transform can be performed on the interferogram to decode the multiplexed information and obtain a Raman spectrum.

In principle the FTIR provides several advantages over convectional dispersive instruments; 1) higher resolution, 2) sensitivity, 3) signal to noise ratio (S/N) and 4) frequency accuracy. The improved S/N ratio facilitates resolution enhancement of observed protein through the application of Fourier self-deconvolution techniques. This is of importance because it offers the possibility of obtaining quantitative information on the conformation and changes in the secondary structure of proteins.
1.10 Conclusion

A review of the literature has indicated that there are gaps in our knowledge in terms of the structure and properties of egg albumen and whey proteins during heating and on high pressure processing. In addition the interaction of egg albumen and whey proteins has not been studied hitherto. As both proteins are used in combination in many dairy and bakery products, it is essential to study the gelation behaviour of egg albumen and its main constituent ovalbumin and whey protein isolate and its main component β-lactoglobulin, in isolation and in mixtures, using the advanced rheological, thermodynamic (Differential Scanning Calorimetry) and spectroscopic techniques (Raman spectroscopy). Moreover the effect of sugar and salt on protein behaviour has only been studied to a limited extent and requires detailed investigation, therefore the objectives of this study are:

I. To determine the structural and physicochemical properties of egg albumen, ovalbumin, whey protein isolate and β-lactoglobulin using small and large deformation rheology, FT Raman spectroscopy, differential scanning calorimetry and microscopy

II. To compare the effect of high pressure, heat and combined high pressure/heat treatment on the structure and texture of egg albumen and whey proteins.

III. To relate the rheological behaviour of single and mixed proteins systems to the structures of gels formed under high pressure and heat treatment and determine the nature of protein-protein interactions.

IV. To investigate the effect of sodium chloride and sugar on the rheological properties and molecular interactions of unheated, heated and high pressure treated egg albumen and whey proteins
Chapter 2. Materials and methods

2.1 Materials

Deuterium oxide, (D, 4561, 99.9% atom D), sucrose (purity 99.94% and calcium content 0.03 wt%), sodium chloride, ovalbumin, β-lactoglobulin, egg albumen was obtained from Sigma - Aldrich Chemical Company, Poole, Dorset, United Kingdom. Whey protein isolate (Bipro) was kindly supplied by Davisco Foods, International, USA.

2.2 Methods

2.2.1 Proximate analysis

2.1.1.1 Protein determination by Kjeldahl method

*The principle*

Kjeldahl method is a chemical method for determining the nitrogen content of solids as well as liquids. The procedure involves the wet combustion of a sample by heating with concentrated sulphuric acid in the presence of a catalyst usually metallic ions, to effect the reduction of organic nitrogen to ammonia. The ammonia is then retained in solution as ammonium sulphate. Addition of sodium hydroxide makes the solution alkaline and the ammonia is released by steam distillation, trapped or collected in boric acid and titrated against a standard acid. The amount of ammonia produced is directly correlated with the nitrogen content of the sample.

*Method*

The method employed for the determination of total protein was according to the AOAC methods (Williams, 1984). 2 g of the protein powders were weighed on Whatman filter paper and placed into digestion tubes (in triplicates). A blank was made by digestion of Whatman filter paper in the absence of a protein sample. Two selenium catalyst tablets (Sigma) and 20 ml of concentrated sulphuric acid were added to each tube and gently swirled before being placed in a Tecator Kjeltec Digestion System 1 apparatus at 420°C for 2-4 hours. Completion of digestion stage was recognised by the formation of a clear solution. Upon cooling 75 ml of distilled water were added to the digested samples. The ammonia was released by addition of
excess sodium hydroxide (40% w/v) and removed by steam distillation in a Tecator Distillation Unit 1002. The ammonia was collected in 25 ml of 4% (w/v) boric acid (Fisons) and titrated with standard 0.1M hydrochloric acid using methylene red as indicator.

**Calculation of the total crude Nitrogen**

Crude protein was calculated using a conversion factor of 6.25 (Egan, 1981). The following equations was used:

\[
\%N = \frac{14.01 \times (\text{Titrant sample (ml)} - \text{Blank titrant (ml)})}{\text{sample (g)} \times 100} \quad \text{Equation 2.1}
\]

\[
\% \text{ Protein} = N \times K \quad \text{Equation 2.2}
\]

The correction factor K depends on the amount of nitrogen present in the protein sample (Table 2.1)

**Table 2.1 Kjeldahl correction factors**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Factor K</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat</td>
<td>5.70</td>
</tr>
<tr>
<td>Whey</td>
<td>6.38</td>
</tr>
<tr>
<td>BSA</td>
<td>6.38</td>
</tr>
<tr>
<td>Egg albumen</td>
<td>6.20</td>
</tr>
</tbody>
</table>

It was necessary to determine the protein content of egg albumen and commercial whey protein isolate in all the experiments undertaken were based on w/w of protein not the weight of the powder.
2.2.1.2 Determination of fat by Soxhlet extraction

*Principle*

Soxhlet procedure was used to extract crude fat from the protein powders of whey and Egg albumin. Soxhlet method is based on a simple reflux concept where the solvent when heated, evaporates and condenses above the sample. The pure condensed solvent drips through the sample back into the flask taking with it any dissolved fat.

\[
\text{% Fat} = \frac{\text{Weight loss on extraction} \times 100}{\text{Weight of Moisture}}
\]

2.2.1.3 Moisture

The concept of moisture determination is important in food processing and quality testing of foods. Since the amount of dry matter in a food is inversely related to the amount of moisture it contains, moisture content is of direct importance to the processor and consumer. The effect of moisture on stability and quality of foods is well known, for example, grain that contains too much water is subject to rapid deterioration from mould growth, heating, insect damage and sprouting (Pomeranz and Meloan 1994).

*Method*

Moisture determination was carried out as per guidelines laid out in AOAC method (Egan 1981) The formula below was used to calculate moisture content:

\[
\text{% moisture} = \frac{\text{weight loss on drying at 100°C}}{\text{weight of sample}} \times 100
\]
2.2.2 Differential scanning calorimetry (DSC)

2.2.2.1 The principle

Differential Scanning Calorimetry is a technique in which the property of a sample is monitored against time or temperature while the temperature of the sample is varied according to a specified programme. The application of heat to a substance can induce a change of state of the substance, for example, melting of a substance when temperature is increased. Change of state of a substance is accompanied by change in energy level. Changes in energy level are manifested by absorption (endothermic) or liberation (exothermic) of heat. The term enthalpy in relation to calorimetry is associated with the changes in heat due to a change of state. Enthalpy is therefore defined as the quantity of heat that is absorbed by a closed isothermal system, if at constant pressure with no work performed, the change of state is associated with volume change (Kilara and Sharkasi, 1996).

In differential scanning calorimetry, the sample and reference material are sealed into metal crucibles, placed on a metal plate connected to a furnace which is a heat sink and the temperature difference of the sample and reference substance is measured. The difference of heat flowing from sink into the sample and reference is proportional to the temperature difference of the sample and the reference substance, differential heat flow (mJ/sec) between the sample and reference are recorded and displayed graphically as a thermogram. On the DSC trace, heat flow in (mJ/sec) is plotted against temperature, a downward peak represents an absorption of heat (endothermic reaction). A thermally induced change such as protein denaturation is an endothermic process and the upward peak indicates a release of heat (Ma and Harwalkar, 1996).

2.2.2.2 Food applications of DSC

In food systems DSC is applied in a number of ways: (1) Under normal biological conditions a knowledge of protein structure and conformation are essential for understanding how protein functions. This knowledge can be applied in optimising conditions for protein isolation and processing of foods. (2) DSC can be used to monitor conformational changes in proteins with the addition of salt or adjustment of
Chapter 2. Materials and methods

pH; such reagents modify protein structure and can provide information on forces involved in protein structure. (3) DSC can be used to monitor changes resulting from processing treatments such as heating or storage. For example when oat globulin was preheated at 100°C and 110°C there was a progressive decrease in enthalpy, indicating partial denaturation, while changes were more pronounced at higher temperatures of preheating and (4) Protein-protein interactions can be assessed by DSC. These interactions can be demonstrated by shifts in (Td) or changes in the shape of the endotherm which suggests interaction between proteins. An example of protein-protein interactions which results in increased stability of the protein complex is the association of bovine trypsin with soybean trypsin inhibitor or egg albumen ovomucoid this is shown by a significant increase in the (Td) of the complex compared with the protein on its own (Barbut and Findlay, 1990). Change in thermal stability also indicates interaction between proteins when only one shows a thermally detectable transition, for example, β-betalactoglobulin denatures easily while κ-casein is thermally inert (Paulsson and Dejmek 1990). The interaction affects the technological behaviour of milk and milk products; for example in the syneresis of yogurt, rennet coagulation time and the stability of milk.

2.2.2.3 Method

Whey protein isolate, β-lactoglobulin, egg albumen and ovalbumin protein solutions (15% w/w) were made in distilled water in duplicates. Each sample (0.80 g) and reference (water) was heated at 0.5°C/min from 10 to 90°C before cooling to 10°C using the Setaram DSC V11 micro-calorimeter (Setaram, Lyons, France)(cycle 1). The sample was reheated and cooled (cycle 2) to establish a baseline. The temperature reached when half of the protein was denatured referred to as (Tm), was measured at the tip of the peak. The total energy required to denature the protein, the enthalpy change (ΔH), was measured by intergrating the area under the peak (Badii and Howell, 2002, 2003).
2.2.3 Transmission electron microscopy

2.2.3.1 The principle

The excellent resolving power of transmission electron microscopy is largely a function of very short wavelength of electrons accelerated under the influence of an applied electric field (Goodhew et al., 1998). Ordinary microscopes use visible light and magnify approximately 1500 times and have a resolution limit of about 0.2 μm, whereas a transmission electron microscope is capable of magnifying approximately 200,000 times and has a resolution limit of about 1 nm (Simpkins, 1989; Goodhew et al., 1998). Transmission electron microscopy requires careful sample preparation; preservation of samples requires initial fixation which can be achieved by rapid freezing or chemical treatment to stabilise and crosslink the protein. The most common fixation agent is glutaraldehyde, which is able to form methylene bridges with side chain amino groups of protein. The fixed tissues are then dehydrated and sectioned.

2.2.3.2 Method

1 mm$^3$ cube samples were cut from gels prepared by heating at 90°C for 30 min and high pressure treated at 600 MPa for 20 min. The gels were fixed according to method developed by (Howell and Comfort, 2002) for 4 hours in 5 ml of 4 % glutaraldehyde (Sigma, E.M grade) in 0.1 M cacodylate buffer (Sigma) at pH 7.4. After fixation the samples were washed four times in cacodylate buffer before being dehydrated. The dehydration process involved passing the samples through 25 % (w/v) ethanol, followed by 50 % (w/v) ethanol, 75 % (w/v) ethanol, 90 % (w/v) ethanol and finally absolute ethanol. Samples were held for at least 10 minutes in each solution, and each solution was used twice. After the second incubation in absolute ethanol, the samples were placed in a 1:1 ratio of absolute ethanol and L.R. White resin for 20 minutes. During this time they were placed on a rotating drum as the two solutions were immiscible. Finally, the samples were incubated in pure L.R. White resin for 4 hours at room temperature in individual capsules (TAAB), and to polymerise the resin samples were incubated for a further 12 hours at 60°C. The resultant embedded samples were sectioned on a microtome and floated onto nickel grids. The samples were then stained with uranyl acetate and viewed on a 400T transmission electron microscope.
CHAPTER 3
Chapter 3. Studies on egg albumen and whey protein interactions by FT-Raman spectroscopy and rheology

3.1 Introduction

Protein-protein interactions which occur during processing and storage of many food products alter the functional properties of food proteins including gelation, emulsification and foaming (Howell, 1992, 1994). In food systems, interaction of globular proteins with other food proteins can lead to synergism, phase separation or precipitation, depending on their structure and physical chemical properties thereby affecting food texture (Howell, 1995). Studies by Howell and Lawrie (1984, 1985) on the gelation of plasma and egg albumen protein showed synergistic interactions which varied with time and temperature of heating and the concentration of each protein in the mixture. In contrast, the combination of lysozyme with either α-lactalbumin or β-lactoglobulin resulted in aggregation due to mainly electrostatic interactions as judged by turbidity measurements, chromatography and molecular modelling (Howell et al., 1995). However, in this case the aggregation was between the basic lysozyme (pI 10) and negatively charged β-lactoglobulin (pI 6). Previous studies by Howell and Lawrie (1984) on mixtures of soya isolate or sodium caseinate and blood plasma proteins showed phase separation. Similar findings were reported by Roefs et al., 1994; Polyakov et al., 1997; Tolstoguzv, 2002). More recently, Comfort and Howell (2002) reported that the gelation of whey and soya protein isolates resulted in phase separation due to incompatible molecular size and configuration of the proteins.

Although, mixtures of globular proteins like plasma and egg albumen exhibited synergistic interactions (Howell and Lawrie, 1984), this hypothesis has not been tested in other similarly charged globular proteins used in food manufacture. In product formulation, synergistic interactions can be used to produce stronger gels with lower concentration of proteins. Whey and egg proteins are globular proteins widely used in meat, dairy and bakery food products to enhance functional properties. Due to the heterogeneity of whey and egg albumen, it is necessary to assess the compatibility and interactions of both the individual proteins and whole protein isolate. To investigate protein-protein interactions, the gelation properties of egg albumen, whey isolate protein and their binary mixtures were studied by large and small deformation rheology. Previously, evidence for protein-protein interactions was provided by viscosity and turbidity measurements, electron microscopy and chemical
modification, for example using urea, SDS or β-mercaptoethanol (Howell and Lawrie, 1985; Howell and Taylor 1991; Murphy and Howell, 1991). However, these methods do not ascertain whether the changes are due to modification of the individual proteins or genuine interactions. With the availability of more direct spectroscopic methods like FT-Raman spectroscopy it is now possible to examine protein-protein interactions non-invasively in more detail. The role of covalent, disulphide bonds as well as electrostatic and hydrophobic interaction in protein–protein interactions in the lysozyme-whey protein aggregates was confirmed previously by Raman spectroscopy (Howell and Li-Chan, 1996; Howell et al., 1999). The advantage of Raman spectroscopy over other spectroscopic techniques or chemical analysis is that it can be used for aqueous solutions, crystal, fibres, gels and films without destruction of samples. The Raman spectra of proteins can also give information regarding peptide backbone conformation as well as the microenvironment around the side chains (Li-Chan, 1996). Thus, Raman spectroscopy provides a more direct way of studying the interactions of individual and combined whey and egg albumin protein gels. The objectives of this study were to test the hypothesis that small similarly charged globular proteins such as whey and egg albumen can form compatible gel networks and undergo synergistic interactions and secondly to relate protein interactions to changes in structure by FT-Raman spectroscopy of unheated and heated egg and whey proteins in isolation and combination.

Chapter 3. Studies on egg albumen and whey protein interactions by FT-Raman spectroscopy and rheology
3.2 Materials

Materials were the same as in section 2.1

3.3 Methods

3.3.1. FT-Raman spectroscopy

3.3.3.1. Sample preparation

The protein content of egg albumen and whey protein isolate were determined and found to be 80% and 95% respectively. These values were used to make up the solution on a w/w protein basis. Solutions of individual whey and egg albumen proteins 15% (w/w) were prepared in D$_2$O. A mixture of 7.5% (w/w) whey protein and 7.5% (w/w) egg albumen protein, in D$_2$O was also prepared. Heated samples of the above protein solutions were made as follows. Protein solution (7 ml) was poured into stainless steel tubes 50 mm long and 15 mm diameter and heated at 90°C for 30 minutes to form gels. The gels were stored overnight at 4°C. Unheated and heated samples were placed in NMR tubes (5 mm diameter, Precision grade, Aldrich Chemical Company Milwaukee USA) and analysed by FT-Raman spectroscopy. The pH was adjusted to 7 using acid/alkali and nor buffer treated in all Raman samples used in this study.

3.3.3.2. F-T Raman spectroscopy, data collection and analysis

Raman spectra were recorded at 4°C on a Perkin Elmer 2000 FT-Raman spectrometer (Beaconsfield, Buckinghamshire, UK). Spectral resolution was set at 4 cm$^{-1}$, laser power 1600 mW and the data presented are based on 128 co-added spectra. Frequency calibration of the instrument was undertaken using the sulphur line at 217 cm$^{-1}$. The spectra were analysed using Grams 32 software (Galactic Industries Corp, Salem NH). The protein spectra obtained were baselined and the intensity was normalised using the phenylalanine peak at 1004 cm$^{-1}$ (Tu, 1986; Howell and Li-Chan, 1996). The major bands in the spectra, related to vibrational motions of various side chains or polypeptide backbone, were assigned by comparison with Raman spectra of proteins which have been reported in the literature (Howell and Li-Chan, 1996; Howell et al., 2001). The interaction of proteins was investigated by the analysis of the difference spectra calculated from the average of the normalised spectra of
individual proteins minus the experimental spectra of the protein mixtures. For the difference spectra the intensity values are recorded on the y axis.

3.3.2. Rheological methods
3.3.2.1. Small deformation testing
Egg albumen and whey isolate protein (15% w/w protein) were dissolved in distilled water on their own and as whey and egg albumen protein mixtures in the ratios 10:5; 7.5:7.5; and 5:10. The samples (0.5 ml) were analysed using a Rheometrics 200 controlled stress rheometer with a 40 mm parallel plate geometry and 0.3 mm gap. Temperature sweeps were recorded from 20°C to 90°C and on cooling from 90°C to 20°C, at a heating rate of 2°C per minute and an oscillation frequency of 1 rad/sec. The applied stress was varied to keep the strain at about 1%. The elastic modulus $G'$ and viscous modulus $G''$ were recorded; the gelation point was taken to be the temperature at which $G'/G''$ crossover occurred. The interaction between the proteins was calculated using the interaction index devised by Howell and Lawrie (1984) as follows;

\[
\text{Actual value-additive value*} \times 100\\
\text{Additive value}
\]

*i.e the value derived from summing the contributions of the component proteins measured in isolation, in proportion to the concentration of each in the mixture.

3.3.2.2. Large deformation testing
Protein solutions (15% w/w) in distilled water were poured into stainless steel tubes, 50 mm long and 15 mm in diameter and heated in a water bath at 90°C for 30 minutes to promote network formation. The heated samples were stored at 4°C overnight to age. The gelled samples were cut into 15 mm long cylinders and were tested on TAXT2 texture analyser (Stable Microsystems, Godalming, Surrey, UK). The force at maximum compression (12 mm) and the Young’s modulus (the gradient of the stress-strain curve) were measured. The test speed was set at 0.2 mm/sec, and the samples were compressed by a distance of 12 mm to a final height of 3 mm.
Statistical analysis

Statistical analysis was performed using the Instat programme (Instatgraph pad Software Inc. 1992-1998). An unpaired $t$-test was calculated using this programme.

3.4 Results and discussion

3.4.1 Rheological analysis

3.4.1.1 Small deformation testing

The initial value of the elastic modulus ($G'$) was $<0.01$ (Pa) at 20°C for whey protein isolate and egg albumen indicating that these proteins do not aggregate easily or interact at room temperature (Table 3.1). For whey protein, $G'$ increased gradually and a major increase was observed at 80°C which could be attributed to the denaturation of $\beta$-lactoglobulin prior to gelation. $\beta$-lactoglobulin constitutes more than 54% of whey proteins (Bottomely et al., 1989) and the denaturation temperature of $\beta$-lactoglobulin ranges between 76-82°C (Paulsson et al., 1990; Aguilera, 1995).

Table 3.1: Small deformation analysis comparing the elastic modulus ($G'$) values for egg albumen (EA), and whey protein isolate (W) at 20°C (beginning of heating cycle), 90°C and 20°C (end of cooling cycle)

<table>
<thead>
<tr>
<th>Protein (15%w/w)</th>
<th>$G'$ (Pa) at 20°C before heating</th>
<th>$G'$ (kPa) at 90°C</th>
<th>$G'$ (kPa) at 20°C after cooling</th>
<th>$G'/G''$ cross-over (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EA</td>
<td>$&lt;0.01$</td>
<td>12.2</td>
<td>73.4</td>
<td>(gel-like at outset)</td>
</tr>
<tr>
<td>W</td>
<td>$&lt;0.01$</td>
<td>10.4</td>
<td>21.6</td>
<td>80</td>
</tr>
<tr>
<td>10:5 W/EA</td>
<td>$&lt;0.01$</td>
<td>2.1</td>
<td>11.7</td>
<td>78</td>
</tr>
<tr>
<td>7.5:7.5 W/EA</td>
<td>$&lt;0.01$</td>
<td>4.1</td>
<td>15.9</td>
<td>69</td>
</tr>
<tr>
<td>5:10 W/EA</td>
<td>$&lt;0.01$</td>
<td>1.9</td>
<td>12.8</td>
<td>63</td>
</tr>
</tbody>
</table>
Egg albumen showed a distinctive gelation pattern different from that of whey protein isolate. The $G'$ value for egg albumen (15% w/w) was higher than the $G''$ value at the outset and throughout the experiment indicating an association of proteins; a $G'/G''$ crossover was not observed. There were transitions at 64°C and 75°C, corresponding to the denaturation of ovotransferrin and ovalbumin respectively (Donovan et al., 1975; Beverage et al., 1985; Hsieh and Regenstein 1992; Hsieh et al., 1993). At the end of the heating cycle (90°C) the storage modulus was 10.4 kPa and 12.2 kPa for whey protein isolate and egg albumen respectively; these results indicate that egg albumen had a stronger and more ordered gel network structure than the whey isolate. Ferry (1948) proposed a two step gelation process which may be used to describe egg and whey protein gelation as shown below.

![Diagram of gelation process](image)

Step 1, the denaturation step, is usually induced by heat and is reversible. Step 2 represents network formation, which may also be induced by heating and or cooling. Later theories by Barbu and Joly (1953) and Kratchovil et al (1962) proposed linear aggregation of partially unfolded globular proteins when repulsion was high and random aggregates or clumping when repulsion was small. More recently, it has been reported that most globular proteins do not unfold completely into linear molecules but rather, form partially unfolded expanded molten globules which link up like a 'string of beads' (Ohgushi and Wada, 1983; Howell, 1995). Howell and Lawrie (1984) reported that at lower temperatures (<79°C) blood plasma proteins formed reversible linear chain gel networks due to non-covalent, hydrogen, hydrophobic and electrostatic linkages whereas at higher temperature (>79°C) non-reversible heat set gels were formed involving covalent disulphide bonds. Rector et al., (1989) also reported reversible gelation of dialysed whey protein isolates and β-lactoglobulin gels.
Major changes were observed in $G'$ on cooling indicating further polymerisation of network strands. At the end of the cooling cycle (20°C) the elastic modulus ($G'$) was 73.4 kPa and 21.6 kPa for egg albumen and whey protein isolate respectively; indicating a greater % increase in $G'$ values for egg albumen proteins on cooling. This concurs with results observed by Comfort and Howell (2002). The mixtures showed a similar trend with an initial low $G'$ value at (<0.01Pa) increasing gradually during heating with a further increase on cooling. $G'$ represents elastic energy stored and recovered per cycle of a sinusoidal deformation and has been correlated with the number of intermolecular cross-links in a network (Phillips et al., 1994).

3.4.1.2. Large deformation testing

Large deformation tests which measure the mechanical properties of a gel indicated that the Young’s modulus (gradient of the stress-strain curve) followed a similar pattern to the storage modulus ($G'$) of the final network formed (Table 3.2). For whey protein isolate and egg albumen the Young’s modulus was 12.1 Pa (± 0.1) and 17.2 Pa (±1.3) respectively, reflecting a stronger egg albumen gel compared with whey protein gels.

In the mixed gels, the highest Young’s modulus value was obtained for the 10:5 whey/egg albumen mixture 43.3 Pa (± 4.0) compared to 19.4 Pa (± 0.6) and 26 Pa (± 1.2) for the 5:10 and 7.5:7.5 mixture of whey and egg albumen respectively. The higher Young’s modulus value of the 10:5 whey/egg albumen mixture is also reflected in the force at maximum (12 mm) compression, 2760 (± 300) compared with 1248 (± 40) for the 5:10 whey/egg albumen mixture and 1610 (± 120) for the 7.5:7.5 whey and egg albumen mixture. This finding indicated synergistic protein-protein interaction of the two proteins, and the corresponding calculated interaction index was highest in the 10:5 whey/egg albumen mixture at 179% (Table 3.2). This is in contrast to the small deformation tests where the interaction was highest in the 7.5:7.5 mixture whey/egg albumen mixture and indicated $G'$ values lower than the expected. This result may be due to differences in gel preparation and measurement between large and small deformation tests.
Chapter 3. Studies on egg albumen and whey protein interactions by FT-Raman spectroscopy and rheology

Table 3.2: Large deformation analysis of egg albumen (EA), whey isolate (W) and their mixtures (W/EA) heated at 90°C for 30 min showing force at maximum compression (12 mm) and Young’s modulus.

<table>
<thead>
<tr>
<th>Protein gel</th>
<th>Force (g) at 12mm compression</th>
<th>Young’s Modulus(Pa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15%EA</td>
<td>1159 (± 90)</td>
<td>17.2 (± 1.30)</td>
</tr>
<tr>
<td>15%W</td>
<td>905 (± 50)</td>
<td>12.1 (± 0.10)</td>
</tr>
<tr>
<td>10:5 W/EA</td>
<td>2760 (± 300)</td>
<td>43.3 (± 4.30)</td>
</tr>
<tr>
<td>Interaction Index</td>
<td>+ 179</td>
<td></td>
</tr>
<tr>
<td>7.5:7.5 W/EA</td>
<td>1610 (± 120)</td>
<td>26.0 (± 1.20)</td>
</tr>
<tr>
<td>Interaction Index</td>
<td>+ 56</td>
<td></td>
</tr>
<tr>
<td>5:10 W/EA</td>
<td>1248 (± 40)</td>
<td>19.4 (± 0.60)</td>
</tr>
<tr>
<td>Interaction Index</td>
<td>+ 16</td>
<td></td>
</tr>
</tbody>
</table>

Figures in parenthesis are standard deviation values based on 5 replicates

Interaction Index = \( \frac{\text{Actual value} - \text{additive value} \times 100}{\text{Additive value}} \)

* i.e. the value derived from summing the contributions of the component proteins measured in isolation, in proportion to the concentration of each in the mixture. (Howell and Lawrie, 1984).
3.4.2. F-T Raman Spectroscopy

3.4.2.1. Comparison of unheated samples of whey and egg albumen proteins

The spectra for whey and egg albumen proteins (Fig.3.1, 3.2, Table 3.3) showed some common principal features. There were well resolved peaks between 900-970 cm\(^{-1}\) region (Amide II alpha helix region). For egg albumen, a strong peak was centred at 960 cm\(^{-1}\) and strong shoulder peaks were observed at 933 cm\(^{-1}\) and 947 cm\(^{-1}\). In contrast, there was a weaker intensity peak at 991 cm\(^{-1}\) representing the beta sheet structure. In whey, strong peaks occurred at 938 cm\(^{-1}\) and 951 cm\(^{-1}\) (alpha-helix region). A higher beta-sheet content was shown by pronounced peaks at 986 cm\(^{-1}\) (Amide II anti-parallel beta-sheet), and the band at 1239 cm\(^{-1}\) (Amide II anti-parallel beta-sheet) whereas a weaker band was observed in egg albumen at 991 cm\(^{-1}\) and no peak was observed at 1239 cm\(^{-1}\) for egg albumen. Similarly, the absence of the beta sheet structure at 1239 cm\(^{-1}\) and presence of a peak at 1320 cm\(^{-1}\) indicated a higher content of helical structure in egg albumen in accordance with literature values (Mine et al., 1990). Whey proteins comprise 50% beta-lactoglobulin and the behaviour of the isolate reflects that of the predominant protein (Bottomely et al., 1990). For whey it has been reported previously that the secondary structure of beta-lactoglobulin has 40-50% beta-structure and less than 10% alpha-helix (Byler and Susi 1986, 1988). In addition, these findings are consistent with previous work, which showed, that in unheated globular proteins the alpha helix structure tends to predominate (Clarke et al., 1981, Li-Chan, 1996; Howell and Li-Chan, 1996).
Chapter 3. Studies on egg albumen and whey protein interactions by FT-Raman spectroscopy and rheology

Figure 3.1: FT-Raman spectra (400-1600 cm\(^{-1}\)) of 15% (w/w) unheated whey protein in D\(_2\)O. The spectra were normalised to the phenylalanine peak at 1004 cm\(^{-1}\).

Figure 3.2: FT-Raman spectra (400-1600 cm\(^{-1}\)) of 15% (w/w) unheated egg albumen protein in D\(_2\)O. The spectra were normalised to the phenylalanine peak at 1004 cm\(^{-1}\).
### Table 3.3: Normalised intensity values for selected regions of the F-T Raman spectra of unheated (U) and 90°C heated (H) egg albumen and whey proteins

<table>
<thead>
<tr>
<th>Peak Assignment</th>
<th>Normalised Intensity*</th>
<th>Egg albumen</th>
<th>Whey</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>U</td>
<td>H</td>
</tr>
<tr>
<td>Cystine S-S</td>
<td></td>
<td>0.11±0.11</td>
<td>0.15±0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(497)</td>
<td>(502)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.13±0.01</td>
<td>0.25±0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(524)</td>
<td>(538)</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.17±0.02</td>
<td>0.12±0.01</td>
<td>0.39±0.02</td>
</tr>
<tr>
<td></td>
<td>(761)</td>
<td>(763)</td>
<td>(758)</td>
</tr>
<tr>
<td>Tyrosine Doublet</td>
<td>0.30±0.02</td>
<td>0.31±0.02</td>
<td>0.26±0.02</td>
</tr>
<tr>
<td></td>
<td>(831)</td>
<td>(827)</td>
<td>(831)</td>
</tr>
<tr>
<td></td>
<td>0.25±0.04</td>
<td>0.25±0.03</td>
<td>0.25±0.01</td>
</tr>
<tr>
<td></td>
<td>(852)</td>
<td>(854)</td>
<td>(851)</td>
</tr>
<tr>
<td>CH bend/Trp</td>
<td>0.40±0.02</td>
<td>0.38±0.03</td>
<td>0.95±0.02</td>
</tr>
<tr>
<td></td>
<td>(1337)</td>
<td>(1337)</td>
<td>(1350)</td>
</tr>
<tr>
<td>COO⁺ (Asp/Glu)</td>
<td></td>
<td></td>
<td>1.91±0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(1418)</td>
</tr>
<tr>
<td>CH₂ bend</td>
<td>0.93±0.02</td>
<td>1.14±0.06</td>
<td>1.90±0.02</td>
</tr>
<tr>
<td></td>
<td>(1454)</td>
<td>(1450)</td>
<td>(1450)</td>
</tr>
<tr>
<td>C-N/C-C stretch</td>
<td>0.11±0.01</td>
<td>0.83±0.04</td>
<td>0.23±0.02</td>
</tr>
<tr>
<td></td>
<td>(1060)</td>
<td>(1061)</td>
<td>(1051)</td>
</tr>
<tr>
<td>Amide 111</td>
<td>0.52±0.06</td>
<td>0.40±0.01</td>
<td>0.63±0.02</td>
</tr>
<tr>
<td></td>
<td>(933)</td>
<td>(929)</td>
<td>(938)</td>
</tr>
<tr>
<td></td>
<td>0.44±0.04</td>
<td>0.46±0.01</td>
<td>0.73±0.01</td>
</tr>
<tr>
<td></td>
<td>(947)</td>
<td>(941)</td>
<td>(951)</td>
</tr>
<tr>
<td></td>
<td>0.33±0.01</td>
<td>0.42±0.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(960)</td>
<td>(960)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.27±0.01</td>
<td>0.51±0.01</td>
<td>0.61±0.03</td>
</tr>
<tr>
<td></td>
<td>(991)</td>
<td>(988)</td>
<td>(986)</td>
</tr>
<tr>
<td>β-sheet structure</td>
<td></td>
<td></td>
<td>0.83±0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(1239)</td>
</tr>
<tr>
<td>α-helix structure</td>
<td>0.46±0.01</td>
<td>0.34±0.03</td>
<td>1.10±0.01</td>
</tr>
<tr>
<td></td>
<td>(1317)</td>
<td>(1323)</td>
<td>(1321)</td>
</tr>
</tbody>
</table>

* intensity at the wavenumber (cm⁻¹) shown in parenthesis, was normalised to the intensity of the phenylalanine band at 1004 cm⁻¹. Spectral data were the average of 128 scans and baseline corrected.
For whey proteins a fairly broad band was observed at 500 cm\(^{-1}\) and 511 cm\(^{-1}\) which may be attributed to the cystine residues. It has been suggested that in whey proteins the S-S stretching frequency lies around 507-510 cm\(^{-1}\) for the gauche – gauche – gauche conformation (Belloco et al., 1972). The location of the S-S stretching band near 524 cm\(^{-1}\) shown in Fig. 1b for egg albumen indicated a gauche – gauche – trans conformation of the disulphide bond. Painter and Koenig (1972) showed that ovalbumin has one disulphide bond and more recently Li-Chan and Qin (1998) reported that the spectral band arising from the disulphide bond is located near 525 cm\(^{-1}\). Egg white is known to be made up of 54% ovalbumin (Nisbet et al., 1981; Nakai et al., 2000), and contains 1 disulphide bond and four free sulphhydryl groups.

Whey protein showed double peaks at 1418 cm\(^{-1}\) and 1450 cm\(^{-1}\), the peak at 1418 cm\(^{-1}\) corresponded to the C=O stretch of dissociated carboxyl group of Asp or Glu and side chain vibrations of the imidazole ring of histidine (Howell and Li-Chan 1996, Li-Chan and Qin, 1998). In contrast, the band at 1418 cm\(^{-1}\) was absent in egg albumen suggesting that these residues might be involved in some kind of interaction within the molecule. Tu (1986) and Nakai et al., (1994) indicated that carboxyl group vibrational bands can be used to monitor the state of ionisation since ionised groups (COO\(^{-}\)) exhibit a band at 1400-1420 cm\(^{-1}\) while the undissociated form of COOH exhibits bands at 1700-1750 cm\(^{-1}\).

A methylene (CH\(_2\) bend) band was observed at 1450 cm\(^{-1}\) in whey and at 1454 cm\(^{-1}\) in egg albumen protein. The Amide 1 band was centred at 1654 cm\(^{-1}\) and 1662 cm\(^{-1}\) for whey and egg albumen respectively. For whey proteins, Nonaka et al., (1993) reported a peak at 1658 cm\(^{-1}\) and Howell and Li-Chan (1996) reported a slightly higher wavenumber centred at 1670 cm\(^{-1}\) where the contribution of the \(\beta\)-sheet is dominant. In egg albumen the Amide 1 band was centred around 1662-65 cm\(^{-1}\) (Painter and Koenig 1972; Li-Chan and Qin, 1998). The Amide 1 band was investigated in section 5.4.1.2 by deconvolution in subsequent studies.
3.4.2.2. Comparison of heated samples of egg albumen and whey proteins

FT-Raman spectra for whey and egg albumen solutions, heated to form gels at 90°C for 30 minutes, are shown in Fig.3.3, 3.4 and data for selected peaks are shown in Table 3.3. Similar changes in the secondary structure of both whey and egg albumen proteins were observed on heating compared with the unheated samples. Compared with unheated samples (0.61 for whey and 0.27 for egg albumen) there were bigger and pronounced peaks in the Amide II region at 988 cm⁻¹, suggestive of the increased anti-parallel β-pleated sheet, in both heated whey (0.88 ± 0.08) and egg albumen (0.51 ± 0.01); (p<0.05) (Kato and Takagi, 1988; Wang and Damodaran, 1991; Boye et al., 1996; Howell and Li-Chan, 1996). Mine et al., (1990) reported that the change in CD spectra of heat denatured egg white was due to an increase in β-sheet structure and reduction of helical structure. In addition, there was a reduction in peak intensity in the Amide III α-helix region at 938 cm⁻¹ and 953 cm⁻¹ (p<0.05) for whey proteins; for egg albumen no significant changes were observed. According to Wang and Damodaran (1991) the transformation of α-helix and aperiodic structure into a critical level of β-structure is required for gel formation.

Compared with unheated whey proteins there was a decrease in the intensity of the band at 1419 cm⁻¹ assigned to the C=O group of Asp and Glu carboxylate groups in heated whey protein, suggesting changes in the ionisation state of this residue. This band was not observed in either unheated or heated egg albumen.

Changes in the hydrophobic groups were also evident on heating these proteins. Whey proteins showed a marked decrease in the intensity of the band at 1449 cm⁻¹, (1.43 ± 0.03); compared to unheated whey protein (1.90 ± 0.02), (p<0.05). The band at 1450 cm⁻¹ assigned to methylene asymmetric bending (H-C-H) or deformation motions of CH₂ and CH₃ groups indicated changes in the environment around the aliphatic and hydrocarbon side chains after heating. Surprisingly, in contrast egg albumen showed a marked increase in peak intensity at 1450 cm⁻¹, 1.14 ± 0.06 compared to 0.93 ± 0.02 in the unheated egg albumen (p<0.05).
Chapter 3. Studies on egg albumen and whey protein interactions by FT-Raman spectroscopy and rheology

Figure 3.3: FT-Raman spectra (400-1600 cm\(^{-1}\)) of 15% (w/w) heated whey protein in D\(_2\)O. The spectra were normalised to the phenylalanine peak at 1004 cm\(^{-1}\).

Figure 3.4: FT-Raman spectra (400-1600 cm\(^{-1}\)) of 15% (w/w) heated egg albumen protein in D\(_2\)O. The spectra were normalised to the phenylalanine peak at 1004 cm\(^{-1}\).
On heating, both whey and egg albumen showed a significant decrease (p<0.05) in the 1346-1348 cm\(^{-1}\) band assigned to the C-H bending or CH\(_3\) symmetric vibrations and/or to the Trp vibrational modes. The level of hydrophobic residues exposed to the outer surface of the protein is increased during heat denaturation as shown by a decrease in the bands represented by hydrophobic residues such as tryptophan. This trend was confirmed by Badii and Howell (2001) in aggregate formation of actomyosin in frozen cod and haddock. These results suggest that changes in the hydrophobic environment around the aliphatic and aromatic side chains, may be used to monitor denaturation and conformational changes in proteins. In this study, further changes including reduced intensity in the aromatic amino acid residues were observed including the tryptophan bands near 754-760 cm\(^{-1}\), 1555 cm\(^{-1}\) and 1585 cm\(^{-1}\). These findings suggest the exposure of tryptophan residues which play a role in hydrophobic interaction in gels (Nonaka \textit{et al.}, 1993; Howell and Li-Chan, 1994).

Raman spectroscopy provides the only direct way of analysing the disulphide region. Howell (1992) suggested that disulphide formation and or disulphide interchange reactions are important for non-reversible heat gelation of most globular proteins. In both whey and egg albumen proteins, changes were observed in the disulphide region (500-540 cm\(^{-1}\)). For heated whey there was a shift in the all gauche peak from 500 to 503 cm\(^{-1}\). Minor bands near 516 cm\(^{-1}\) and 535 cm\(^{-1}\) were reported in \(\beta\)-lactoglobulin to represent disulphide bands in the gauche-gauche trans and trans-gauche-trans conformations respectively (Kitagawa \textit{et al.}, 1979; Nakanishi, 1974). However, Byler \textit{et al} (1983) suggested that tryptophan might contribute to the band near 540 cm\(^{-1}\). In the heated egg albumen a strong peak appeared at 538 cm\(^{-1}\) in the heated sample indicating a conformational shift around the single cystinyl disulphide. Li-Chan and Qin (1998) observed a similar conformational shift from 525 to 540 cm\(^{-1}\) in the ovalbumin molecule after heating at 80°C.
3.4.2.3. Comparison of unheated mixtures of egg albumen and whey

FT-Raman spectra of mixtures of unheated egg albumen and whey proteins are shown (Figs. 3.5, 3.6). The mixtures of egg albumen and whey formed Milky white dispersions. In contrast, studies by Howell et al., (1995) reported that mixtures of lysozyme and \(\alpha\)-lactalbumin or \(\beta\)-lactoglobulin yielded white precipitates due to electrostatic interactions between basic lysozyme (pI 10.1) and negatively charged whey proteins (pI 5.5). In addition, to the experimental spectra, the theoretical spectra (not shown) which would be expected in the absence of interactions, were calculated as an average of the component spectra measured for the individual proteins. The difference between the theoretical average and actual experimental spectra are shown as the difference spectra. The appearance of positive or negative peaks in the difference spectra are indicative of interactions between component proteins in the mixtures (Howell and Li-Chan, 1996). Quantitative data for the normalised intensity peaks in the experimental and calculated spectra are shown in Table 3.4.

For the unheated mixture of egg albumen and whey, sharp peaks at 504 cm\(^{-1}\) and 514 cm\(^{-1}\) representing the disulphide S-S stretching region were observed for the experimental spectra and at 507 cm\(^{-1}\) and at 518 cm\(^{-1}\) for the calculated spectra. These conformational shifts in the experimental spectra indicate changes in the disulphide region. Howell and Li-Chan (1996) also reported changes in the disulphide region in their study of unheated \(\beta\)-lactoglobulin-lysozyme mixture.

The band near 758 cm\(^{-1}\) assigned to the Trp indole ring was higher in the experimental spectrum than in the calculated spectrum, indicating that Trp residues were more buried in the interior of the protein in the unheated mixture (Howell and Li-Chan, 1996). The intensity at 1450 cm\(^{-1}\) assigned to the CH\(_2\) bend motion of the aliphatic side chain was higher in the experimental than the calculated spectra in the unheated 7.5:7.5 whey/egg albumen mixture. These results suggest the involvement of aliphatic amino acid residues in hydrophobic interactions in the mixtures (Howell and Li-Chan, 1996).
Chapter 3. Studies on egg albumen and whey protein interactions by FT-Raman spectroscopy and rheology

Figure 3.5: FT-Raman experimental (a) and difference (b) spectra (400-1700 cm$^{-1}$) of unheated whey and egg albumen mixture (50:50) 15% (w/w) dissolved in D$_2$O. Spectra were baselined and normalised to the phenylalanine peak at 1004 cm$^{-1}$.

Figure 3.6: FT-Raman experimental (a) and difference (b) spectra (400-1700 cm$^{-1}$) of heated whey and egg albumen mixture (50:50) 15% (w/w) dissolved in D$_2$O. Spectra were baselined and normalised to the phenylalanine peak at 1004 cm$^{-1}$. 
Chapter 3. Studies on egg albumen and whey protein interactions by FT-Raman spectroscopy and rheology

Table 3.4: Normalised intensity values at selected regions of the experimental (bold font) and calculated (italics) spectra of unheated (U) and 90°C heated (H) binary mixtures of whey and egg albumen proteins (7.5:7.5 w/w)

<table>
<thead>
<tr>
<th>Peak</th>
<th>Normalised Intensity*</th>
<th>Assignment</th>
<th>whey/Egg albumen</th>
<th>whey/Egg albumen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>U</td>
<td>H</td>
<td>U</td>
<td>H</td>
</tr>
<tr>
<td>Cystine S-S</td>
<td>0.12 ±0.02</td>
<td>0.17 ±0.06</td>
<td>0.12 ±0.01</td>
<td>0.11 ±0.01</td>
</tr>
<tr>
<td></td>
<td>(490)</td>
<td>(491)</td>
<td>(499)</td>
<td>(490)</td>
</tr>
<tr>
<td></td>
<td>0.14 ±0.02</td>
<td>0.19 ±0.06</td>
<td>0.10 ±0.01</td>
<td>0.13±0.01</td>
</tr>
<tr>
<td></td>
<td>(504)</td>
<td>(501)</td>
<td>(507)</td>
<td>(509)</td>
</tr>
<tr>
<td></td>
<td>0.10±0.01</td>
<td>0.14±0.01</td>
<td>0.10±0.01</td>
<td>0.20±0.01</td>
</tr>
<tr>
<td></td>
<td>(514)</td>
<td>(518)</td>
<td>(521)</td>
<td>(521)</td>
</tr>
<tr>
<td>TrpTophan</td>
<td>0.14 ±0.02</td>
<td>0.12±0.08</td>
<td>0.22±0.03</td>
<td>0.17±0.05</td>
</tr>
<tr>
<td></td>
<td>(756)</td>
<td>(753)</td>
<td>(757)</td>
<td>(758)</td>
</tr>
<tr>
<td>Tyrosine Doublet</td>
<td>0.28 ±0.04</td>
<td>0.33±0.08</td>
<td>0.30±0.02</td>
<td>0.29±0.01</td>
</tr>
<tr>
<td></td>
<td>(828)</td>
<td>(829)</td>
<td>(828)</td>
<td>(829)</td>
</tr>
<tr>
<td></td>
<td>0.21 ± 0.03</td>
<td>0.21 ± 0.08</td>
<td>0.28 ±0.01</td>
<td>0.28±0.01</td>
</tr>
<tr>
<td></td>
<td>(853)</td>
<td>(853)</td>
<td>(853)</td>
<td>(853)</td>
</tr>
<tr>
<td>CH bend/Try</td>
<td>0.54 ± 0.04</td>
<td>0.46 ± 0.04</td>
<td>0.40 ±0.01</td>
<td>0.66±0.01</td>
</tr>
<tr>
<td></td>
<td>(1349)</td>
<td>(1346)</td>
<td>(1347)</td>
<td>(1347)</td>
</tr>
<tr>
<td>COO*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CH$_2$ bend</td>
<td>1.24 ±0.03</td>
<td>1.3 ±0.08</td>
<td>0.94 ±0.01</td>
<td>1.40±0.03</td>
</tr>
<tr>
<td></td>
<td>(1449)</td>
<td>(1450)</td>
<td>(1451)</td>
<td>(1450)</td>
</tr>
<tr>
<td>C-N or C-C stretch</td>
<td>0.12±0.02</td>
<td>0.13±0.04</td>
<td>0.12±0.02</td>
<td>0.35±0.04</td>
</tr>
<tr>
<td></td>
<td>(1059)</td>
<td>(1052)</td>
<td>(1049)</td>
<td>(1051)</td>
</tr>
<tr>
<td>Amide 111</td>
<td>0.52±0.03</td>
<td>0.46±0.07</td>
<td>0.49±0.01</td>
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<td>(937)</td>
<td>(938)</td>
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<tr>
<td></td>
<td>0.43±0.03</td>
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<td>0.41±0.01</td>
<td>0.44±0.01</td>
</tr>
<tr>
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<td>(957)</td>
<td>(959)</td>
<td>(955)</td>
<td>(953)</td>
</tr>
<tr>
<td></td>
<td>0.40±0.03</td>
<td>0.60±0.03</td>
<td>0.26±0.01</td>
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</tr>
<tr>
<td></td>
<td>(991)</td>
<td>(987)</td>
<td>(986)</td>
<td>(987)</td>
</tr>
<tr>
<td>β-sheet structure</td>
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<td>-</td>
<td>0.36±0.01</td>
<td>0.66±0.02</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>(1239)</td>
<td>(1239)</td>
</tr>
<tr>
<td>α-helix structure</td>
<td>0.56±0.02</td>
<td>0.45±0.07</td>
<td>0.41±0.02</td>
<td>0.34±0.02</td>
</tr>
<tr>
<td></td>
<td>(1320)</td>
<td>(1323)</td>
<td>(1320)</td>
<td>(1320)</td>
</tr>
</tbody>
</table>

* intensity at the wavenumber (cm$^{-1}$) shown in parenthesis, normalised to the phenylalanine band at 1004 cm$^{-1}$. Spectral data were an average of 128 scans and baseline corrected.
In the unheated mixture of whey and egg albumen a lower tyrosine doublet ratio 850 cm\(^{-1}\)/830 cm\(^{-1}\) (0.70) in the experimental spectra was observed compared with the calculated spectra (0.93). These results indicate either increasing buriedness or involvement of tyrosine residues as strong hydrogen bond donors in the mixture (Li-Chan, 1994; Nonaka et al., 1993).

### 3.4.2.4. Comparison of heated mixtures of egg albumen and whey

The spectra for the heated mixture of egg albumen and whey are shown in Fig.3.6. Changes were observed in the experimental compared with the calculated spectra for Raman bands assigned to \(\alpha\)-helical and \(\beta\)-sheet structures. There was a higher peak intensity of the \(\beta\)-sheet structure at 987 cm\(^{-1}\) (\(p<0.05\)) in the experimental spectra indicating that egg-whey protein interactions caused further formation of \(\beta\)-sheet type structures during gelation. There was no difference between the calculated spectra and experimental spectra in the \(\alpha\)-helix region.

There was a notable change in the experimental heated mixture, in the doublet at 1450 and 1421 cm\(^{-1}\) typical of CH\(_2\) bending and COO' moiety (of Asp and Glu). The peak at 1421 cm\(^{-1}\) in the experimental heated mixture was not clearly observed compared with the unheated mixture which may suggest that these acidic residues play a role in electrostatic interaction involved during the gelation process. The stronger peak in the experimental compared with calculated spectra 1450 cm\(^{-1}\) suggest heat induced hydrophobic interactions (Howell and Li-Chan, 1996). These findings are in concurrence with Howell and Li-Chan (1996) and Kitagawa et al., (1979) who indicated that exposure of buried tryptophan residues in proteins is observed by the decrease in the peak intensity around 760 cm\(^{-1}\) and the exposed Trp may play a role in hydrophobic interactions. In addition, stronger and higher peak intensity for Trp, CH and CH\(_2\) vibrations were observed in the experimental compared with the calculated spectra. These results show that heating of whey and egg albumen protein mixtures promoted further hydrophobic interactions of previously exposed aromatic and aliphatic residues (Howell and Li-Chan, 1996).
The 850 cm\(^{-1}\)/830 cm\(^{-1}\) tyrosine doublet ratio was higher (0.96) for the calculated spectra compared with the experimental spectra (0.64); indicating the exposure of tyrosine residues and a more polar environment due to protein-protein interactions during heat gelation.

3.5 Conclusion

In this study we have shown by large deformation rheology that synergistic interactions occur in whey and egg albumen mixtures. Howell and Lawrie (1984) proposed that the synergistic interactions between compatible small globular proteins (plasma and egg albumen) depended on the degree of unfolding of the individual proteins in the mixture which governed optimum exposure of specific groups and thereby optimum interaction. Synergistic protein-protein interactions between globular proteins can be used to advantage in product formulation to achieve stronger gels and new textures.

Involvement of specific groups can be monitored closely by F-T Raman spectroscopy in unheated and heated proteins. Changes in disulphide bonds were observed which contributed to the formation of strong irreversible gels on heating. Additionally, the increase in β-sheet structure may contribute to the hydrogen bonding required for increased polymerisation of both proteins on cooling. There was also clear evidence of exposure of hydrophobic groups on heating which contributed to the gel network stability. The changes observed in the native and heat-treated egg albumen and whey proteins described in this chapter enable comparison with pressure-treated proteins as shown in chapter 4.
CHAPTER 4
Chapter 4. A comparative study of heat and high pressure induced gels of whey and egg albumen proteins and their binary mixtures

4.1 Introduction

Protein gelation is used to obtain or improve the consistency of a food product. The use of whey and egg albumen proteins in food systems as gelling and thickening agents and texture modifiers has been extensively reviewed in the literature (Howell and Lawrie, 1985; Kinsella and Whitehead; 1989; Morr and Ha, 1993; Nakamura and Doi 2000; Ngarize et al., 2003).

Traditionally, gel formation is induced by application of heat to the protein solution. However, foods are rarely homogenous and are composed of a mixture of proteins with carbohydrates and fats. Heat treatment of proteins and other nutrients can induce various chemical reactions such as Maillard browning which may lead to nutritional, sensory and safety deterioration in certain foods (de Man, 1999; Tauscher, 1995; Hayashi et al., 1989; Hayashi and Balny, 1996; Knorr et al., 2002). Consequently, there is now an increasing trend towards the application of non-thermal technologies which may prolong shelf-life without these detrimental effects. Non-thermal technologies such as high pressure processing are of interest to the food industry, because they not only provide alternatives to conventional methods of thermal processing but also offer opportunities for creating new ingredients and textures.

High pressure processing, involves subjecting food material to a high hydrostatic pressure and holding it isobarically for a specified period of time before release. Typical commercial processes are in the order of 100-1000 MPa and holding times vary from 10-30 min. In order to achieve the necessary level of processing in terms of microbial inactivation, sterilisation and extension of shelf-life, high pressure needs to be applied at elevated temperatures (>70°C) whereas induction of molecular changes can occur at lower pressures (Mertens and Deplace, 1993; Heinz and Knorr, 2002).

High pressure technology is popular because it can be used to obtain stable products with minimal effects on flavour, colour and nutritional value or to create novel texture and taste (Cheftel, 1995). Although the effect of high pressure on individual proteins have been reviewed (Balny and Masson, 1993; Gross and Janicke, 1994; Galazka and
Ledward, 1998; Heremans, 2002), the way high pressure affects protein interactions in mixed systems has not been adequately studied. Therefore, in this present study, whey and egg albumen protein-protein interactions were investigated using both heat and pressure denaturation. Previous studies chapter 3, Ngarize et al., 2003 revealed synergistic interaction in the treated binary mixtures of whey and egg albumen proteins especially when mixed in the ratio of 10%whey/5% egg albumen. In this study, heated individual whey and egg albumen protein gels and the mixed gels were compared with pressure treated gels using a range of pressures (400-800 MPa).

Both heat and pressure are known to denature proteins resulting in aggregation and gelation (Hayakawa et al., 1992; Van Camp and Huyghebaert, 1995; Hayakawa et al., 1996; Sikorski, 2001; Ngarize et al., 2003). The denaturation process results in changes in protein conformation, structure and hence functionality. The mechanism of heat and pressure protein denaturation is reported to differ and consequently affects the type of gels formed (Hayashi et al., 1989; Galazka and Ledward., 1998; Ledward 2000). Pressure denatured proteins are reported to involve rupture of hydrophobic and electrostatic interactions which result from a decrease in volume of the protein solution while heat denaturation of the protein is caused by violent movement of molecules that can destroy heat labile hydrogen bonds. In addition, heat denaturation is accompanied by destruction and formation of covalent bonds which enhances the production of off-flavours or toxic compounds (Knorr et al., 2002). In pressure denaturation, the rearrangement of water molecules surrounding amino acids residues promotes increased glossiness and transparency (Okamoto et al., 1990).

The mechanism involved in heat and high pressure gels has been investigated mostly by chemical modification, chromatography, electrophoresis, solubility, enzymatic digestion, immunology, thermal analysis and microscopy. In recent years, several spectroscopic techniques have been used to obtain a more highly resolved picture of molecular structures of aggregates and gels formed during heating and by application of pressure. These techniques include nuclear magnetic resonance (NMR) (Zhang et al., 1995), ultraviolet (UV) spectroscopy (Mombelli et al., 1997), as well as fluorescence and infrared spectroscopy with small angle X-ray scattering (Panick et al., 1999). Circular dichroism (CD) has also been used to study heat denaturation of
several proteins (Clark et al., 1981a, Clark and Lee-Tuffnell, 1986; Wang and Damodaran, 1991) but like most of the above methods its application is limited to dilute solutions. Furthermore, CD cannot be used to study pressure denaturation for technical reasons, the correction that might be required to account for pressure-induced birefringence in the windows will be much larger than the expected changes in the protein (Heremans et al. 1997, Heremans 2002).

The effect of pressure on protein-protein interactions has been studied by light scattering or turbidity methods (Gorovits and Horowitz, 1998). However, these methods give information on the degree of association and aggregation, but no information on conformational changes or the nature of intermolecular interactions. Recent reports have indicated differences between the structural changes induced by temperature and pressure as judged from Fourier transform infrared spectroscopy (Smeller et al., 1999). The use of Raman spectroscopy to study heat denaturation and to a limited extent pressure denaturation has been reported (Clark et al., 1981a; Nakai et al., 1994; Howell and Li-Chan, 1996; Howell et al., 1999; Heremans and Wong 1985). As discussed previously, FT-Raman spectroscopy allows a detailed investigation of solid gels as well as direct measurement of disulphide bonds. In the present study, a range of complementary techniques including large deformation rheology, FT-Raman spectroscopy and transmission electron microscopy were used to provide a detailed comparison of the mechanism of gelation and properties of heat and pressure-treated whey and egg albumen gels. In addition, the hypothesis that globular proteins such as whey and egg albumen can display synergistic interactions under high pressure, similar to those indicated in heat treated gels was tested (Ngarize et al., 2003, Howell and Lawrie, 1984).
Chapter 4. A comparative study of heat and high pressure induced gels of whey and egg albumen proteins and their binary mixtures

4.2 Materials
Materials were the same as in section 2.1

4.3 Methods

4.3.1. FT Raman spectroscopy

4.3.1.1. Sample preparation and analysis
Sample preparation for FT Raman spectroscopy, data collection and analysis was the same as in section 3.3.1

4.3.2. Large deformation testing

4.3.2.1. Heat treated samples
Protein solutions (15% w/w) in distilled water were poured into stainless steel tubes, 50 mm long and 15 mm in diameter and heated in a water bath at 90°C for 30 minutes to promote network formation (Howell and Lawrie, 1984). The heated samples were stored at 4°C overnight to age. The gelled samples were cut into 15 mm long cylinders and were tested on TA-XT2 texture analyser (Stable Microsystems, Godalming, Surrey, UK). The force at maximum compression (12 mm) and the Young’s modulus (the gradient of the stress-strain curve) were measured. The test speed was set at 0.2 mm/sec, and the samples were compressed by a distance of 12 mm to a final height of 3 mm.

4.3.2.2. Pressure treated samples

The solutions for high pressure treated gels were prepared by the same method as heat treated gels except the samples were placed in visking tubing tied at both ends, heat sealed in a high pressure polythene bag (Crayovac W.R Grace Limited Cromwell Road, Cambridge, United Kingdom). The samples were introduced into the working chamber (300ml capacity) of a Stansted Food Lab high pressure rig (Stansted Fluid Power, Ltd, 70 Bentfield road, Stansted, CM24 8HT, Essex, UK.) containing the low compressibility fluid (2:8 mixture of castor oil:ethanol). The equipment was installed in an air conditioned laboratory at 20°C. The temperature in the pressurisation chamber increased during compression reaching a maximum after 2 minutes (the maximum temperature achieved in all treatments was 30°C, corresponding to the
Chapter 4. A comparative study of heat and high pressure induced gels of whey and egg albumen proteins and their binary mixtures

treatment at 400 MPa, 500 MPa, 600 MPa), then decreased to 20°C in 0.5 min and remained stable at 20°C until the end of the cycle. The compression and decompression times were about 1-2 minutes and 0.5 min respectively. The samples were stored at 4°C similar to the heat-treated ones. For samples less than 10 ml, the Stansted Micro Food-Lab, Model S-FL-085-9W high pressure rig was used.

4.3.2.3. Transmission Electron Microscopy
The samples were prepared and analysed by the method of Comfort and Howell (2002) described in section 2.2.3.2
4.4 Results and Discussion

4.4.1. Large deformation testing

There were significant differences in appearance and textural properties between heat and pressure induced gels (Table 4.1). Pressure induced whey and egg albumen gels were glossy and smooth in appearance with a rubbery texture compared with heat-treated gels which were less glossy but were harder and more brittle. In addition, pressure-treated gels (in commonly used range 400-600 MPa) had significantly lower values of force at maximum compression and Young’s modulus than heat-treated gels and tended to lose water easily upon compression. This is consistent with transmission electron microscopy results in this study which showed that pressure treated gels formed larger aggregates with irregular pores compared with heat treated gels (Plates 4.1-4.4). Studies by (Okamoto et al., 1990) on egg albumen and yolk, paste of rabbit meat, fish and soya protein reported similar findings with pressure-treated gels being characterised by soft glossy gels while heat-treated gels were harder.

The gel strength increased with increasing pressure for both whey and egg albumen proteins (15% w/w in distilled water). For whey protein both force at maximum compression and Young’s modulus were significantly higher (P < 0.05) than those of egg albumen proteins in all the pressure treatments used in this study (400-800 MPa) (see Table 4.1). Egg albumen proteins showed a similar trend to whey proteins except that no gelation was observed below 500 MPa, for this reason values were not determined. Previous reports have indicated that egg albumen does not gel below 500 MPa (Bridgeman, 1914; Ledward and Galazka 1998; Strohalm et al., 2000). These results reflect the differences between the two proteins; whey protein gels are characterized by a higher number of crosslinks and stronger interactions between individual molecules producing greater gel strength values, while values for egg albumen were lower; this can be attributed to weaker interactions achieved between neighbouring egg albumen protein molecules. It is well established that the greater the number of crosslinks within a gel network, the higher the gel strength, (Van Camp and Huyghebaert, 1995; Cheftel and Dumay, 1996). Funtenberger et al., (1997) and Iametti et al., (1998) related gel strength in high pressure treated β-lactoglobulin and ovalbumin to the extent of disulphide/sulphydryl (SS/SH)
## Table 4.1: Comparison of large deformation analysis of egg albumen and whey proteins either heated at 90°C for 30 min or high pressure treated (400-800 MPa for 20 min) showing force at maximum compression and Young’s modulus

<table>
<thead>
<tr>
<th>Type of treatment</th>
<th>Force (g) at maximum compression</th>
<th>Young’s modulus (Pa)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Egg Albumen</td>
<td>Whey</td>
</tr>
<tr>
<td>Pressure (MPa)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>400</td>
<td>ND</td>
<td>341</td>
</tr>
<tr>
<td></td>
<td>(±40)</td>
<td></td>
</tr>
<tr>
<td>450</td>
<td>ND</td>
<td>450</td>
</tr>
<tr>
<td></td>
<td>(±25)</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>389</td>
<td>524</td>
</tr>
<tr>
<td></td>
<td>(±30)</td>
<td></td>
</tr>
<tr>
<td>550</td>
<td>425</td>
<td>616</td>
</tr>
<tr>
<td></td>
<td>(±20)</td>
<td></td>
</tr>
<tr>
<td>600</td>
<td>633</td>
<td>702</td>
</tr>
<tr>
<td></td>
<td>(±36)</td>
<td></td>
</tr>
<tr>
<td>650</td>
<td>680</td>
<td>1000</td>
</tr>
<tr>
<td></td>
<td>(±25)</td>
<td></td>
</tr>
<tr>
<td>700</td>
<td>701</td>
<td>1261</td>
</tr>
<tr>
<td></td>
<td>(±50)</td>
<td></td>
</tr>
<tr>
<td>750</td>
<td>792</td>
<td>1415</td>
</tr>
<tr>
<td></td>
<td>(±20)</td>
<td></td>
</tr>
<tr>
<td>800</td>
<td>830</td>
<td>1418</td>
</tr>
<tr>
<td></td>
<td>(±40)</td>
<td></td>
</tr>
<tr>
<td>Heated (90°C)</td>
<td>1159</td>
<td>905</td>
</tr>
<tr>
<td></td>
<td>(±90)</td>
<td></td>
</tr>
</tbody>
</table>

ND means Not determined

Figures in parenthesis are standard deviation values based on 5 replicates
interchange during pressurisation and (Tani et al., 1997; Yoshinori, 1996) observed the same findings in heat-treated β-lactoglobulin and ovalbumin. In the heat induced gels, egg albumen had significantly higher force at maximum compression 1159 ± 90, compared with whey 905 ± 50, (p <0.05). Young’s modulus followed a similar pattern 17.2 (Pa) and 12.1(Pa) for egg albumen and whey respectively (Table 4.1). These values for heat set gels were similar to high pressure treated whey protein values for force at maximum compression at very high pressures namely, 650, 700, 750, and 800 MPa. The Young’s modulus was equally comparable at 12.4, 16, 18.4, 20.2 (Pa) respectively. These results indicate that gel strength depends on the amount of pressure used, and that gel strength values similar to heat treatment can be obtained at pressures above 650 MPa.

It is interesting to note that in this study whey proteins pressurised at 700-800 MPa had significantly higher gel strength values compared with the heat-set gel. This could have technological and practical implications depending on the type of texture and product required. Textural attributes of processed food such as cheese, custard and meat products depend on the type of protein used and the way the gels are prepared (Barbut, 1996). There is an optimum pressure level for each globular protein such that during processing consideration needs to be given to the type of protein and the end product. For example, for whey proteins α-lactalbumin is not readily denatured by pressure, denaturation started at >400 MPa, although it can undergo disulphide/sulphhydryl (SS/SH) interchange reactions to aggregate with β-lactoglobulin (Law et al., 1998). β-Lactoglobulin denaturation has been reported to start at >100 MPa and nearly complete at 400 MPa (Huppertz et al., 2002). This difference in stability is accounted for by the fact that β-lactoglobulin contains two disulphide bonds (S-S) and one sulphhydryl residue (SH) per mole while, α-lactalbumin contains four disulphide bonds and no free sulphhydryl groups. In this study, egg albumen did not gel below 500 MPa but gel strength increased with increasing pressure up to maximum of 800 MPa, however, high pressure treated gels still remained significantly (830 ± 40 Pa at 800MPa) less strong than heated ones (1159 ± 90 Pa) (Table 4.1). Many globular proteins under appropriate conditions and
high concentrations, may denature irreversibly to form gels or precipitates (Galazka and Ledward, 1998). Based on results of the present study, consideration to optimum processing pressure is required for each protein.

In the mixed gels, the highest of Young’s modulus values were obtained for the 10:5 whey/egg albumen mixture for both heated and high pressure treated proteins (Table 4.2). A similar trend was also observed for force at maximum compression at 400 MPa, 500 MPa and 600 MPa. This finding indicated synergistic protein-protein interactions for the two proteins and similarly the corresponding calculated interaction index showed highest and positive values for the 10:5 whey/egg albumen mixture in the high pressure treated samples. Although the synergistic protein-protein interactions have been reported for heat-set gels (Howell and Lawrie, 1985, Matsudomi et al., 1994; Aryana et al., 2002; Ngarize et al. 2003) these have not been investigated for pressure-treated gels to date. The presence of negative values for interaction index in high pressure treated mixtures at 7.5:7.5 and 5:10 whey/egg albumen indicates that these mixtures are not favourable for interaction under pressure and therefore give weaker gel strength. Negative interactions in mixed protein gels of blood plasma and egg albumen were also reported by Howell and Lawrie (1985) for certain protein concentrations.
Chapter 4. A comparative study of heat and high pressure induced gels of whey and egg albumen proteins and their binary mixtures

Table 4.2: Comparison of large deformation analysis of egg albumen (EA) and whey (W) protein mixtures either heated at 90°C for 30 min or high pressure treated (400-600 MPa for 20 min) showing force at maximum compression and Young’s modulus

<table>
<thead>
<tr>
<th>Type of treatment</th>
<th>Force at maximum compression (g)</th>
<th>Young’s modulus (Pa)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pressure (MPa)</strong></td>
<td>10:5 W/EA 7.5:7.5 W/EA 5:10 W/EA</td>
<td>10:5 W/EA 7.5:7.5 W/EA 5:10 W/EA</td>
</tr>
<tr>
<td>400</td>
<td>733 (± 40) 91 (± 5) 140 (± 10)</td>
<td>12.0 (± 0.7) 1.2 (± 0.1) 1.9 (± 0.4)</td>
</tr>
<tr>
<td>Interaction index</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>500</td>
<td>887 (± 70) 222 (± 20) 176 (±5)</td>
<td>20.0 (±1.3) 3.0 (± 0.3) 2.0 (± 0.6)</td>
</tr>
<tr>
<td>Interaction index</td>
<td>+85</td>
<td>-80</td>
</tr>
<tr>
<td>600</td>
<td>1267 (± 65) 714 (± 60) 353 (± 30)</td>
<td>35.0 (±2.9) 11.0 (± 1.0) 6.0 (± 0.6)</td>
</tr>
<tr>
<td>Interaction index</td>
<td>+86</td>
<td>+7</td>
</tr>
</tbody>
</table>

**Heated at (90°C)**

|                               | 2760 (± 300) 1610 (± 120) 1248 (± 40) | 43.3 (± 4.3) 26.0 (± 1.2) 19.4 (± 0.6) |
| Interaction index             | +179                             | +56                  |

Figures in parenthesis are standard deviation values based on five replicates. Interaction index = (Actual value-additive value)/additive value x100. The additive value is derived from the contributions of the component proteins measured in isolation, in proportion to concentration of each in the mixture (Howell and Lawrie, 1984).
4.4.2. Gel microstructure: transmission electron microscopy

There were notable differences in the gel microstructure of high pressure and heat-treated gels of egg albumen and whey proteins both individually and in their mixtures. High pressure treated (500 MPa for 20 min) egg albumen was characterized by a porous, aggregate network structure (Plate. 4.1). Similarly, high pressure treated whey proteins (500 MPa for 20 min) showed a continuous fine stranded network (Plate. 4.2). Large deformation studies of both high pressure-treated egg albumen and whey samples (500 MPa for 20 min) indicated gel strength values in a similar range (389 g and 524 g respectively), and were accompanied by loss of fluid from the gel network structure. Van Camp and Huyghebaert (1995) reported that high pressure-treated whey protein concentrate gels were characterized by a porous, finely stranded network with a lower gel strength and were surrounded by non-incorporated liquid (NIL, indicating poor water holding capacity) compared with heat treated samples.

Heat treated (90°C 30 min) egg albumen and whey protein gels were both characterized by a less porous, more compact network structure (Plates 4.3, 4.4). Van Camp and Huyghebaert (1995) reported similar findings for whey proteins and indicated that there are marked intermolecular network bondings between adjacent polypeptide side chains and that the hollow spacing (diameter 1.5-4.5 μm) form separate entities capable of maintaining the liquid enclosed during deformation. In addition in this study, the formation of more continuous network structure with larger number of contact points were found for heat induced gels compared with pressure-induced gels thus correlated with the high gel strength 1159 g and 905 g for heat-treated egg albumen and whey protein gels respectively.

Marked differences were observed between the microstructure of 15% w/w protein gels of heated and pressure treated egg albumen and whey protein mixed in the ratio 7.5:7.5. Pressure-treated mixtures produced gels with fewer smaller aggregates whereas heat treated gels indicated large dense aggregates (Figs. 4.5, 4.6). This structural difference reflects the stronger heat-set gels compared with the pressure-treated softer gels.
Chapter 4. A comparative study of heat and high pressure induced gels of whey and egg albumen proteins and their binary mixtures

Plate 4.1: TEM of egg albumen protein gel (15% w/w) high pressure treated (500 MPa for 30 min) (x 22 000)

Plate 4.2: TEM of whey protein gel (15% w/w) high pressure treated (500 MPa for 30 min) (x 22 000)
Chapter 4. A comparative study of heat and high pressure induced gels of whey and egg albumen proteins and their binary mixtures

Plate 4.3: TEM of egg albumen protein gel (15% w/w) heated at 90°C for 30 min (x22 000)

Plate 4.4: TEM of whey protein gel (15% w/w) heated at 90°C for 30 min (x22 000)
Chapter 4. A comparative study of heat and high pressure induced gels of whey and egg albumen proteins and their binary mixtures

Plate 4.5: TEM of mixed protein gel 7.5:7.5 (15% w/w) high pressure treated (500 Mpa for 30 min) (x22 000)

Plate 4.: TEM of mixed protein gel 7.5:7.5whey/egg albumen (15% w/w) heated at 90°C for 30 min (x22 000)
4.4.3. FT Raman spectroscopy

4.4.3.1. Comparison of heated and high pressure treated samples of egg albumen and whey proteins

FT Raman spectra for heated (90°C for 30 min) and high pressure-treated (600 MPa for 20 min) whey and egg albumen protein gels are shown in figs.4.1 and 4.2 and figs. 4.3, and 4.4 respectively and data for selected peaks are shown in Table 4.3. Changes in the disulphide region exhibited a significant decrease in relative intensity of the 500-540 cm\(^{-1}\) region for both high pressure- treated whey and egg albumen (0.13 ± 0.01, 0.05 ± 0.01 at 500 cm\(^{-1}\) the all-gauche conformation) (p < 0.05) respectively compared with much higher peak intensities in heated samples (0.23 ± 0.02, 0.15 ± 0.06) (p < 0.05) respectively. Peaks in the trans-gauche-trans conformation were observed at 538 cm\(^{-1}\) and 532 cm\(^{-1}\) in heated and pressure-treated egg albumen, while whey proteins exhibited all gauche conformation at 516 cm\(^{-1}\) and 513 cm\(^{-1}\) for heated and pressure treated respectively.

These results suggest that disulphide bonds are significantly altered by both treatments with heat denaturation having a more marked effect compared with pressure treatment at 600 MPa for 20 min. Involvement of disulphide bonds in heat denaturation and gel formation has already been documented (Kitagawa et al. 1979, Li-Chan and Qin, 1998; Ngarize et al., 2003). However, during high pressure treatment, the present study indicated that the involvement of disulphide bonds is less extensive as shown by the decreased peak intensity (Table 4.3). As mentioned earlier, the gel strength values for heat-treated proteins were higher than for pressure treated (400-600 MPa) samples. The present study confirms disulphide bonds play a major role in gel formation and determine the elastic nature of both heat and pressure treated gels through SH/SS interchange reactions. Several authors have reported that the buried sulfhydryl groups are exposed to the solvent upon heating and application of pressure. The exposure of these groups promote SH/SS interchange and therefore also enhance the aggregation process (Shimada and Cheftel, 1989; Howell and Lawrie, 1985; Funtenberger et al., 1997; Qi et al., 1997; Kanno et al., 1998).
Chapter 4. A comparative study of heat and high pressure induced gels of whey and egg albumen proteins and their binary mixtures

Figure 4.1: FT Raman spectra (400-1800 cm\(^{-1}\)) for heated (90°C, 30 min) whey protein 15% (w/w) dissolved in D\(_2\)O. The spectra were baselined and normalised to the phenylalanine peak at 1004 cm\(^{-1}\).

Figure 4.2: FT Raman spectra (400-1800 cm\(^{-1}\)) for high pressure treated (600 MPa for 20 min) whey protein 15% (w/w) dissolved in D\(_2\)O. The spectra were baselined and normalised to the phenylalanine peak at 1004 cm\(^{-1}\).
Chapter 4. A comparative study of heat and high pressure induced gels of whey and egg albumen proteins and their binary mixtures

Figure 4.3: FT Raman spectra (400-1800 cm\(^{-1}\)) for heated (90°C, 30 min) egg albumen protein 15% (w/w) dissolved in D\(_2\)O. The spectra were baselined and normalised to the phenylalanine peak at 1004 cm\(^{-1}\).

Figure 4.3: FT Raman spectra (400-1800 cm\(^{-1}\)) for high pressure treated (600 MPa for 20 min) egg albumen protein 15% (w/w) dissolved in D\(_2\)O. The spectra were baselined and normalised to the phenylalanine peak at 1004 cm\(^{-1}\).
## Chapter 4. A comparative study of heat and high pressure induced gels of whey and egg albumen proteins and their binary mixtures

Table 4.3: Normalised intensity values in selected regions of the (400-1500 cm⁻¹) FT Raman spectra of 90°C heated (H) and high pressure treated (HP) (600 MPa for 20 min) egg albumen and whey proteins

<table>
<thead>
<tr>
<th>Peak Assignment</th>
<th>Normalised Intensityᵃ</th>
<th>Egg albumen</th>
<th>whey protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H</td>
<td>HP</td>
<td>H</td>
</tr>
<tr>
<td>Cystine S-S</td>
<td>0.15 ± 0.06 (502)</td>
<td>0.05 ± 0.01 (514)</td>
<td>0.23 ± 0.01 (503)</td>
</tr>
<tr>
<td></td>
<td>0.25 ± 0.01 (538)</td>
<td>0.05 ± 0.02 (532)</td>
<td>0.23 ± 0.02 (516)</td>
</tr>
<tr>
<td>Cystine S-S</td>
<td>0.12 ± 0.01 (763)</td>
<td>0.12 ± 0.01 (757)</td>
<td>0.21 ± 0.04 (754)</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.31 ± 0.02 (827)</td>
<td>0.18 ± 0.02 (827)</td>
<td>0.28 ± 0.04 (827)</td>
</tr>
<tr>
<td></td>
<td>0.25 ± 0.03 (854)</td>
<td>0.12 ± 0.03 (850)</td>
<td>0.21 ± 0.05 (852)</td>
</tr>
<tr>
<td>Tryrosine Doublet</td>
<td>0.38 ± 0.03 (1337)</td>
<td>0.32 ± 0.02 (1343)</td>
<td>0.76 ± 0.14 (1348)</td>
</tr>
<tr>
<td>CH bend/Trp</td>
<td>0.38 ± 0.03 (1337)</td>
<td>0.32 ± 0.02 (1343)</td>
<td>0.76 ± 0.14 (1348)</td>
</tr>
<tr>
<td>COO⁻</td>
<td>—</td>
<td>—</td>
<td>1.55 ± 0.04 (1419)</td>
</tr>
<tr>
<td>CH₂ bend</td>
<td>1.14 ± 0.06 (1450)</td>
<td>1.03 ± 0.07 (1453)</td>
<td>1.43 ± 0.03 (1449)</td>
</tr>
<tr>
<td>C-N/C-C stretch</td>
<td>0.83 ± 0.04 (1061)</td>
<td>0.04 ± 0.01 (1056)</td>
<td>0.29 ± 0.05 (1046)</td>
</tr>
<tr>
<td>Amide 111'</td>
<td>0.40 ± 0.01 (929)</td>
<td>0.40 ± 0.03 (938)</td>
<td>0.50 ± 0.05 (938)</td>
</tr>
<tr>
<td></td>
<td>0.46 ± 0.01 (941)</td>
<td>0.36 ± 0.01 (955)</td>
<td>0.52 ± 0.05 (956)</td>
</tr>
<tr>
<td></td>
<td>0.42 ± 0.03 (960)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Amide 111</td>
<td>0.51 ± 0.01 (988)</td>
<td>0.30 ± 0.02 (989)</td>
<td>0.88 ± 0.08 (988)</td>
</tr>
<tr>
<td>β-sheet</td>
<td>—</td>
<td>—</td>
<td>0.81 ± 0.03 (1238)</td>
</tr>
<tr>
<td>α-helix</td>
<td>0.34 ± 0.03 (1323)</td>
<td>0.27 ± 0.02 (1318)</td>
<td>0.90 ± 0.03 (1319)</td>
</tr>
</tbody>
</table>

Intensity at wavenumber (cm⁻¹) shown in parenthesis, was normalized to the intensity of the phenylalanine band at 1004 cm⁻¹. Spectral data were the average of 128 scans and baseline corrected.
Amide I bands indicated a significant increase in β-sheet structure (980-988 cm⁻¹) for both heated whey (0.88 ± 0.08) and egg albumen (0.51 ± 0.01) (p < 0.05) compared with high pressure treated samples. There were no significant changes in the α-helix region (900-950 cm⁻¹) of heated and pressure treated samples. However, unlike whey proteins, heated egg albumen exhibited changes in random structure at 960 cm⁻¹ showing that egg white was substantially denatured (Howell and Li-Chan, 1996), whilst no peaks were present in the high pressure treated egg albumen and whey proteins in this region. The absence of the 960 cm⁻¹ peak in all high pressure treated samples reinforces the view that high pressure is a milder form of treatment compared with heat. In addition, the Amide I band at 1240 cm⁻¹ typical of β-sheet structures (Howell and Badii, 2002), was absent in heated egg albumen and all high pressure treated samples, with the exception of heated whey.

The amide I band at 1320 cm⁻¹ showed significant but lower values in α-helix in high pressure treated whey protein 0.27 ± 0.02 (HP), compared with heated whey 0.90 ± 0.01 (H) (p < 0.05). Similar findings were obtained for high pressure treated egg albumen 0.27 ± 0.02 (HP) compared with heated egg albumen 0.34 ± 0.03 (H) (p < 0.05). Increase in the β-sheet structure for both heated and high pressure treated whey and egg albumen protein suggest that β-sheet formation is a necessary part of gelation, (Clark et al., 1981; Clark and Lee-Tuffnell, 1986; Galazka and Ledward, 1998). Recent studies using FTIR reported that protein unfolding for pressure denaturation is less extensive than temperature denaturation (Panniclc et al., 1999, Heremans, 2002). Under the conditions used in this study β-sheet formation was less extensive in high pressure treated proteins compared with heat treated proteins.

In addition, the band near 1060-1080 cm⁻¹ assigned to the C-C and C-N skeletal stretching vibrations was more distinct and had significantly higher peak intensity (p < 0.05) in the heated samples for both whey and egg albumen. This band represents intermolecular sheet interactions and supports the above view in relation to increased β-sheet structure in heated samples (Li -Chan and Qi, 1998; Parker, 1983).
There were differences in the tyrosine doublet ratio 850/830 cm\(^{-1}\) for both heated and high pressure treated whey and egg albumen samples. In egg albumen a lower intensity ratio was observed for the high pressure treated sample (0.67) compared with the heated sample (0.80). In contrast, a high ratio of 0.90 was observed for high pressure treated whey samples compared with 0.75 for heated samples. A lower intensity ratio observed in high pressure treated egg albumen supports the involvement of tyrosine residues in hydrogen bond formation. A decrease in the intensity ratio \(I_{850}/I_{830}\) suggests an increase in "buriedness" or involvement as strong hydrogen bond donors (Li Chan et al., 1994; Howell and Li-Chan, 1996). Other workers have proposed that involvement of tyrosine residues in hydrogen bond formation possibly accounts for intermolecular hydrogen bond formation between \(\beta\)-sheets acting as junction zones in stabilizing the gel network (Wang and Damodaran 1991; Li-Chan et al., 1994). The high intensity ratio observed in high pressure treated whey indicates increasing exposure of tyrosine residues to a polar environment (Li-Chan and Qi, 1998).

The difference in tyrosine doublet ratio 850/830 cm\(^{-1}\) between egg albumen and whey proteins might reflect the differences in protein unfolding under heat and pressure. The above results suggest that, pressure denaturation in whey tends to disrupt hydrophobic interactions via increased exposure of hydrophobic amino acids to the surface of the protein; this would explain the higher tyrosine doublet ratio in the high pressure treated whey sample compared with heat treated whey. It has been previously reported that hydrophobic interactions are initially favoured by increasing temperature, but disrupted by increasing pressure (Ledward and Galazka, 1998; Needs, 2002).

The specific involvement of the CH\(_2\) band at 1450-1460 cm\(^{-1}\) assigned to the H-C-H methylene asymmetric bending or deformation of CH\(_2\) and CH\(_3\) (Howell and Li-Chan, 1996) was different for heated and high pressure treated whey proteins. High pressure treated whey showed a significantly higher peak intensity (1.56 ± 0.07) compared with heated whey samples (1.43 ± 0.03) \((p < 0.05)\) suggesting marked changes in the environment around aliphatic residues or hydrocarbon chains after high pressure treatment. In contrast, egg albumen showed no significant differences between heated
Chapter 4. A comparative study of heat and high pressure induced gels of whey and egg albumen proteins and their binary mixtures

and high pressure treated samples around the 1450 cm\(^{-1}\) band; this difference in behaviour indicates strong hydrophobic interactions in high pressure treated whey compared with egg albumen. Previous studies (Galazka et al., 1999a) have reported that changes in conformation of high pressure treated whey proteins is due to increased surface activity and hydrophobicity in β-lactoglobulin and bovine serum albumin (BSA), while, in whey protein mixtures the changes in the conformation of β-lactoglobulin seem to be the rate limiting factor for heat induced aggregation (Law, 1996; Relkin et al., 1997).

In addition, the 1336 cm\(^{-1}\) band assigned to C-H bending or CH\(_3\) symmetric vibrations and/or Trp vibrational modes showed a significantly higher peak intensity (p <0.05) for both heated whey and egg albumen samples compared with high pressure treated samples. These results indicate marked changes in this hydrophobic region for heated samples, Howell and Li-Chan (1996) also reported a marked increase in this band after heating α-lactalbumin. Further changes showed increased exposure of aromatic residues in both high pressure treated whey and egg albumen, namely Trp bands around 1555 and 1585 cm\(^{-1}\) (Li Chan et al., 1994).

However, it was interesting to note that a new sharp and broad peak at 874-880 cm\(^{-1}\) assigned to Trp residue was more pronounced in both high pressure treated samples of whey and egg albumen suggesting an increased exposure of aromatic residues (Li - Chan, 1996). A broad sharp single peak at 1207-1210 cm\(^{-1}\) assigned to Tyr residues was more pronounced in high pressure treated samples compared with heat-treated samples indicating exposure and further disruption of hydrophobic groups during pressure treatment. Involvement of hydrophobic interactions in gel formation has previously been reported (Ngarize 2003 et al., Howell et al., 1999). This study indicates clearly that hydrophobic interactions predominate in high pressure treated gels compared with heated gels.
4.4.3.2. Comparison of heated and high pressure treated mixtures of whey and egg albumen

FT Raman spectra of heated and high pressure treated mixtures of whey and egg albumen (7.5:7.5) are shown in fig. 4.5 and 4.6. In addition to the experimental spectra, the theoretical (not shown) which would be expected in the absence of interactions, were calculated as an average of the component spectra measured for the individual proteins. The difference between the theoretical average and actual experimental spectra are shown as the difference spectra. The appearance of positive and negative peaks in the difference spectra are indicative of interactions between the two component protein in the mixtures (Howell and Li-Chan, 1996). Quantitative data for the normalised intensity peaks in the experimental and calculated spectra are shown in Table 4.4.

There were some common findings in both heated and high pressure treated mixtures in the amide 11' band at 980-990 cm⁻¹ assigned to the β-sheet structures, although the peak intensity was more pronounced in the heated mixture. An additional broad sharp peak at 1239 cm⁻¹ (β-sheet structure) was found in the heated mixture difference spectra but was absent in the high pressure treated difference spectra; this is supported by the lower β-sheet structure content observed in the individual high pressure treated egg albumen and whey protein samples. This trend is closely related to the lower gel strength values observed in large deformation studies of high pressure treated samples compared with heat-treated samples.

Marked peak intensities were observed in high pressure treated difference spectra for Trp at 757 cm⁻¹, 874 cm⁻¹, and Tyr at 1207 cm⁻¹ while negative peaks were observed for the CH bend/trp 1346 cm⁻¹ and CH₂ bend at 1450 cm⁻¹. These results suggest that high pressure treatment induced further hydrophobic interactions between aromatic and aliphatic residues in the mixtures of proteins which had been previously exposed upon pressure unfolding of the protein. Both heated and pressure treated samples indicated similar peaks in the difference spectra at 1207 cm⁻¹, 874-880 cm⁻¹ suggesting further hydrophobic interactions in the protein mixtures.
Chapter 4. A comparative study of heat and high pressure induced gels of whey and egg albumen proteins and their binary mixtures

Figure: 4.5. Experimental and difference FT Raman spectra (400-1800 cm\(^{-1}\)) for heated (90°C, 30 min) whey and egg albumen protein mixture (7.5:7.5 w/w) dissolved in D\(_2\)O. Spectra were baselined and normalised to the phenylalanine peak at 1004 cm\(^{-1}\).

Figure: 4.6. Experimental and difference FT Raman spectra (400-1800 cm\(^{-1}\)) for whey and egg albumen protein mixture (7.5:7.5 w/w) high pressure treated (600 MPa for 20 min) dissolved in D\(_2\)O. Spectra were baselined and normalised to the phenylalanine peak at 1004 cm\(^{-1}\).
Table 4.4: Normalised intensity values for selected regions (400-1500 cm\(^{-1}\)) of the experimental (bold font) and calculated (italics) FT-Raman spectra of 90°C heated (H) and high pressure treated (HP) (600 MPa for 20 min) egg albumen and whey proteins

<table>
<thead>
<tr>
<th>Assignment</th>
<th>Whey/egg albumen</th>
<th>whey/egg albumen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H</td>
<td>HP</td>
</tr>
<tr>
<td>Cystine S-S</td>
<td>0.17 ± 0.06 (491)</td>
<td>0.04 ± 0.01 (489)</td>
</tr>
<tr>
<td></td>
<td>0.19 ± 0.06 (501)</td>
<td>0.08 ± 0.02 (500)</td>
</tr>
<tr>
<td></td>
<td>0.14 ± 0.01 (518)</td>
<td>0.06 ± 0.03 (519)</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.12 ± 0.08 (753)</td>
<td>0.16 ± 0.03 (753)</td>
</tr>
<tr>
<td></td>
<td>0.33 ± 0.08 (829)</td>
<td>0.25 ± 0.02 (828)</td>
</tr>
<tr>
<td></td>
<td>0.21 ± 0.08 (853)</td>
<td>0.20 ± 0.03 (852)</td>
</tr>
<tr>
<td>CH bend/Trp</td>
<td>0.46 ± 0.04 (1346)</td>
<td>0.50 ± 0.05 (1342)</td>
</tr>
<tr>
<td>COO’</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>CH(_2) bend</td>
<td>1.3 ± 0.08 (1450)</td>
<td>1.62 ± 0.05 (1452)</td>
</tr>
<tr>
<td>C-N/C-C stretch</td>
<td>0.13 ± 0.04 (1052)</td>
<td>0.08 ± 0.05 (1052)</td>
</tr>
<tr>
<td>Amide 111*</td>
<td>0.46 ± 0.07 (938)</td>
<td>0.48 ± 0.02 (937)</td>
</tr>
<tr>
<td></td>
<td>0.50 ± 0.04 (959)</td>
<td>0.51 ± 0.04 (951)</td>
</tr>
<tr>
<td></td>
<td>0.60 ± 0.03 (987)</td>
<td>0.49 ± 0.02 (984)</td>
</tr>
<tr>
<td>Amide 111</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>β-sheet</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>α-helix</td>
<td>0.45 ± 0.07 (1323)</td>
<td>0.47 ± 0.04 (1321)</td>
</tr>
</tbody>
</table>

Intensity at wavenumber (cm\(^{-1}\)) shown in parenthesis, was normalized to the intensity of the phenylalanine band at 1004 cm\(^{-1}\). Spectral data were the average of 128 scans and baseline corrected.
4.4. Conclusion

Large deformation testing revealed higher gel strength and Young’s modulus values in heat-treated (90°C, 30 min) whey and egg albumen protein samples compared with high pressure treatment at 400-600 MPa, however, at higher pressures (>650 MPa) the gel strength values for whey protein isolate were comparable to heat-treated protein gels. Transmission electron microscopy showed that temperature-induced gels had a dense structure and a higher number of cross-links, while high pressure treated gels were porous with fewer cross-links. Raman spectroscopy allowed monitoring of specific groups during protein gelation. Changes in disulphide bonds were clearly demonstrated to be less extensive in high pressure treated (400-600 MPa) gels compared with heated samples. There were few changes in the secondary structure and less formation of β-sheet structures in high pressure treated samples compared with heat-treated samples. In contrast, hydrophobic interactions played a major role in protein gelation during both heat and pressure treatment but particularly for pressure processed gels. Synergistic protein-protein interactions giving higher than expected gel strength values occured in both temperature induced gels and pressure-induced gels, particularly at 10:5 whey/egg albumen protein combination.
CHAPTER 5


5.1. Introduction

Protein function plays an important role in the development of new food products; this is strongly determined by the protein structure. During processing changes occur which alter protein structure and hence functionality (Kumosinski and Farrell, 1993, Howell and Li-Chan, 1996; Howell et al., 1999). Unfortunately, measurement of the actual changes in secondary structure in foods is sometimes difficult due to the requirements of sample preparation for certain methods, including medium conditions (pH, presence of minerals, concentration, type of solvent). Moreover, there is a lack of reliable methodologies for observing protein structural changes in real food samples.

Secondary structures are characterized by periodic motifs such α-helices, β-sheets, turns and disordered structures. The gold standard method is X-ray crystallography which requires the molecules to form well-ordered crystals. This is not always possible for all proteins and indeed, most proteins used in the food industry cannot be crystallised as the desired functionality for the molecule most likely exists in solution, gel or in a colloidal state. In addition, X-ray crystallography requires that a suitable heavy metal be incorporated without distorting the crystal (Cantor and Schimmel, 1980).

An alternative to X-ray crystallography is multidimensional nuclear magnetic resonance (NMR) spectroscopy which allows structure determination in solution. However, the interpretation of the NMR spectra of large proteins is very complex and limited to small proteins (15-25 kDa) and the technique is very costly and time consuming. These limitations have spurred the development of alternative methods that are not able to generate structures at atomic resolution but provide structural information on proteins (especially on secondary structures) at a molecular level. These methods include circular dichroism (CD) and vibrational (infrared and Raman) spectroscopy. The CD technique measures the difference in absorbance between left and right circular polarized light in the far ultraviolet region, mainly in 230-185 nm (wavelength dependence on ellipticity). CD bands gives rise to either positive or
negative ellipticity produced by optically active peptide bonds for each of the standard conformational states (α-helix, β-sheet and random coil). However, a limitation of the CD method is that, measurements are made on highly dilute, optically clean solutions (any scattering components will affect results). Although CD is better at following changes in the α-helix region, non-periodic, β-tum structures cannot be distinguished by this method (Kavanagh et al., 2000; Kumosinski and Farrell, 1993).

Vibrational techniques such as Fourier transform infrared (FTIR) and Raman spectroscopies may be used in the study of food proteins that are turbid or particulate either in nature or as a result of processing. The two techniques are complementary because infrared absorption requires a change in the intrinsic dipole moment with molecular vibrations and Raman scattering depends on changes in polarizability of the molecule (Li-Chan and Qin, 1998). Polar functional groups such as C=O and O-H have strong infrared stretching vibrations, whereas intense Raman lines are associated with nonpolar groups such as (C=C, S-S).

The use of FTIR analysis in the estimation of protein secondary structure in lyophilised samples or D₂O solutions is limited by the formation of possible artefacts and errors (Jackson and Mantsch, 1992, 1995; Wilder et al., 1992). Water has a strong infrared absorption band (1650 cm⁻¹) which interferes in the infrared spectrum (Amide I band region) of aqueous protein samples and therefore specialised sampling techniques and careful baseline subtraction are required. In contrast, water is a poor Raman scatterer, and therefore direct analysis of aqueous samples and foods is feasible (Li-Chan, 1996).

The use of Raman spectroscopy in the determination of secondary structures is based on the premise that proteins and peptides structures contain specific absorption bands particularly in the Amide I region (1600-1700 cm⁻¹) which are due to contributions from C=O stretching vibration of the amide group, coupled with the in plane N-H bending and C-N stretching vibration (Surewicz and Mantsch, 1988). The exact
frequency of the Amide I band depends on the nature of hydrogen bonding between C=O and N-H moieties. In proteins, each of the amide groups is involved in the secondary structure of some type, either a helix, β-sheet or random structure. Because each of these secondary structural motifs is associated with a characteristic hydrogen bonding pattern between amide C=O and N-H groups, it follows that each type of secondary structure will give rise to characteristic amide I absorptions. It is this separation of Amide I absorptions that underlies the determination of protein secondary structure by Raman spectroscopy.

The relationship between the position of the Amide I band and the type of secondary structure may be best observed from the infrared spectra of homopolypeptides that adopt a well-defined and often highly homogenous secondary structures (Susi et al., 1967; Timasheff et al., 1967). However, in heteropolypeptides and in real food protein systems, there are a variety of domains containing polypeptide fragments in different conformations (Jackson and Mantsch, 1995). Therefore, the Amide I band contours of proteins are usually complex composites that consist of a number of overlapping component bands representing α-helices, β-sheets, turns and random structures.

Resolution-enhancement techniques by band narrowing allow the identification of these otherwise hidden component bands. Such analysis requires that the correspondence between the resolved component of the Amide I band and specific polypeptide structures be established. A basis for this has been provided by the analysis of X-ray crystallography data and corresponding infrared spectra of proteins of known three-dimensional structure, making the assignment of band conformation possible (Krimm and Bandekar, 1986). Studies by Susi and Byler (1986), Olinger et al., 1986; Dong et al., (1996); Lefevre and Subirade (2001) have revealed that the composite Amide I band can be broken down into a number of individual components. Several mathematical procedures such as Fourier self deconvolution (FSD) and derivativisation have been developed to allow visualisation of overlapping bands.
Chapter 5. Comparison of changes in secondary structure of unheated, heated and high pressure treated β-lactoglobulin and ovalbumin proteins using Fourier Transform Raman spectroscopy and self deconvolution

following manipulation of spectrum (Cameron and Moffatt, 1984; Mantsch and Moffatt, 1993).

FSD is used together with curve fitting analysis which not only enables resolution of individual component bands but calculation of the area of each of the bands. Although in many cases the deconvoluted Amide I bands have been used to determine secondary structure by curve fitting, it should be recognized that resolution enhanced spectra, especially derivative spectra do not reproduce true band intensities and relative component fractions cannot be obtained directly from them. Despite these shortcomings, both methods are extremely useful for identifying component frequencies in complex spectra.

The effect of different conditions (salt concentration, pH, effect of pressure, disulphide bond exchange) on the secondary structure of β-lactoglobulin during gel formation has been reported using circular dichroism, (Matsuura and Manning, 1994) and infrared spectroscopy during pressure and temperature treatment (Pannick et al., 1999; Boye et al., 1996; Casal et al., 1988). For ovalbumin, changes in secondary structure using different methods have been reported. Painter and Koenig (1976) showed an increase in the inter-molecular β-sheet structure during thermal aggregation using Raman spectroscopy. A similar observation by an infrared and laser Raman spectroscopic study during heat-induced gelation of a number of globular proteins has been reported (Clark et al., 1981). However, these findings are based on qualitative data and more recent studies by (Kato and Takagi, 1988; Mine et al., 1990; Smith et al., 2000) used CD and FTIR spectroscopy to quantitatively estimate various secondary fractions.

Although deconvolution studies of the CH-stretch region for heated β-lactoglobulin and lysozyme have been reported (Howell et al., 1999), no studies to date exist on the use of Raman spectroscopy to analyse the secondary structure by deconvolution of Amide I band of whey and egg albumen proteins using temperature and pressure denaturation. In this study, quantitative changes in the secondary structure of β-
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β-lactoglobulin and ovalbumin were investigated through the deconvolution of the Amide I band, whilst the overall secondary structure of both β-lactoglobulin and ovalbumin has been established by X-ray crystallography (Papiz et al., 1986, and Stein et al., 1991). The objectives of this study were to establish the role of Raman spectroscopy in the quantitative determination of changes in protein secondary structure during processing.
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5.2 Materials

Materials were the same as in section 2.1

5.3 Methods

5.3.1. Sample preparation

Solutions of ovalbumin and β-lactoglobulin 15% (w/w) were prepared in D$_2$O. Heat treated samples of the above protein solutions were made as follows. Protein solution (7 ml) was poured into stainless steel tubes 50 mm long and 15 mm diameter and heated at 90°C for 30 minutes to form gels. The gels were stored overnight at 4°C to promote network formation (Howell and Lawrie, 1984). Unheated and heated samples were placed in NMR tubes (5 mm diameter, Precision grade, Aldrich Chemical Company Milwaukee USA) and analysed by FT-Raman spectroscopy.

5.3.1.1. Pressure treated samples

The solutions for high pressure treated gels were prepared by the same method as heat treated gels except the samples were placed in visking tubing tied at both ends, heat sealed in a high pressure polythene bag (Crayovac W.R Grace Limited Cromwell Road, Cambridge, United Kingdom). The samples were introduced into the working chamber (300 ml capacity) of a Stansted Food Lab high pressure rig (Stansted Fluid power, Ltd, 70 Bentfield road, Stansted, CM24 8HT, Essex, UK.) containing low compressibility fluid (2:8 mixture of castor oil:ethanol). The equipment was installed in an air conditioned laboratory at 20°C. The temperature in the pressurisation chamber increased during compression reaching a maximum after 2 minutes. The maximum temperature achieved in all treatments was 30°C, corresponding to the treatment at 400 MPa, 500 MPa, 600 MPa), then decreased to 20°C in 0.5 minutes and remained stable at 20°C until the end of the cycle. The compression and decompression times were about 1-2 minutes and 0.5 min respectively. The samples were stored at 4°C similar to the heat-treated ones. For samples less than 10 ml, the Stansted Micro Food-Lab, Model S-FL-085-9W high pressure rig was used.
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5.3.2. FT Raman spectroscopy, data collection and analysis

Raman spectra were recorded at 4°C on a Perkin Elmer 2000 FT-Raman spectrometer (Beaconsfield, Buckinghamshire, UK). Spectral resolution was set at 4 cm⁻¹, laser power 1600mW and the data presented are based on 128 co-added spectra. Frequency calibration of the instrument was undertaken using the sulphur line at 217 cm⁻¹. The spectra were analysed using Grams 32 software (Galactic Industries Corp, Salem NH). The protein spectra obtained were baselined and the intensity was normalised using the phenylalanine peak at 1004 cm⁻¹ (Tu 1986, Howell and Li-Chan 1996). The major bands in the spectra, related to vibrational motions of various side chains or polypeptide backbone, were assigned by comparison with Raman spectra of proteins which have been reported in the literature (Howell and Li-Chan 1996, Howell et al 2001). To calculate the secondary structure components, the Amide I region (1600-1700 cm⁻¹) was truncated and deconvoluted using a non-linear least squares curve fitting subroutine which included mixed Gaussian and Lorenztian components. The percentage of each secondary structure component ie; α-helix, β-sheet, β-turns and random coil (unspecific) were determined by the method of Williams, (1986).

\[
\% \text{ of secondary structure} = \frac{\text{area of secondary structure}}{\text{area of Amide I band}}
\]
Chapter 5. Comparison of changes in secondary structure of unheated, heated and high pressure treated \( \beta \)-lactoglobulin and ovalbumin proteins using Fourier Transform Raman spectroscopy and self deconvolution

5.4 Results and discussion

5.4.1. FT Raman spectroscopy, analysis of secondary structure

The results of deconvolution of the Amide I bands of unheated, heated and high pressure treated (600 MPa for 20 min) ovalbumin are shown in (Fig. 5.1, 5.2 and 5.3) and (fig. 5.4, 5.5 and 5.6) for \( \beta \)-lactoglobulin respectively.

5.4.1.1. Native protein samples

There were marked differences in the secondary structures of the proteins subjected to various treatments. The spectra of unheated ovalbumin exhibited 12 major bands related to secondary structure; the assignment of the bands were made on the basis of previous infrared studies for over 50 proteins in water solutions (Susi and Byler, 1988; Dong and Caughey, 1994; Dong et al., 1990). In ovalbumin the bands at 1636 cm\(^{-1}\), 1632 cm\(^{-1}\), 1625 cm\(^{-1}\) and 1620 cm\(^{-1}\) can be assigned to low frequency \( \beta \)-sheets and 1682 cm\(^{-1}\) and 1673 cm\(^{-1}\) to \( \beta \)-turns whereas the band at 1690 cm\(^{-1}\) is associated with high frequency \( \beta \)-turns. The presence of the 1625 cm\(^{-1}\) band has been reported by several workers (Takeda et al., 1995; Dong et al., 2000). The \( \alpha \)-helix band was centered at 1648 and 1652 cm\(^{-1}\). The two low frequency bands near 1605 and 1614 cm\(^{-1}\) are caused by aromatic side chains of the individual amino acids (Susi and Byler, 1988) and do not contribute to the secondary structure.

Quantitative analysis revealed that unheated ovalbumin had a composition corresponding to 41% \( \alpha \)-helix, 34% \( \beta \)-sheet, 12% \( \beta \)-turns and 13% random coil. Circular dichroism (CD) studies provided a close match of these results which showed that native ovalbumin is made up of 40-49% \( \alpha \)-helix, 30% \( \beta \)-sheet and 20% unordered structure (Kato and Tagaki, 1988; Mine et al., 1990). On the other hand values determined by X-ray crystallography showed 45% \( \beta \)-sheet and 35% \( \alpha \)-helix (Stein et al., 1991; Wright et al., 1990) and FTIR in D\(_2\)O by factor analysis indicated 44% \( \beta \)-sheet and 35% \( \alpha \)-helix (Perkin et al. 1992).
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β-lactoglobulin also exhibited bands at 1643 cm\(^{-1}\), 1637 cm\(^{-1}\), 1632 cm\(^{-1}\), 1626 cm\(^{-1}\) and 1622 cm\(^{-1}\) assigned to low frequency β-sheets and at 1682 cm\(^{-1}\) and 1673 cm\(^{-1}\) assigned to β-turns. The band at 1690 cm\(^{-1}\) is associated with high frequency β-turns. Studies by Susi and Byler (1986, 1988) support these findings. Quantitative analysis of unheated β-lactoglobulin showed 54% β-sheet, 15% α-helix and 25% turns and 6% random coil; this is consistent with previous Raman studies (~54% β-sheet, ~10% α-helix, 32% unspecified (Susi and Byler, 1986; Berjot et al., 1987). CD and Infrared studies suggest an α-helix content of 10-15%, β-sheet content of approximately 50%, with β-turns accounting for 20% and remaining 15% representing amino acid residues in a random non-repetitive arrangement without a well defined structure (Timasheff et al., 1966; Susi and Byler, 1986; Casal et al., 1988). These results indicate that the secondary structure of β-lactoglobulin is dominated by a β-sheet structure while ovalbumin is dominated by an α-helix secondary structure. (Table 5.1 shows tentative peak assignments for Amide 1 band).

Table 5.1: Tentative peak assignments of deconvoluted Amide I components, data from (Kavanagh et al., 2000; Byler and Susi, 1988; Casal et al., 1988)

<table>
<thead>
<tr>
<th>Structure</th>
<th>Amide 1 Wavenumber (cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggregated strands</td>
<td>1610-1620</td>
</tr>
<tr>
<td>β-Sheet (low frequency)</td>
<td>1620-1640</td>
</tr>
<tr>
<td>β-Sheet (high frequency)</td>
<td>1670-1680</td>
</tr>
<tr>
<td>β-Turns</td>
<td>1658-1670</td>
</tr>
<tr>
<td>α-Helix</td>
<td>1650-1660</td>
</tr>
<tr>
<td>Random coil</td>
<td>1660-1670</td>
</tr>
</tbody>
</table>
Chapter 5. Comparison of changes in secondary structure of unheated, heated and high pressure treated β-lactoglobulin and ovalbumin proteins using Fourier Transform Raman spectroscopy and self deconvolution

Figure. 5.1. FT-Raman spectra of unheated ovalbumin; Amide I region (1600-1700 cm\(^{-1}\)), the peaks were fitted after deconvolution.

Figure. 5.2. FT-Raman spectra of heated ovalbumin; Amide I region (1600-1700 cm\(^{-1}\)), the peaks were fitted after deconvolution.
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Figure. 5.3. FT-Raman spectra of high pressure treated (600 MPa for 20 min) ovalbumin; Amide I region (1600-1700 cm⁻¹), the peaks were fitted after deconvolution.

Figure. 5.4: FT-Raman spectra of native β-lactoglobulin; Amide I region (1600-1700 cm⁻¹), the peaks were fitted after deconvolution.

Figure. 5.5: FT-Raman spectra of heated β-lactoglobulin; Amide I region (1600-1700 cm⁻¹), the peaks were fitted after deconvolution.
Chapter 5. Comparison of changes in secondary structure of unheated, heated and high pressure treated β-lactoglobulin and ovalbumin proteins using Fourier Transform Raman spectroscopy and self deconvolution

Figure 5.6: FT-Raman spectra of high pressure treated (600 MPa for 20 min) of β-lactoglobulin; Amide I region (1600-1700 cm⁻¹), the peaks were fitted after deconvolution.
Chapter 5. Comparison of changes in secondary structure of unheated, heated and high pressure treated β-lactoglobulin and ovalbumin proteins using Fourier Transform Raman spectroscopy and self deconvolution

Table 5.2: Percentage breakdown of different fractions of secondary structures in unheated, heated (90°C for 30 min) and high pressure (600 MPA for 20 min) treated ovalbumin and β-lactoglobulin proteins.

<table>
<thead>
<tr>
<th>% Secondary Structures</th>
<th>α-helix</th>
<th>β-Sheet</th>
<th>β-turns</th>
<th>random coil</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ovalbumin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>41</td>
<td>34</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>Heated</td>
<td>16</td>
<td>51</td>
<td>20</td>
<td>13</td>
</tr>
<tr>
<td>High pressure treated</td>
<td>34</td>
<td>30</td>
<td>25</td>
<td>11</td>
</tr>
<tr>
<td><strong>β-lactoglobulin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>15</td>
<td>54</td>
<td>25</td>
<td>6</td>
</tr>
<tr>
<td>Heated</td>
<td>10</td>
<td>70</td>
<td>14</td>
<td>6</td>
</tr>
<tr>
<td>High pressure treated</td>
<td>10</td>
<td>37</td>
<td>24</td>
<td>30</td>
</tr>
</tbody>
</table>
Chapter 5. Comparison of changes in secondary structure of unheated, heated and high pressure treated β-lactoglobulin and ovalbumin proteins using Fourier Transform Raman spectroscopy and self deconvolution

Figure. 5.8. FT Raman spectra (500-1800 cm⁻¹) of (a) native (b) heated (c) high pressure treated (600 MPa for 20 min) β-lactoglobulin (15% w/w) dissolved in D₂O. The spectra were baselined and normalised to the phenylalanine peak at 1004 cm⁻¹.

5.4.1.2. Processed samples

In this study, major changes were observed in the heated samples of ovalbumin and β-lactoglobulin, especially a decrease in the band at 1648-1658 cm⁻¹ which is attributed to the α- helical structure (Table 6.2). Previous studies reported similar findings (Casal et al., 1988; Susi and Byler, 1988; Howell and Li-Chan, 1996). For heated ovalbumin the composition was as follows 51% β-sheet, 16% α- helix, 20% turns and 13% random structure. Heated β-lactoglobulin had 70% β-sheet, 14% β-turns and 10% α- helix and 6% random coil. This decrease in helical structures has been observed by other workers (Herald and Smith, 1992; Kato and Takagi, 1988; Howell and Li-Chan, 1996). There was a relatively weak band in β-lactoglobulin in the Amide III' at 940 cm⁻¹, this may be due to low α- helix content of this protein; approximately one tenth of the total residues are involved in α- helix structures (Fogolari et al., 1998). There was a substantial increase in the 1674 cm⁻¹ band
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The FT Raman spectra for native, heated (90°C for 30 min) and high pressure treated (600 MPa for 20 min) ovalbumin and β-lactoglobulin are shown in (Fig. 5.7. a, b, c) and (Fig.5.8. a, b, c). The high helical content in unheated proteins is also reflected in the amide III’ region 900-970 cm⁻¹ in both ovalbumin and β-lactoglobulin. In addition, the amide III’ α- helix band at 1318 cm⁻¹ had a higher peak intensity in both ovalbumin and β-lactoglobulin while the peak at 1239 cm⁻¹ Amide III’ β-sheet band had a higher peak intensity in β-lactoglobulin (0.79) compared to ovalbumin (0.13) (p<0.05). The high peak intensity at 1239 cm⁻¹ Amide III’ β-sheet band supports the observation above that β-lactoglobulin has a more beta- sheet structure; previous work on heated whey isolate and egg albumen is consistent with these findings (Ngarize et al., 2003) (chapter 3).

Figure 5.7. FT Raman spectra (500-1800 cm⁻¹) (a) native (b) heated and (c) high pressure treated (600 MPa for 20 min) of ovalbumin (15% w/w) dissolved in D₂O. The spectra were baselined and normalised to the phenylalanine peak at 1004 cm⁻¹.
some workers (Casal et al., 1988; Susi and Byler, 1988) and to turns by others (Krimm and Bandekar, 1986).

In addition, there was an increase in protein aggregation bands at 1684 cm\(^{-1}\) and 1618 cm\(^{-1}\) attributed to the intermolecular hydrogen bonded \(\beta\)-sheet structures (Ismail et al., 1992; Dong et al., 2000). The presence of the band at 1618-1620 cm\(^{-1}\) in both heated ovalbumin and \(\beta\)-lactoglobulin indicated the disruption of hydrogen bonds within some secondary structure and formation of new stronger hydrogen bond, which is associated with aggregation, that is the formation of intermolecular hydrogen bonds (Subirade et al., 1998). The development of such a band has been observed with the formation of gels in some proteins such as serum albumin (Clark et al., 1981) and in glycinin (Nagano et al., 1994), indicating the unfolding of the compact structure and the formation of \(\beta\)-aggregation. The presence of the 1680 cm\(^{-1}\) band suggests that these aggregates consist of anti-parallel \(\beta\)-strands. There was a decrease in the peak intensity at the 1318 cm\(^{-1}\) Amide III' band which corresponds to \(\alpha\)-helix structure in both ovalbumin and \(\beta\)-lactoglobulin; a similar trend was observed in all heated proteins in our previous study (Ngarize et al., 2003). The absence of the band at 1240 cm\(^{-1}\) (Amide III' beta-sheet) in both high pressure treated ovalbumin and \(\beta\)-lactoglobulin indicates subtle differences in protein conformation may be responsible for the presence of aggregation bands (Heremans and Wong, 1985).

In this study, comparison of native, heated and high pressure treated ovalbumin and \(\beta\)-lactoglobulin show major differences. Quantitative analysis of high pressure treated ovalbumin indicated 30 % \(\beta\)-sheet, 34% \(\alpha\)-helix, 25% \(\beta\)-turns and 11% random structure, these data differ from native ovalbumin above (41% \(\alpha\)-helix, 34% \(\beta\)-sheet, 12% \(\beta\)-turns and 13% random coil). There is twice the number of \(\beta\)-turns in the high pressure treated ovalbumin compared with the native form.

Compared with unheated ovalbumin there was an increase in \(\beta\)-sheet structure from 34% to 51% in heated ovalbumin and no increase in random coil. In addition, there was some difference in the \(\alpha\)-helix content of native (41%) and high pressure treated ovalbumin (34%, \(p<0.05\)). Some reports have indicated that the spectrum of high
pressure denatured protein has more features of the native protein than that of the temperature denatured protein (Wong and Heremans, 1988). This suggests that the unfolding for pressure denaturation for treatments tested (400-800 MPa) is less extensive than unfolding for temperature denaturation (90°C for 30 min). Rheological studies on whey and egg albumen, heat and pressure treated support this view (Ngarize et al., 2003) (chapter 1). Pressure induced denaturation with gel formation has been observed in a number of studies (Van Camp and Huyghebaert, 1995; Heremans et al., 1997; Smeller et al., 1999).

Comparison of native β-lactoglobulin with high pressure treated samples showed that pressure induced more changes in β-lactoglobulin compared with ovalbumin. High pressure treated β-lactoglobulin had 40% β-sheet, 23% β-turns, 10% α-helix and 30% random structure compared with 54% β-sheet, 15% α-helix and 25% turns and 6% random coil in the native form. The increase in random structure in high pressure treated β-lactoglobulin at the expense of β-sheet and α-helix has been reported in literature (Pannick et al., 1999). However, heat denaturation indicated 70% β-sheet, 14% β-turns, 10% α-helix and 6% random coil (p<0.05). This indicates that heat denaturation results in the increased formation of β-sheet structures compared with 37% in high pressure treated β-lactoglobulin. The increased β-sheet structure is a major contributory factor to increased gelling properties of heated β-lactoglobulin and ovalbumin compared with pressure treated samples as shown by large deformation studies (Ngarize et al., 2003 submitted).
Chapter 5. Comparison of changes in secondary structure of unheated, heated and high pressure treated β-lactoglobulin and ovalbumin proteins using Fourier Transform Raman spectroscopy and self deconvolution

5.4 Conclusion

This study has shown that by using Fourier transform Raman spectroscopy and self deconvolution the Amide I band of unheated, heated and high pressure treated ovalbumin and β-lactoglobulin can be resolved into component bands. The results showed some similarities in the conformation of Amide I band; the bands around 1636 cm\(^{-1}\) (low frequency beta sheets) were present in both heated and pressure treated ovalbumin and β-lactoglobulin. Interestingly, like most proteins there was more than one β-sheet component; in the 1680-1690 cm\(^{-1}\) region (high frequency beta sheets) indicative of protein aggregation and in hydrogen bonding between β-strands. Heat-induced gelation produced a greater increase in β-sheet structure in both heated ovalbumin and β-lactoglobulin compared with high pressure treated samples. In addition, the absence of the Amide III’ beta-sheet structure at 1239 cm\(^{-1}\) in both high pressure treated ovalbumin and β-lactoglobulin samples gave further evidence that the involvement of β-sheets structures is less intense in high pressure treated proteins gels this may explain why pressure treated gels were found to be weaker in previous studies by (Ngarize et al., 2003). There was a trend towards an increase in beta-sheet structure at the expense of α-helix structures in all the heated samples, while in high pressure treated samples, changes in secondary structures showed an increase in random structure in β-lactoglobulin and a decrease in the α-helix. On the other hand ovalbumin showed an increase in β-turns without much change in α-helix and β-sheets; these differences ovalbumin and β-lactoglobulin may reflect the differences in the way the two proteins respond to pressure treatment. This study has highlighted that heat and pressure unfolding and denaturation differed with respect to secondary changes.
6.1 Introduction

Proteins and sugars are common ingredients in many food systems and are often used together to control structure and texture of food products. Egg albumen and whey proteins are major functional ingredients in many baked products and contribute to texture formation by their ability to foam and gel at elevated temperatures (Barbut and Findlay, 1990). The functional properties of proteins may be changed depending on the nature and strength of their interactions with other ingredients such as sugars and solvent conditions (Galazka et al., 2000). The addition of sugar has been demonstrated to play a crucial role in obtaining an acceptable texture in various cakes. For example in angel food cake, recommended levels of sucrose (approximately 40%) ensure a simultaneous denaturation of egg white proteins and gelatinisation of starch (Donovan, 1977).

Previous studies have shown that sugars can increase the denaturation temperature of whey and egg albumen proteins and various other globular proteins (Harwalker and Ma 1989; Arntfield et al., 1990; Timasheff, 1993; Boye et al., 1996; Jou and Harper, 1996; Kulmyrzaev et al., 2000; Dickinson and Merino, 2002). The protection against globular protein unfolding that is conferred by high sugar levels not only results in heat stability but offers stability against pressure-induced denaturation (Dumay et al., 1994). The proposed mechanisms for increased thermal stability and pressure induced denaturation of proteins has been documented in the literature. Firstly, the direct contact between protein and water is considered thermodynamically unfavourable in the presence of sugars (Arakawa and Timasheff, 1982; Lee and Timasheff, 1981); some researchers have correlated this with an enhancement of hydrophobic interactions (Philips et al., 1994).

Secondly, the presence of concentrated sugars results in diminished water activity and is considered to make water-protein interactions less effective (Barone et al., 1992; Parsegian et al., 1995). In other words, the presence of sugars decreases the thermodynamic affinity of protein molecules for the solvent.
Chapter 6. Effects of sugars on rheological properties and molecular interactions of unheated, heated and high pressure treated whey and egg albumen protein

The non-specific sugar-protein interactions, which occur during heating or high pressure treatment of foods should be distinguished from more specific reaction such as Maillard reaction, which is a sugar-amine reaction that occurs during heat processing. The Maillard reaction is a non-enzymic browning reaction involving the carbonyl group of reducing sugars, e.g., glucose and the amino group of an amino acid (de Man, 1999). The Maillard reaction is a sugar-amine interaction unlike caramelisation that occurs when sugars are heated on their own to high temperatures, but there is no interaction with amino compounds and no nitrogenous compounds can result. The Maillard reaction is of prime importance to the food manufacturer because it occurs when almost all foods are heated and is responsible for the flavours and colours that develop during heating (Ames, 1998), for example, in the manufacture of coffee and bakery products.

The Maillard reaction is pH and temperature dependent (Ames, 1990; Ledl and Schleicher, 1990). Few studies have investigated the effect of high pressure on the Maillard reaction, Tamaoka et al., (1991) reported that the development of brown colour was greatly suppressed when pressures of 200-400 MPa were applied to a xylose-lysine system at 50°C in sodium hydrogen carbonate solution at pH 8.2. More recently, Hill et al., (1996) using a glucose-lysine system at pH 8 and 10.1 observed that pressure treatment at 600 MPa enhanced Maillard browning; however, at pH 5.1 and 6.5, application of pressure depressed the rate of browning, it was postulated that at lower pH such as 6.5, the system is buffered mainly by the carboxylic acid group of the amino acid, and high pressure favoured the ionic form of this group resulting in pressure induced pH decrease of about 0.2 of a pH unit for every 100 MPa pressure applied (Heremans, 1995). It is beyond the remit of this study to investigate texture related changes during Maillard reactions which is unlikely to occur due to the use of sucrose.

The influence of sugars in terms of globular protein gelation has been documented, there is considerable evidence supporting the role of protein-protein interactions and protein aggregation. Kulmyrzaev et al., (2000) reported increased gel rigidity in whey proteins when cooled to 30°C, which can be attributed to enhancement of protein-protein...
interactions in the presence of sugars. Other workers have reported stronger attractive electrostatic interactions in casein molecules in the presence of sugars (Mora-Gutierrez et al., 1997). More recently, Abbasi and Dickinson (2001) reported existence of critical ranges of sugar concentration for producing pressure-induced gels from dispersions of skimmed milk powder at micellar casein concentration down to approximately 2% w/v.

Pioneering studies by Howell and Lawrie (1984) on plasma-egg albumen proteins interactions in gels was undertaken in 45% (w/w) sucrose solution in a model system resembling a high ratio cake. The effect of high sugar in these systems was to increase the gel strength by binding of moisture by sugar leaving the protein highly concentrated. Although, the stabilising effect of sugar on proteins is well documented in literature, little is known about the actual mechanism with respect to protein gel formation. In this study, the gelation of whey and egg albumen protein and their mixtures, following heat and pressure denaturation has been studied using FT-Raman spectroscopy and large deformation testing in the presence of 20-40% (wt%) sucrose solution. Such interactions may be important in bakery products where the temperature range of thermal events like protein denaturation and starch gelatinisation should be in a specific range to obtain the desired texture for the final product (Donovan, 1977).
6.2 Materials
Materials used were the same as in section 2.1

6.3 Methods

6.3.1. FT Raman spectroscopy

6.3.1.1. Sample preparation
Solutions 15% (w/w) of individual whey or β-lactoglobulin and egg albumen or ovalbumin proteins were prepared in D2O. A mixture of 7.5% (w/w) whey protein or β-lactoglobulin and 7.5% (w/w) egg albumen protein or ovalbumin, in D2O was also prepared. Sucrose 20% (w/w) was added to the above protein solutions. Heated samples of the above protein solutions were made as follows. Protein solution (7 ml) was poured into stainless steel tubes 50 mm long and 15 mm diameter and heated at 90°C for 30 minutes to form gels. The gels were stored overnight at 4°C. Unheated and heated samples were placed in NMR tubes (5mm diameter, Precision grade, Aldrich Chemical Company Milwaukee USA) and analysed by FT-Raman spectroscopy.

6.3.1.2. FT Raman spectroscopy, data collection and analysis
Raman spectra were recorded at 4°C on a Perkin Elmer 2000 FT-Raman spectrometer (Beaconsfield, Buckinghamshire, UK). Spectral resolution was set at 4 cm⁻¹, laser power 1600 mW and the data presented are based on 128 co-added spectra. Frequency calibration of the instrument was undertaken using the sulphur line at 217 cm⁻¹. The spectra were analysed using Grams 32 software (Galactic Industries Corp, Salem NH). The protein spectra obtained were baselined and the intensity was normalised using the phenylalanine peak at 1004 cm⁻¹ after subtraction of the sucrose spectra from the protein spectra (Tu, 1986; Howell and Li-Chan, 1996). The major bands in the spectra, related to vibrational motions of various side chains or polypeptide backbone, were assigned by comparison with Raman spectra of proteins which have been reported in the literature (Howell and Li-Chan, 1996; Howell et al., 2001). The interaction of proteins was investigated by the analysis of the difference spectra calculated from the average of the
normalised spectra of individual proteins minus the experimental spectra of the protein mixtures. For the difference spectra the intensity values are recorded on the y axis.

6.3.2. Large deformation testing
6.3.2.1. Heat treated samples
Protein solutions (15% w/w) in distilled water were poured into stainless steel tubes, 50 mm long and 15 mm in diameter and heated in a water bath at 90°C for 30 minutes to promote network formation (Howell and Lawrie, 1984). The above samples were made in 10% and 20% (w/w) sucrose solutions, with additional samples in 40% (w/w) sucrose solution. The heated samples were stored at 4°C overnight to age. The gelled samples were cut into 15 mm long cylinders and were tested on TA-XT2 texture analyser (Stable Microsystems, Godalming, Surrey, UK). The force at maximum compression (12 mm) and the Young’s modulus (the gradient of the stress-strain curve) were measured. The test speed was set at 0.2 mm/sec, and the samples were compressed by a distance of 12 mm to a final height of 3 mm. The interaction between the proteins was calculated as the percentage change using the interaction index (Howell and Lawrie, 1984) as follows:

Interaction index = \frac{Actual value - additive value}{additive value} \times 100

Additive value

* the value derived from summing the contributions of the component proteins measured in isolation, in proportion to the concentration of each in the mixture.
6.3.2.2. Pressure treated samples

The solutions for high pressure treated gels were prepared by the same method as heat treated gels except the samples were placed in visking tubing tied at both ends, heat sealed in a high pressure polythene bag (Crayovac W.R Grace Limited, Cromwell Road, Cambridge, United Kingdom). The samples were introduced into the working chamber (300 ml capacity) of a Stansted Food Lab high pressure rig (Stansted Fluid power, Ltd, 70 Bentfield Road, Stansted, CM24 8HT, Essex, UK.) containing the low compressibility fluid (2:8 mixture of castor oil:ethanol). The equipment was installed in an air conditioned laboratory at 20°C. The temperature in the pressurisation chamber increased during compression, reaching a maximum after 2 minutes (the maximum temperature achieved in all treatments was 30°C, corresponding to the treatment at 400 MPa, 500 MPa and 600 MPa), then decreased to 20°C in 0.5 min and maintained at 20°C until the end of the cycle. The compression and decompression times were about 1-2 minutes and 0.5 min respectively. The samples were stored at 4°C similar to the heat-treated ones. For samples less than 10ml, the Stansted Micro Food-Lab, Model S-FL-085-9W high pressure rig was used.
6.4 Results and Discussion

6.4.1. Rheological studies

6.4.1.1. Large deformation testing

The effect of sugars on egg albumen and whey protein gelation is discussed in terms of the influence on protein-protein interactions, (table 6.1 and 6.2). The presence of 10% sucrose in the heated (90°C for 30 min) egg albumen and whey protein gels gave significantly different readings for Young’s modulus of; 10.7 Pa (± 0.80) and 12.5 Pa (± 0.20) respectively (P<0.05). Our previous studies (Ngarize et al., 2003) indicated that a high Young’s modulus was related to the extent of crosslinks in the gel network structure and therefore in gel strength. The values for force at maximum compression were 835 (± 40) and 1082 (± 40) for egg albumen and whey protein gels indicating a significant difference (p<0.05).

In 20% sucrose solution, egg albumen gels were stronger with a Young’s modulus of 19.7 Pa (± 1.40) compared with 16.8 Pa (±1.20) for whey protein (p<0.05). The force at maximum compression was 1248 (±100) and 1219 (±90) for egg albumen and whey protein gels respectively. Evidence from previous studies supports the role of sugar as an enhancer of protein–protein interactions and protein aggregation. Kulmyrzaez et al., 2000 a, b) reported increased attraction between unfolded whey protein molecules in the presence of sucrose, Antipova et al., (1999) indicated a corresponding strengthening of protein–protein attractive interactions in caseinate gels. This finding was previously reported by van Vliet et al., (1989) who showed that interactions in casein gels are probably due to hydrophobic and electrostatic interactions. This view is further supported by Phillips et al., (1994) who attributed the main effect of sugars to the promotion of hydrophobic interactions through the modification of water structure surrounding proteins.
Table 6.1: Large deformation analysis of egg albumen protein (15% w/w) heated at 90°C and 95°C for 30 min and high pressure treated (HP) (600 MPa for 20 mins) in the presence of 10-40% (w/w) sucrose

<table>
<thead>
<tr>
<th>Egg albumen (15% w/w)</th>
<th>Protein gel</th>
<th>Force (g) at 12mm compression</th>
<th>Young’s modulus (Pa)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Heat Processing conditions</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heated at 90°C</td>
<td>1158</td>
<td>17.2</td>
<td>(±100) (±1.30)</td>
</tr>
<tr>
<td>Heated at 95°C</td>
<td>1222</td>
<td>18.5</td>
<td>(±120) (1.20)</td>
</tr>
<tr>
<td>10% sucrose at 90°C</td>
<td>835</td>
<td>10.7</td>
<td>(±40) (0.80)</td>
</tr>
<tr>
<td>20% sucrose at 90°C</td>
<td>1248</td>
<td>19.7</td>
<td>(±100) (±1.40)</td>
</tr>
<tr>
<td>40% sucrose at 90°C</td>
<td>879</td>
<td>140.5</td>
<td>(±790) (±13.0)</td>
</tr>
<tr>
<td>10% sucrose at 95°C</td>
<td>986</td>
<td>11.5</td>
<td>(±20) (±0.40)</td>
</tr>
<tr>
<td>20% sucrose at 95°C</td>
<td>1452</td>
<td>20.0</td>
<td>(±60) (±1.00)</td>
</tr>
<tr>
<td>40% sucrose at 95°C</td>
<td>838</td>
<td>12.8</td>
<td>(±50) (±0.60)</td>
</tr>
<tr>
<td><strong>High pressure treatment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HP with 10% sucrose</td>
<td>502</td>
<td>3.56</td>
<td>(±40) (±0.30)</td>
</tr>
<tr>
<td>HP with 20% sucrose</td>
<td>220</td>
<td>2.50</td>
<td>(±18) (±0.30)</td>
</tr>
</tbody>
</table>

Figures in parenthesis are standard deviation values based on five replicates.
Chapter 6. Effects of sugars on rheological properties and molecular interactions of unheated, heated and high pressure treated whey and egg albumen protein

Table 6.2: Large deformation analysis of whey protein (15% w/w) heated at 90°C and 95°C for 2 min and high pressure treated (HP) (600 MPa for 20 mins) in the presence of 10-40% (w/w) sucrose

<table>
<thead>
<tr>
<th>Whey (15% w/w)</th>
<th>Protein gel Force (g) at 12mm compression</th>
<th>Young's modulus (Pa)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Heat Processing conditions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heated at 90°C</td>
<td>905</td>
<td>12.1</td>
</tr>
<tr>
<td>(±50)</td>
<td>(±0.10)</td>
<td></td>
</tr>
<tr>
<td>Heated at 95°C</td>
<td>2024</td>
<td>25.1</td>
</tr>
<tr>
<td>(±200)</td>
<td>(±1.5)</td>
<td></td>
</tr>
<tr>
<td>10% sucrose at 90°C</td>
<td>1082</td>
<td>12.5</td>
</tr>
<tr>
<td>(±40)</td>
<td>(±0.20)</td>
<td></td>
</tr>
<tr>
<td>20% sucrose at 90°C</td>
<td>1219</td>
<td>16.8</td>
</tr>
<tr>
<td>(±90)</td>
<td>(±1.20)</td>
<td></td>
</tr>
<tr>
<td>40% sucrose at 90°C</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>10% sucrose at 95°C</td>
<td>1182</td>
<td>13.0</td>
</tr>
<tr>
<td>(±30)</td>
<td>(±1.00)</td>
<td></td>
</tr>
<tr>
<td>20% sucrose at 95°C</td>
<td>2340</td>
<td>27.0</td>
</tr>
<tr>
<td>(±100)</td>
<td>(±2.20)</td>
<td></td>
</tr>
<tr>
<td>40% sucrose at 95°C</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>40% sucrose at 95°C (1hr holding time)</td>
<td>824</td>
<td>11.7</td>
</tr>
<tr>
<td>(±60)</td>
<td>(±0.80)</td>
<td></td>
</tr>
</tbody>
</table>

**High pressure treatment**

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>HP with 10% sucrose</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>HP with 20% sucrose</td>
<td>158</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>(±8.6)</td>
<td>(±0.01)</td>
</tr>
</tbody>
</table>

Figures in parenthesis are standard deviation values based on five replicates.
### Table 6.3: Large deformation analysis of mixtures of whey and egg albumen protein (15% w/w) heated at 90°C for 30 min in 10-20% (w/w) sucrose

<table>
<thead>
<tr>
<th>Protein gel</th>
<th>Force (g) at 12mm compression</th>
<th>Young's modulus (Pa)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>10% sucrose at 90°C</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10:5 W/EA</td>
<td>1143 (±100)</td>
<td>10.7 (±1.00)</td>
</tr>
<tr>
<td>Interaction index</td>
<td>+24.5</td>
<td></td>
</tr>
<tr>
<td>7.5:7.5 W/EA</td>
<td>1015 (±50)</td>
<td>9.8 (±1.50)</td>
</tr>
<tr>
<td>Interaction index</td>
<td>+5.9</td>
<td></td>
</tr>
<tr>
<td>5:10 W/EA</td>
<td>980 (±20)</td>
<td>7.9 (±0.20)</td>
</tr>
<tr>
<td>Interaction index</td>
<td>+6.8</td>
<td></td>
</tr>
<tr>
<td><strong>20% sucrose at 90°C</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10:5 W/EA</td>
<td>1742 (±90)</td>
<td>18.6 (±3.60)</td>
</tr>
<tr>
<td>Interaction index</td>
<td>+42</td>
<td></td>
</tr>
<tr>
<td>7.5:7.5 W/EA</td>
<td>1340 (±50)</td>
<td>15.3 (±2.50)</td>
</tr>
<tr>
<td>Interaction index</td>
<td>+8.6</td>
<td></td>
</tr>
<tr>
<td>5:10 W/EA</td>
<td>1280 (±20)</td>
<td>14.1 (±2.20)</td>
</tr>
<tr>
<td>Interaction index</td>
<td>+3.4</td>
<td></td>
</tr>
<tr>
<td><strong>10% sucrose at 95°C</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10:5 W/EA</td>
<td>1220 (±60)</td>
<td>13.0 (±1.90)</td>
</tr>
<tr>
<td>Interaction index</td>
<td>+9.2</td>
<td></td>
</tr>
<tr>
<td>7.5:7.5 W/EA</td>
<td>1084 (±80)</td>
<td>10.0 (±1.50)</td>
</tr>
<tr>
<td>Interaction index</td>
<td>+4.8</td>
<td></td>
</tr>
<tr>
<td>5:10 W/EA</td>
<td>1034 (±50)</td>
<td>9.8 (±0.9)</td>
</tr>
<tr>
<td>Interaction index</td>
<td>+1.6</td>
<td></td>
</tr>
<tr>
<td><strong>20% sucrose at 95°C</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10:5 W/EA</td>
<td>1850 (±40)</td>
<td>19.0 (±1.90)</td>
</tr>
<tr>
<td>Interaction index</td>
<td>-9.5</td>
<td></td>
</tr>
<tr>
<td>7.5:7.5 W/EA</td>
<td>1435 (±70)</td>
<td>13.50 (±1.50)</td>
</tr>
<tr>
<td>Interaction index</td>
<td>-24.3</td>
<td></td>
</tr>
<tr>
<td>5:10 W/EA</td>
<td>1378 (±30)</td>
<td>12.9 (±0.9)</td>
</tr>
<tr>
<td>Interaction index</td>
<td>-21.2</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 6. Effects of sugars on rheological properties and molecular interactions of unheated, heated and high pressure treated whey and egg albumen protein

Table 6.4: Large deformation analysis of mixtures of whey (W) and egg albumen (EA) protein (15% w/w) high pressure treated (HP) (600 MPa for 20 mins) in 10-20% (w/w) sucrose

<table>
<thead>
<tr>
<th>Protein gel</th>
<th>Force (g) at 12mm compression</th>
<th>Young's modulus (Pa)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HP with 10% sucrose</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10:5 W/EA</td>
<td>871 (±70)</td>
<td>8.90 (±1.60)</td>
</tr>
<tr>
<td>Interaction index</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7.5:7.5 W/EA</td>
<td>647 (±90)</td>
<td>7.1 (±0.90)</td>
</tr>
<tr>
<td>Interaction index</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5:10 W/EA</td>
<td>466 (±80)</td>
<td>4.5 (±0.60)</td>
</tr>
<tr>
<td>Interaction index</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>HP with 20% sucrose</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10:5 W/EA</td>
<td>170 (±20)</td>
<td>2.9 (±0.40)</td>
</tr>
<tr>
<td>Interaction index</td>
<td>+262</td>
<td></td>
</tr>
<tr>
<td>7.5:7.5 W/EA</td>
<td>276 (±30)</td>
<td>4.9 (±0.7)</td>
</tr>
<tr>
<td>Interaction index</td>
<td>+54</td>
<td></td>
</tr>
<tr>
<td>5:10 W/EA</td>
<td>162 (±30)</td>
<td>2.6 (±0.30)</td>
</tr>
<tr>
<td>Interaction index</td>
<td>-19</td>
<td></td>
</tr>
</tbody>
</table>

Figures in parenthesis are standard deviation values based on five replicates.

Interaction Index = \( \frac{\text{Actual value} - \text{additive value}}{\text{Additive value}} \times 100 \)

* i.e. the value derived from summing the contributions of the component proteins measured in isolation, in proportion to the concentration of each in the mixture. (Howell and Lawrie, 1985).
In 40% sucrose solution and on heating at 90°C for 30 min, egg albumen protein gave gels with a high Young's modulus of 140 Pa (± 13) with a corresponding high force at maximum compression of 8789 (± 790) whereas whey proteins did not form a gel. In addition, gels were not formed with whey proteins even at 95°C with 40% sucrose. However, for whey proteins heated (with 40% sucrose) at 95°C for 1 hour, a gel was formed with a Young’s modulus of 11.7 Pa (± 0.80) and force at maximum compression of 824 (± 60).

In the presence of 10% sucrose (95°C for 30 min) whey proteins showed a significantly higher force at maximum compression at 1182 (± 30) compared with egg albumen at 986 (± 20); the Young’s modulus followed a similar trend at 13.0 (± 1.00) and 11.5 (± 0.40) for whey and egg albumen protein respectively (p<0.05). In the presence of 20% sucrose (95°C for 30 min) for egg albumen a significantly higher force at maximum compression was obtained 1248 (± 100) compared with whey at 1219 (± 90)(p<0.05).

These results indicate that sucrose has greater effect at high concentrations particularly on whey protein compared with egg albumen protein, with the absence of gelation in 40% sucrose solution both at 90°C and 95°C (for 30 min) for whey protein. Bryan and McClements (2000) have reported the varying of sugar concentration in terms of whether or not sugars retard or enhance the gelation of proteins using preferential exclusion data. In this approach, sugars were classified as additives (co-solvents) that increase the stability of the folded state and decrease solubility and are therefore excluded from the protein surface, as their concentration near the protein surface is lower than that in the bulk solvent. This generates an osmotic stress that favours a decrease in contact area between the proteins and the surrounding solution. The apparent negative binding of sugar causes the protein to be what has been termed preferential hydrated (Lee and Timasheff, 1981; Timasheff, 1998). The increased osmotic stress influenced both the thermal stability and aggregation of protein molecules, such that, increasing sucrose concentration stabilised the native globular protein which meant that the solutions had to be heated to higher temperature before protein molecules unfolded. This explains why in
Chapter 6. Effects of sugars on rheological properties and molecular interactions of unheated, heated and high pressure treated whey and egg albumen protein

This study, whey proteins did not gel at 90°C, but when the temperature was increased to 95°C (with 1 hr holding time) the increase in osmotic stress also meant that once the protein molecules did unfold they had a greater tendency to aggregate (because aggregates had a lower contact area than individual molecules), which accounts for gel formation and increased gel rigidity (Bryan and Mclements, 2000). These results indicated that in the presence of sugars the type of protein affects the gelation process and therefore egg albumen and whey protein both behaved in a different manner at high sugar concentrations despite the same levels of sucrose concentration and temperature conditions.

The increased stability of the protein in sugar solution has practical implications, depending on the use of the protein in food systems. Reported examples include the use of high sucrose levels in angel food cake batter such that the denaturation temperature for the main egg albumen protein, ovalbumin, coincides with the gelatinisation temperature of wheat starch in the batter (Donovan, 1977). The degree to which sugars influence gelation of proteins depends on whether they are reducing or non-reducing. Reducing sugars such as ribose or xylose have been reported to increase the gel strength of whey protein gels because of their ability to react with amine groups in proteins via Maillard reaction, leading to extensive cross-linking of proteins (Hill et al., 1992; Rich and Foegeding, 2000). However, other studies reported a decrease in gel strength (Boye et al., 1996; Soeda, 1997). It seems in the former studies proteins had sufficient time to unfold, while in the later they did not.

For the high pressure treated samples (Tables 6.1 and 6.2), in 10% sucrose solution, gelation did not occur for whey proteins while egg albumen formed a gel with a Young’s modulus of 3.6 Pa (± 0.30) and force at maximum compression of 502 (± 40). In 20% sucrose solution, egg albumen had a significantly higher Young’s modulus of 2.5 Pa (±0.30) with force at maximum compression of 220 (± 18) compared with 1.8 Pa (± 0.01) and 158 (± 8.6) for whey protein (p <0.05) respectively. Sugars have been found to alter the properties of gels formed by application of high pressure to solutions of globular proteins. Recently, studies have reported the existence of critical ranges of sugar
concentration for producing pressure-induced gels from dispersion of skimmed milk powder at micellar-casein concentrations of 2% w/v (Abbasi and Dickinson, 2001). In addition, high sugar concentrations (>50 wt%) inhibited pressure-induced gelation of casein micellar systems completely. Similarly, present results at high sucrose concentration (40 wt%) demonstrated the importance of minimum sugar-protein ratios for promoting protein gelation.

In the mixed gels containing 10% sucrose (Table 6.3 and 6.4) a high Young’s modulus value was obtained for the heated (90°C for 30min) samples for the 10:5 whey/egg albumen mixture, 10.7 Pa (±1.00) compared with 9.8 Pa (±1.50) and 7.9 Pa (±0.20) for 7.5:7.5 and 5:10 mixtures of whey and egg albumen proteins. The high Young’s modulus value of the 10:5 whey/egg albumen protein mixture was also reflected in the force at maximum compression, 1143 (±100) compared with 1015 (±50) for 7.5:7.5 and 980 (±20) for 5:10 whey and egg albumen mixtures (p<0.05). This finding indicated synergistic interactions of the two proteins and the corresponding calculated interaction index was highest for the 10:5 whey/egg albumen mixture. These results are consistent with our previous work on whey and egg protein interactions in the absence of sucrose (Ngarize et al., 2003), indicating that the presence of sucrose did not alter the interaction mechanism between whey and egg albumen proteins.

In the presence of 20 % sucrose, the highest Young’s modulus was also found for 10:5 whey/egg albumen protein mixture at 18.6 Pa (± 3.60) and a corresponding high force at maximum compression 1742 (±90) compared with 1340 (±50) and 1280 (±20) for the 7.5:7.5 whey/egg albumen mixture and 5:10 mixture of whey and egg albumen respectively. The interaction index showed a similar trend with the 10:5 whey/egg albumen mixture showing the highest reading at (+42). These results show that synergistic interactions occurred even in the presence of sucrose, suggesting that the sugars did not inhibit protein-protein interactions. Similar findings were observed by Howell and Lawrie (1985) in plasma and egg albumen protein gels in the presence of 45% sucrose, especially when heated at low temperature (85°C and 90°C for 30 min) for
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shorter periods of time (15 and 30 min) compared with 95°C for 60 min. In the present study a similar trend was observed in the whey/egg albumen protein mixtures in 10% sucrose solution heated at 95°C, however, in 20% sucrose solution at 95°C negative interaction indices were observed for all three mixtures, suggesting that at this high temperature whey and egg albumen proteins were not interacting.

In the high pressure treated samples, in the presence of 10% sucrose, force at maximum compression was highest for the 10:5 whey/egg albumen mixture at 871 (±70) with a corresponding Young's modulus of 12.0 Pa (±1.60), compared with 647 (±90) and 466 (±80) and Young's modulus of 7.1 Pa (±0.90) and 4.5 (±0.60) for the 7.5:7.5 and 5:10 mixture of whey and egg albumen protein respectively. The interaction index was not determined for these mixtures since, whey protein did not gel in 10% sucrose solution; the effect of sugar concentration has been discussed already in the above section. In 20% sucrose solution, the 7.5:7.5 mixture gave the highest Young's modulus at 4.9 Pa (±0.7) with a corresponding higher force at maximum compression of 276 (±30), compared with 170 (± 20) for 10:5 whey/egg albumen mixture and 162 (±30) for 5:10 whey/egg albumen mixture.

Surprisingly, the interaction index for the pressure-treated 10:5 whey/egg albumen mixture was highest at +262 compared with the heated mixture in 10% sucrose solution. The unfolding of the protein molecules and subsequent gelation appears to be lower for the pressure treated whey/egg albumen protein mixtures (400-700 MPa) compared with the heat-treated mixture (90°C for 30 min) (Ngarize et al 2003). Thus the conclusion arrived by Howell and Lawrie (1985) that interaction was greatest between proteins when they were only partially unfolded, as in milder heat treatments, would support the above finding. In this study, the mechanism of interaction has been elucidated by FT Raman spectroscopy. Although, Raman spectra were obtained for whey and egg albumen proteins as well as β-lactoglobulin and ovalbumin only the latter are reported as similar results were obtained for the former.
6.4.2. FT Raman spectroscopy

6.4.2.1. Comparison of unheated samples of ovalbumin and \( \beta \)-lactoglobulin in the presence of 20% sucrose

The FT Raman spectra for unheated samples of ovalbumin and \( \beta \)-lactoglobulin with 20% sucrose are shown in (Fig. 6.1, 6.2) and data for selected peaks are shown in Table 6.5. The location of the disulphide band at 525 cm\(^{-1}\) for unheated ovalbumin indicated the gauche-gauche-trans conformation around the single cystinyl disulphide bond. This has been reported by other workers Li-Chan and Qi (1998) and in previous study on whey and egg albumen proteins (Ngarize et al., 2003, chapter 3). Similarly, in \( \beta \)-lactoglobulin, S-S stretching bands were observed at 502 cm\(^{-1}\) (gauche-gauche-gauche) and the band at 518 cm\(^{-1}\); location of the disulphide bands around 500-510 has been reported Bellocq et al., (1972) and Ngarize et al., (2003). It appears that the presence of 20% sucrose did not affect this region in ovalbumin. The presence of sugars has been found to stabilise proteins. It has been suggested that sugars cause preferential hydration of the protein, as the solute is preferentially excluded from the surface environment of the protein. Lee and Timasheff (1981) and MacClements (2002) attributed the stabilisation of the protein to the increase in free energy required to form the cavity in the solvent accommodating the protein.

Major changes were observed in both ovalbumin and \( \beta \)-lactoglobulin, for the tyrosine doublet at 850/830 cm\(^{-1}\), the doublet was replaced by one broad peak at 830 cm\(^{-1}\) with the shifting of the band at 850 cm\(^{-1}\). The peak intensity for the 830 cm\(^{-1}\) band was 1.00 ± 0.01 and 1.57 ± 0.20 for ovalbumin and \( \beta \)-lactoglobulin respectively, these values are higher than those previously reported in a study of egg albumen and whey protein in the absence of sucrose (Ngarize et al., 2003, chapter 3). These changes in conformation of the proteins suggest that sucrose may modify protein conformation, via a mechanism that involves hydrogen bonding with tyrosine residues.
### Table 6.5: Normalised intensity values at selected regions of the Raman spectra of unheated (U) and 90°C heated (H) ovalbumin and β-lactoglobulin with 20% sucrose

<table>
<thead>
<tr>
<th>Peak Assignment</th>
<th>Normalised intensity*</th>
<th>Ovalbumin</th>
<th>β-lactoglobulin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>U</td>
<td>H</td>
</tr>
<tr>
<td>Cystine S-S</td>
<td>0.22±0.08</td>
<td>0.29±0.01</td>
<td>0.29±0.02</td>
</tr>
<tr>
<td></td>
<td>(498)</td>
<td>(496)</td>
<td>(502)</td>
</tr>
<tr>
<td></td>
<td>0.26±0.09</td>
<td>0.46±0.02</td>
<td>0.30±0.05</td>
</tr>
<tr>
<td></td>
<td>(523)</td>
<td>(514)</td>
<td>(518)</td>
</tr>
<tr>
<td>Trpophan</td>
<td>0.17±0.01</td>
<td>0.15±0.01</td>
<td>0.36±0.02</td>
</tr>
<tr>
<td></td>
<td>(762)</td>
<td>(755)</td>
<td>(759)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1.00±0.02</td>
<td>0.56±0.08</td>
<td>1.57±0.20</td>
</tr>
<tr>
<td></td>
<td>(830)</td>
<td>(827)</td>
<td>(828)</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>0.29±0.06</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>(850)</td>
<td>(851)</td>
<td>(850)</td>
</tr>
<tr>
<td>CH bend/Trp</td>
<td>0.24±0.01</td>
<td>0.37±0.02</td>
<td>0.74±0.07</td>
</tr>
<tr>
<td></td>
<td>(1339)</td>
<td>(1340)</td>
<td>(1346)</td>
</tr>
<tr>
<td>CH2</td>
<td>1.46±0.02</td>
<td>1.13±0.03</td>
<td>2.5±0.20</td>
</tr>
<tr>
<td></td>
<td>(1461)</td>
<td>(1457)</td>
<td>(1457)</td>
</tr>
<tr>
<td>COO-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>C-N/C-C stretch</td>
<td>0.22±0.02</td>
<td>0.33±0.03</td>
<td>0.66±0.08</td>
</tr>
<tr>
<td></td>
<td>(1050)</td>
<td>(1052)</td>
<td>(1055)</td>
</tr>
<tr>
<td>Amide 111'</td>
<td>0.28±0.01</td>
<td>0.29±0.02</td>
<td>0.27±0.04</td>
</tr>
<tr>
<td></td>
<td>(935)</td>
<td>(935)</td>
<td>(937)</td>
</tr>
<tr>
<td></td>
<td>0.25±0.01</td>
<td>0.30±0.02</td>
<td>0.41±0.07</td>
</tr>
<tr>
<td></td>
<td>(961)</td>
<td>(945)</td>
<td>(959)</td>
</tr>
<tr>
<td>ND</td>
<td>0.34±0.02</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>(972)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.06±0.01</td>
<td>0.48±0.02</td>
<td>0.27±0.01</td>
</tr>
<tr>
<td></td>
<td>(984)</td>
<td>(988)</td>
<td>(993)</td>
</tr>
</tbody>
</table>

* intensity at the wavenumber (cm\(^{-1}\)) shown in parenthesis, normalised to the intensity of the phenylalanine band at 1004 cm\(^{-1}\) for spectral data which were average of 128 scans and baseline corrected.
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Table 6.6: Normalised intensity values at selected regions of the Raman spectra of heated (H) at 90°C and high pressure (HP) treated ovalbumin and β-lactoglobulin with 20% sucrose

<table>
<thead>
<tr>
<th>Peak Assignment</th>
<th>Ovalbumin</th>
<th>β-lactoglobulin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H</td>
<td>HP</td>
</tr>
<tr>
<td>Cystine S-S</td>
<td>0.29±0.01</td>
<td>0.15±0.01</td>
</tr>
<tr>
<td></td>
<td>(496)</td>
<td>(496)</td>
</tr>
<tr>
<td>Trpophan</td>
<td>0.46±0.02</td>
<td>0.35±0.03</td>
</tr>
<tr>
<td></td>
<td>(514)</td>
<td>(522)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.15±0.01</td>
<td>0.15±0.01</td>
</tr>
<tr>
<td></td>
<td>(755)</td>
<td>(752)</td>
</tr>
<tr>
<td>Trpophan</td>
<td>0.56±0.08</td>
<td>0.76±0.02</td>
</tr>
<tr>
<td></td>
<td>(827)</td>
<td>(828)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.29±0.06</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>(851)</td>
<td></td>
</tr>
<tr>
<td>CH bend/Trp</td>
<td>0.37±0.02</td>
<td>0.43±0.01</td>
</tr>
<tr>
<td></td>
<td>(1340)</td>
<td>(1342)</td>
</tr>
<tr>
<td>CH₃</td>
<td>1.13±0.03</td>
<td>1.18±0.03</td>
</tr>
<tr>
<td></td>
<td>(1457)</td>
<td>(1461)</td>
</tr>
<tr>
<td>COO</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>C-N/C-C-stretch</td>
<td>0.33±0.03</td>
<td>0.31±0.02</td>
</tr>
<tr>
<td></td>
<td>(1052)</td>
<td>(1061)</td>
</tr>
<tr>
<td>Amide 111’</td>
<td>0.29±0.02</td>
<td>0.24±0.02</td>
</tr>
<tr>
<td></td>
<td>(935)</td>
<td>(936)</td>
</tr>
<tr>
<td></td>
<td>(945)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(972)</td>
<td>(964)</td>
</tr>
<tr>
<td></td>
<td>0.48±0.02</td>
<td>0.20±0.02</td>
</tr>
<tr>
<td></td>
<td>(988)</td>
<td>(985)</td>
</tr>
</tbody>
</table>

* intensity at the wavenumber (cm⁻¹) shown in parenthesis, normalised to the intensity of the phenylalanine band at 1004cm⁻¹ for spectral data which were average of 128 scans and baseline corrected.
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Figure 6.1: FT Raman spectra (400-1800 cm\(^{-1}\)) for unheated ovalbumin 15% (w/w) dissolved in D\(_2\)O with 20 wt % sucrose. The spectra were baselined and normalised to the phenylalanine peak at 1004 cm\(^{-1}\)

Figure 6.2: FT Raman spectra (400-1800 cm\(^{-1}\)) for unheated \(\beta\)-lactoglobulin 15% (w/w) dissolved in D\(_2\)O with 20 wt % sucrose. The spectra were baselined and normalised to the phenylalanine peak at 1004 cm\(^{-1}\)
Both proteins showed a marked increase in the intensity of the band at 1450-60 cm\(^{-1}\) assigned to the H-C-H methylene asymmetric bending or deformation of the CH\(_2\) and CH\(_3\), suggesting changes in the environment around aliphatic or hydrocarbon side chains after the addition of sucrose. The peak intensities were 1.46 ± 0.02 and 2.5 ± 0.20 for ovalbumin and β-lactoglobulin respectively (p<0.05). In addition, the 1336 cm\(^{-1}\) band assigned to the CH/Trp bend or CH\(_3\) symmetric vibrations was higher for β-lactoglobulin (0.74 ± 0.07) compared with ovalbumin (0.24 ± 0.02) (p<0.05), clearly indicating changes in hydrophobic interactions in the presence of sugars.

Sucrose promotes hydrophobic interactions through the modification of the water structure surrounding proteins (Philips et al., 1994; Dickinson and Merino, 2002). The stabilisation of protein structure by sugars has been attributed to the strengthening of hydrophobic interactions, mediated by the solvent water rather than by the direct interaction between the added compound and protein. Other workers (Hellman et al., 1983; MacClements, 2000) have attributed stabilisation of proteins by sucrose to increased surface tension in sucrose–water solutions resulting in preferential exclusion of sucrose from the protein domain, causing greater cohesive force in the highly structured sucrose-water matrix. Further changes were observed in aromatic amino acid residues of Trp at 760, 1555, 1585, 1180, 880 cm\(^{-1}\) and tyrosine residues at 1208-1210 cm\(^{-1}\), peak intensities were higher for β-lactoglobulin compared with ovalbumin, indicating increased hydrophobic interactions between β-lactoglobulin in the presence of sucrose, compared with ovalbumin.

The peak at 1420 cm\(^{-1}\) attributed to the C=O stretch of dissociated carboxyl groups of Asp or Glu and side chain vibrations of the imidazole ring of histidine (Howell and Li-Chan, 1996; Li-Chan and Qi, 1998) was absent in both unheated ovalbumin and β-lactoglobulin, suggesting that these residues were involved in protein-protein or protein-sucrose interaction. In the Amide III\(^{+}\) region no further changes were observed compared with the native samples without sucrose (Ngarize et al., 2003).
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6.4.2.2. Comparison of heated samples of ovalbumin and β-lactoglobulin in the presence of 20% sucrose

FT Raman spectra for ovalbumin and β-lactoglobulin in 20% sucrose solutions heated to form gels at 90°C for 30 min (pH 7), are shown in Fig. 6.3, 6.4 and data for selected peaks are shown in Table 6.6. Upon heating, there were changes in the secondary structure of both ovalbumin and β-lactoglobulin. Compared with unheated samples (0.06 for ovalbumin and 0.27 for β-lactoglobulin) there were bigger and pronounced peaks in the Amide III’ region at 988 cm⁻¹ and 985 cm⁻¹ suggestive of increased anti-parallel β-sheet, in both heated ovalbumin (0.48 ± 0.02) and β-lactoglobulin (0.81 ± 0.07); (p<0.05). These findings are in agreement with previous work on egg albumen and whey in the absence of sucrose (Ngarize et al., 2003, chapter 3).

In ovalbumin, the appearance of a new band at 972 cm⁻¹ attributed to the Amide III' random secondary structure, has been observed previously (Howell and Li-Chan, 1996). It appears that the denaturation of ovalbumin in the presence of sugars affects the secondary structure fractions. In contrast, no such band was observed in β-lactoglobulin, suggesting that the two proteins denature in a different way in the presence of sucrose. The solvent/solute conditions including sugars, have been reported to affect the protein conformation and secondary structure of proteins (Boye et al., 1996; Roddriguez-Nino et al., 1997).

Disulphide-sulphydryl interchange is a necessary pre-requisite for the formation of protein gels and has been known to accompany the heat gelation of most globular proteins (Mulvihill and Donovan, 1987; Howell, 1992). In this study, Raman spectroscopy allowed direct analysis of the disulphide region following heat treatment in the presence of sugars. Changes were observed in both ovalbumin and β-lactoglobulin in the disulphide region (500-540 cm⁻¹). For heated ovalbumin, there was a shift in the band at 523 cm⁻¹ (gauche-gauche-gauche) to 514 cm⁻¹ with a higher peak intensity 0.46 ± 0.02
compared with 0.26 ± 0.09 in the unheated sample (p<0.05). An additional disulphide peak was observed at 532 cm\(^{-1}\) (trans-gauche-trans) conformation in ovalbumin. For β-lactoglobulin bands were observed at 503 cm\(^{-1}\) (all gauche), 517 cm\(^{-1}\) (gauche-gauche-trans) and 532 cm\(^{-1}\) (trans-gauche-trans). Peak intensity was significantly higher for the disulphide band at 517 cm\(^{-1}\), 0.66 ±0.07 compared with 0.30 ±0.05 in the unheated sample (p<0.05). These bands have been reported in our previous study on egg albumen and whey proteins in the absence of sucrose (Ngarize et al., 2003) and by other workers (Shimada et al., 1989; Shimida and Cheftel, 1989).
Figure 6.3: FT Raman spectra (400-1800 cm$^{-1}$) for heated (90°C, 30 min) ovalbumin 15% (w/w) dissolved in D$_2$O with 20 wt % sucrose. The spectra were baselined and normalised to the phenylalanine peak at 1004 cm$^{-1}$.

Figure 6.4: FT Raman spectra (400-1800 cm$^{-1}$) for heated (90°C, 30 min) $\beta$-lactoglobulin 15% (w/w) dissolved in D$_2$O with 20 wt % sucrose. The spectra were baselined and normalised to the phenylalanine peak at 1004 cm$^{-1}$.
Both proteins showed a decrease in peak intensity in the band at 1450 cm\(^{-1}\) assigned to the H-C-H methylene asymmetric bending or deformation of the CH\(_2\) and CH\(_3\). The peak intensities were 1.13 ± 0.03 and 1.69 ± 0.13 compared with the unheated samples 1.46 ± 0.02 and 2.5 ± 0.20 \((p<0.05)\) for ovalbumin and \(\beta\)-lactoglobulin respectively. The decrease in peak intensity suggests that the protein had unfolded and protein-protein interactions were evident, despite the presence of sucrose, reflecting the gelation studies as described earlier. Protein unfolding is an integral part of the gelation process because it leads to the exposure of non-polar amino acids that were originally located in the interior of the globular protein molecule (Mulvihill and Kinsella, 1987, 1988; Howell, 1992). Upon exposure, these non-polar groups cause a strong hydrophobic attraction between neighbouring protein molecules which can lead to aggregation (Bryant and McClements, 1998, Ngarize et al., 2003). Further hydrophobic interactions were exhibited by the 1336 -1350 cm\(^{-1}\) band assigned to the CH/Trp bend or CH\(_3\) symmetric vibrations which showed increased peak intensity for ovalbumin from 0.24 ± 0.01 to 0.37 ± 0.02 and in contrast a decrease in peak intensity for \(\beta\)-lactoglobulin from 0.74 ± 0.07 to 0.58 ± 0.05 was observed.

There were significant changes in the C-C and C-N skeletal stretching vibrations which are typical of intermolecular sheet interactions (Lin-Vien et al., 1991). For \(\beta\)-lactoglobulin there was marked decrease in peak intensity from 0.66 ± 0.08 to 0.19 ± 0.01 \((p<0.05)\). This decrease in intermolecular interactions may be correlated with reduced gel strength in the rheological studies discussed above, due to the presence of sugars. Interestingly, for ovalbumin, a slight increase from 0.22 ± 0.02 to 0.33 ± 0.02 was observed (Table 6) \((p<0.05)\). Changes in tertiary structure were observed; this included the increased exposure of buried tryptophan residues in the heated gels as indicated by the decrease in intensity and sharpness of the band at 760 cm\(^{-1}\) for both ovalbumin and \(\beta\)-lactoglobulin.
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The involvement of tyrosine residues in gel formation was monitored by measuring the ratio of the intensity of the doublet 850/830 cm\(^{-1}\) which is a good indicator of hydrogen bonding and environment of the phenolic group. The tyrosine doublet ratio for both heated ovalbumin and β-lactoglobulin was 0.62 and 0.65 respectively. A decrease in the intensity of the tyrosine ratio 850/830 cm\(^{-1}\) suggests an increase in “buriedness” or involvement as strong hydrogen bond donors (Li-Chan and Nakai, 1994). This low tyrosine ratio observed in the heated samples supports the involvement of tyrosine residues in hydrogen bond-formation between intermolecular β-sheets acting as junction zones in stabilising the gel network structure (Wang and Damodaran, 1990; Howell and Li-Chan, 1996). In contrast to unheated samples, the peak at 850 cm\(^{-1}\) appeared as a shoulder and therefore the tyrosine ratio was not calculated.

6.4.2.3. Comparison of high pressure treated samples of ovalbumin and β-lactoglobulin in the presence of 20% sucrose

The FT Raman spectra of high pressure treated (600 MPa, 20 min) ovalbumin and β-lactoglobulin are shown in Fig. 6.5, 6.6 and Table 6.6. The involvement of the disulphide region 500-540 cm\(^{-1}\) following pressure treatment was observed in both proteins. The peak intensity for both ovalbumin and β-lactoglobulin was significantly less compared with the heated samples namely; 0.35 ± 0.03 at 522 cm\(^{-1}\) for ovalbumin and 0.29 ± 0.09 at 520 cm\(^{-1}\) for β-lactoglobulin in high pressure treated samples and for heat treated ovalbumin, 0.46 ± 0.02 and for heated-treated β-lactoglobulin 0.66 ± 0.07 (p<0.05). The decreased peak intensity observed in both proteins suggests a minor role of disulphide bonds during pressure-induced gelation which correlates with the weaker gel strength observed in high pressure treated whey and egg albumen proteins in this study. Studies by Iametti et al., (1997, 1998) have reported the involvement of disulphide interchange in the gelation of β-lactoglobulin and ovalbumin during pressure treatment. It appears that the addition of sucrose in our present study did not change the involvement of disulphide residues. Sucrose has been shown to act as a baroprotectant during pressure denaturation of proteins (Dumay et al., 1994).
Chapter 6. Effects of sugars on rheological properties and molecular interactions of unheated, heated and high pressure treated whey and egg albumen protein

Figure 6.5: FT Raman spectra (400-1800 cm\(^{-1}\)) for high pressure treated ovalbumin 15% (w/w) in D\(_2\)O with 20 wt % sucrose. The spectra were baselined and normalised to the phenylalanine peak at 1004 cm\(^{-1}\)

Figure 6.6: FT Raman spectra (400-1800 cm\(^{-1}\)) for high pressure treated β-lactoglobulin 15% (w/w) dissolved in D\(_2\)O with 20 wt % sucrose. The spectra were baselined and normalised to the phenylalanine peak at 1004 cm\(^{-1}\)
Both proteins displayed a significant increase in peak intensity in the 1450-1460 cm\(^{-1}\) band, 1.18 ±0.03 for ovalbumin and 2.08 ±0.15 for β-lactoglobulin compared with the heat treated samples (Table 6) (p<0.05); this suggests changes in the environment around aliphatic or hydrocarbon side chains during pressure-induced gelation. Similarly, there was also an increase in peak intensity in the 1340-1350 cm\(^{-1}\) band for the CH/Trp bend indicating increased hydrophobic interactions. Further changes were noted including increased peak intensities in the aromatic amino acid residues including Trp at 760, 1555 and 1585 cm\(^{-1}\) and Tyr residue at 1208-1210 cm\(^{-1}\); the latter band was broader in all samples with sucrose. The increased peak intensity suggests that these groups are more buried in the interior of the proteins, therefore high pressure treatment in the presence of sucrose is dominated by hydrophobic interactions. Previous workers have reported that hydrophobic interactions are enhanced by pressure (Mozhaev et al., 1995; Ledward, 2000), however, these studies were carried out in the absence of sucrose.

Amide III' bands of the Raman spectra indicated a significant decrease in the β-sheet structure of both ovalbumin (0.20 ± 0.02) and β-lactoglobulin (0.67 ± 0.04) compared with the higher values for heated samples above (p<0.05). These changes in the low levels of β-sheet structure were notable and are correlated with the weak gel strength in the high pressure treated samples. Involvement of β-sheet structure in heat-induced gelation was reported in a previous study on egg albumen and whey proteins (Ngarize et al., 2003, chapter 3) and by other workers (Howell and Li-Chan, 1996; Li-Chan, 1996). There were no significant changes in the amide III' α-helix region in these samples (p>0.05).
6.4.2.4. Comparison of unheated mixtures of ovalbumin and β-lactoglobulin in the presence of 20% sucrose

FT Raman spectra of mixtures of unheated ovalbumin and β-lactoglobulin in the presence of 20% sucrose (w/w) are shown in Fig. 6.7, 6.8 and Table 6.7. In addition, to the experimental spectra, the theoretical spectra (not shown) which would be expected in the absence of interactions were calculated as an average of the component spectra measured for the individual proteins. The difference between the theoretical average and actual experimental spectra are shown as difference spectra. The appearance of positive or negative peaks in the difference spectra indicates interactions between component proteins in the mixtures (Howell and Li-Chan, 1996). Quantitative data for the normalised intensity peaks in the experimental and calculated spectra are shown in Table 6.7.

The mixtures of ovalbumin and β-lactoglobulin in 20% sucrose solution formed a clear solution. For the unheated mixture of ovalbumin and β-lactoglobulin peaks were noted at 503 and 519 cm\(^{-1}\) attributed to the disulphide region and similarly at 503 and 520 cm\(^{-1}\) for the calculated spectra. Lower peak intensity was observed in the experimental spectra, compared with the calculated spectra, indicating changes in the environment surrounding disulphide region. Howell and Li-Chan (1996) and Ngarize et al., (2003) reported similar changes in their study on unheated β-lactoglobulin-lysozyme mixture and whey and egg albumen respectively.

Changes in the hydrophobic region were indicated by the more pronounced peak intensity in the calculated spectra compared with the experimental at 1450 cm\(^{-1}\) and 1340 cm\(^{-1}\). These results indicate changes in the environment affecting CH and CH\(_3\) bending vibrations of aliphatic residues in the mixtures. Further changes were also noted in the hydrophobic environment around aromatic residues of 758 cm\(^{-1}\) and 1555 cm\(^{-1}\) typical of Trp in residues in a less polar environment.
6.4.2.5. Comparison of heated and high pressure treated mixtures of ovalbumin and β-lactoglobulin in the presence of 20% sucrose

Spectra for unheated, heated and high pressure treated ovalbumin and β-lactoglobulin in the presence of 20% sucrose (w/w) are shown in Fig. 6.7, 6.8, 6.9 and data for selected peaks are shown in Table 6.7 and 6.8. Changes were observed in the disulphide region for both heated and high pressure treated ovalbumin and β-lactoglobulin samples. Higher peak intensities were noted for both heated experimental and calculated spectra for the disulphide region at 500-520 cm\(^{-1}\) compared with high pressure treated samples. Low disulphide peak intensities observed in high pressure treated samples correlate well with low gel strength of the mixtures observed in this study.

Both heating and high pressure treatment resulted in changes in the secondary structure as noted in the Amide III' band. For heated mixtures there was significantly increased β-sheet structure as shown by increased peak intensity 0.58 ± 0.02 for experimental and 0.82 ± 0.04 for calculated spectra at 988 cm\(^{-1}\) and 0.36 ± 0.04 and 0.37 ± 0.06 for the high pressure treated mixtures. These results suggest that heat treatment promoted increased formation of β-sheet structure compared with high pressure treatment, regardless of the presence of sucrose. The role of sucrose in stabilising proteins from both heat and pressure denaturation has been documented (Arakawa and Timasheff, 1982 and MacClements, 2002). Comparison of the experimental and calculated spectra of heated and high pressure treated samples showed changes in the α-helix region 936 cm\(^{-1}\) and 960 cm\(^{-1}\); the peak intensity values for heated proteins were 0.33 ± 0.04 and 0.39 ± 0.01 for the band at 936 cm\(^{-1}\) and 0.35 ± 0.03 and 0.52 ± 0.01 for experimental and calculated spectra respectively. For the high pressure treated samples, at 936 cm\(^{-1}\) and 960 cm\(^{-1}\) the peak intensities were 0.29 ± 0.02 and 0.35 ± 0.10 and 0.32 ± 0.01 and 0.49 ± 0.03 for experimental and calculated respectively. These results suggest both heating and high pressure treatment did not significantly alter the helical regions although heated mixtures show a slightly higher figure. However, the values for the experimental result were lower.
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Figure: 6.7. Experimental and difference FT Raman spectra (400-1800 cm\(^{-1}\)) for unheated ovalbumin and \(\beta\)-lactoglobulin mixture (7.5:7.5 w/w) dissolved in D\(_2\)O. Spectra were baselined and normalised to the phenylalanine peak at 1004 cm\(^{-1}\)

Figure: 6.8. Experimental and difference FT Raman spectra (400-1800 cm\(^{-1}\)) heated (90°C, 30min) ovalbumin and \(\beta\)-lactoglobulin mixture (7.5:7.5 w/w) dissolved in D\(_2\)O. Spectra were baselined and normalised to the phenylalanine peak at 1004 cm\(^{-1}\)
Figure: 6.9. Experimental and difference FT Raman spectra (400-1800 cm\(^{-1}\)) high pressure treated (600 MPa for 20min) ovalbumin and \(\beta\)-lactoglobulin mixture (7.5:7.5 w/w) dissolved in D\(_2\)O. Spectra were baselined and normalised to the phenylalanine peak at 1004 cm\(^{-1}\).
Chapter 6. Effects of sugars on rheological properties and molecular interactions of unheated, heated and high pressure treated whey and egg albumen protein

Table 6.7: Normalised intensity values at selected regions of the experimental (bold font) and calculated (italics) spectra of unheated (U) and 90°C heated (H) binary mixtures of ovalbumin and beta-lactoglobulin with 20% sucrose.

<table>
<thead>
<tr>
<th>Peak Assignment</th>
<th>Normalised Intensity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ovalbumin/β-lactoglobulin</td>
</tr>
<tr>
<td></td>
<td>U</td>
</tr>
<tr>
<td>Cysteine S-S</td>
<td>0.18±0.03</td>
</tr>
<tr>
<td></td>
<td>(503)</td>
</tr>
<tr>
<td></td>
<td>0.23±0.05</td>
</tr>
<tr>
<td></td>
<td>(519)</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.26±0.08</td>
</tr>
<tr>
<td></td>
<td>(760)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.96±0.05</td>
</tr>
<tr>
<td></td>
<td>(827)</td>
</tr>
<tr>
<td></td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>(849)</td>
</tr>
<tr>
<td>CH bend/Trp</td>
<td>0.45±0.06</td>
</tr>
<tr>
<td></td>
<td>(1346)</td>
</tr>
<tr>
<td>CH2 bend</td>
<td>1.62±0.02</td>
</tr>
<tr>
<td></td>
<td>(1460)</td>
</tr>
<tr>
<td>C-Nor C-Cstretch</td>
<td>0.21±0.07</td>
</tr>
<tr>
<td></td>
<td>(1056)</td>
</tr>
<tr>
<td>COO⁻</td>
<td>ND</td>
</tr>
<tr>
<td>Amide 111⁰</td>
<td>0.28±0.02</td>
</tr>
<tr>
<td></td>
<td>(935)</td>
</tr>
<tr>
<td></td>
<td>0.27±0.05</td>
</tr>
<tr>
<td></td>
<td>(958)</td>
</tr>
<tr>
<td></td>
<td>0.13±0.05</td>
</tr>
<tr>
<td></td>
<td>(991)</td>
</tr>
</tbody>
</table>

Intensity at the wavenumber (cm⁻¹) shown in parenthesis, normalised to the Phenylalanine band at 1006cm⁻¹ for spectral data which were the average of 128 scans and baseline corrected.
Chapter 6. Effects of sugars on rheological properties and molecular interactions of unheated, heated and high pressure treated whey and egg albumen protein

Table 6.8: Normalised intensity values at selected regions of the experimental (bold font) and calculated (italics) spectra heated (U) at 90°C and high pressure treated (HP) binary mixtures of ovalbumin and beta-lactoglobulin with 20% sucrose.

<table>
<thead>
<tr>
<th>Peak Assignment</th>
<th>Normalised Intensity*</th>
<th>ovalbumin/β-lactoglobulin</th>
<th>ovalbumin/β-lactoglobulin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H</td>
<td>HP</td>
<td>H</td>
</tr>
<tr>
<td>Cysteine S-S</td>
<td>0.27±0.01</td>
<td>0.17±0.03</td>
<td>0.27±0.01</td>
</tr>
<tr>
<td></td>
<td>(500)</td>
<td>(505)</td>
<td>(506)</td>
</tr>
<tr>
<td></td>
<td>0.31±0.07</td>
<td>0.24±0.03</td>
<td>0.63±0.01</td>
</tr>
<tr>
<td></td>
<td>(520)</td>
<td>(521)</td>
<td>(516)</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.22±0.04</td>
<td>0.24±0.05</td>
<td>0.24±0.01</td>
</tr>
<tr>
<td></td>
<td>(752)</td>
<td>(755)</td>
<td>(756)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.50±0.04</td>
<td>0.89±0.08</td>
<td>0.95±0.02</td>
</tr>
<tr>
<td></td>
<td>(827)</td>
<td>(828)</td>
<td>(828)</td>
</tr>
<tr>
<td></td>
<td>0.34±0.02</td>
<td>ND</td>
<td>0.61±0.01</td>
</tr>
<tr>
<td></td>
<td>(849)</td>
<td></td>
<td>(852)</td>
</tr>
<tr>
<td>CH bend/Trp</td>
<td>0.47±0.04</td>
<td>0.52±0.05</td>
<td>0.50±0.02</td>
</tr>
<tr>
<td></td>
<td>(1350)</td>
<td>(1344)</td>
<td>(1345)</td>
</tr>
<tr>
<td>CH$_2$ bend</td>
<td>1.31±0.08</td>
<td>1.58±0.02</td>
<td>1.76±0.07</td>
</tr>
<tr>
<td></td>
<td>(1459)</td>
<td>(1460)</td>
<td>(1456)</td>
</tr>
<tr>
<td>C-Nor C-Cstretch</td>
<td>0.12±0.02</td>
<td>0.30±0.02</td>
<td>0.16±0.01</td>
</tr>
<tr>
<td></td>
<td>(1044)</td>
<td>(1054)</td>
<td>(1055)</td>
</tr>
<tr>
<td>COO$^-$</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Amide 111'</td>
<td>0.33±0.04</td>
<td>0.29±0.02</td>
<td>0.39±0.01</td>
</tr>
<tr>
<td></td>
<td>(936)</td>
<td>(936)</td>
<td>(936)</td>
</tr>
<tr>
<td></td>
<td>0.35±0.03</td>
<td>0.32±0.02</td>
<td>0.52±0.01</td>
</tr>
<tr>
<td></td>
<td>(948)</td>
<td>(961)</td>
<td>(960)</td>
</tr>
<tr>
<td></td>
<td>0.58±0.02</td>
<td>0.36±0.04</td>
<td>0.82±0.04</td>
</tr>
<tr>
<td></td>
<td>(988)</td>
<td>(992)</td>
<td>(987)</td>
</tr>
</tbody>
</table>

Intensity at the wavenumber (cm$^{-1}$) shown in parenthesis, normalised to the Phenylalanine band at 1006cm$^{-1}$ for spectral data which were the average of 128 scans and baseline corrected.
Chapter 6. Effects of sugars on rheological properties and molecular interactions of unheated, heated and high pressure treated whey and egg albumen protein

than expected (Table 6.7 and 6.8), indicating increased changes in the secondary structure in protein mixtures.

The involvement of the tyrosine residues was different for the heated proteins compared with high pressure treated mixtures. The tyrosine doublet ratio 850/830 cm\(^{-1}\) was 0.68 and 0.64 for the heated experimental and calculated spectra respectively, suggesting that in the presence of sucrose the tyrosine residues are involved as hydrogen donors. The peak at 850 cm\(^{-1}\) appeared as a shoulder in the high pressure treated samples and therefore no tyrosine doublet ratio was calculated.

Higher peak intensities were noted in the experimental spectra at 1344 cm\(^{-1}\) (CH/Trp bend) and 1460 cm\(^{-1}\) (CH\(_2\) bend), Trp at 1180, Trp at 760 cm\(^{-1}\), and at 1555 cm\(^{-1}\) of high pressure treated mixtures compared with heat treated ones. These results suggest high pressure promoted hydrophobic interactions involving aromatic and aliphatic residues, previously exposed upon denaturation of ovalbumin and β-lactoglobulin during gel formation. Howell and Li-Chan (1996) reported similar findings in β-lactoglobulin-lysozyme precipitated complex.
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6.5 Conclusion

In this study, we have shown by large deformation rheology that synergistic interactions were evident in egg albumen and whey mixtures, in the presence of 10-20% sucrose solution and that at higher sucrose concentration up to 40%, gelation was inhibited, depending on the protein type. The synergistic interactions were more pronounced in heated samples than high pressure treated samples. High pressure treated whey protein samples in 10% sucrose solution did not gel and consequently no interaction index was calculated. FT Raman spectroscopy provided a powerful tool for monitoring changes in protein conformation in unheated, heated and high pressure processed proteins and their binary mixtures. Changes in disulphide bonds were more pronounced for heated samples compared with high pressure treated samples. Marked changes were apparent in the β-sheet structure for heated compared with high pressure treated samples suggesting less protein unfolding during pressure treatment which is therefore a milder form of treatment. In contrast, there was more pronounced involvement of hydrophobic interactions in high pressure treated samples than heated ones. The whey/egg albumen protein mixtures indicated negative interaction index suggesting no interaction between the protein in presence of sucrose except for the high pressure treated protein mixtures in 20% sucrose. Further changes in the mixtures were also noted for hydrophobic groups.
CHAPTER 7
Chapter 7. Effects of salt on the rheological properties and molecular interactions of unheated, heated and high pressure treated ovalbumin and β-lactoglobulin

7.1 Introduction

One of the important functional property of egg albumen and whey proteins is their ability to form heat induced and pressure induced gels capable of immobilizing large quantities of water and other food components (Comfort and Howell, 2003; Kuhn and Foegeding, 1991; Ledward and Galazka, 1998). Both heat and pressure induced gels can be used in the food industry for structure development and improvement. Gel structure is strongly influenced by physical conditions and the textural properties of gels depend on the type of protein, its concentration, pH, ionic environment, heating time and interactions with other ingredients (Howell and Lawrie, 1985; Shimada and Matsushita, 1980; Mastudomi et al., 1991; Langton and Hermansson, 1992; Foedeging et al., 1995; Verheul and Roefs, 1998).

Hydrogen, disulphide bonds and hydrophobic and electrostatic interactions stabilise proteins in aqueous solutions; these bonds also play a role in heat and pressure induced gelation (Howell, 1992). Manipulation of these forces by altering pH, ionic strength, gelling time and temperature produces gels with either fine stranded, mixed or particulate microstructures. In this study, the effect of salt on gelling properties will be investigated. Salt concentrations have been reported to produce maximum or minimum gel strength values according to the protein type, composition of protein, type and concentration of salt (Foegeding et al., 1995). Maximum gel hardness was obtained with 200 mM NaCl or 11.1 mM CaCl₂ and decreased with higher salt concentration for whey protein concentrates (Schmidt et al., 1978,1979). Mulvihill and Kinsella (1988) reported that maximum hardness values for β-lactoglobulin gels were obtained with 200 mM NaCl or 10 mM CaCl₂ and that CaCl₂ appeared to be more effective than NaCl in increasing gel strength, since, lower levels were required to produce similar gel hardness. Hegg et al., (1979) and Croguennec et al., (2002) reported similar findings for ovalbumin and egg white gels respectively in the presence of NaCl or CaCl₂. Overall, different gel structures can be obtained by varying ionic concentration and ion type within the same protein system.
Proteins are amphoteric molecules and therefore, carry both positive and negative charges. At low salt concentration, solubility of the protein usually increases slightly (salting in) and at high salt concentrations the solubility of the proteins drops sharply (salting out). The initial salting in at low salt concentrations is explained by the Debye-Huckel theory which proposes that proteins are surrounded by the salt counter ions (ions of opposite net charge to that of the protein). This screening results in decreasing electrostatic free energy of the protein and increasing activity of the solvent which in turn leads to increasing solubility (Tanford, 1961). The behaviour of proteins solutions at high salt concentrations was explained by Kirkwood (1943), the abundance of salt ions decreases the solvating power of the salt ions, thereby decreasing the solubility of the proteins and precipitations results.

The effect of salt on protein is related to the effect of the specific ion as well as ion type. Cations and anions have different effects on the orientation of water molecules which surround them. In the hydration shell of the cation, the hydrogen atoms of water are directed out, whereas in the anion they are directed inwards (Damodaran and Kinsella, 1982). This different orientation of charges causes anions to decrease the polarity of water to a greater extent than cations. The degree to which water structure is affected depends on the size and charge density of the ions. Both cations and anions tend to arrange themselves in a series known as the Hofmeister series, which represents the relative effectiveness of various anions to precipitate proteins, for example; for anions, the extent of structure breaking effect on water progressively follows the order \( \text{Cl}^- < \text{Br}^- < \text{NO}_3^- \) and with cations the series is as follows, \( \text{Li}^+ < \text{Na}^+ < \text{K}^+ \). Hofmeister classification also depends on whether the salt is kosmotropic (stabilizes water structure) or chaotropic (disrupts water structure).

Ions with largest ionic radius and lowest charge density have greatest ability to break the hydrogen bonded structure of water Schnepf (1992). Since hydrophobic interactions have an important role to play in the conformation of many proteins, the destabilizing effect of ions on water structure has an effect on protein conformation. In this respect salts, can
exert an influence on protein stability by direct interaction with groups involved in protein structure or by direct interaction with the aqueous phase (Somers, 1997).

Most studies have focused on the effect of salt concentration on protein gel texture and showed that transparent gels with fine stranded structure are formed when there is a large electrostatic repulsion between proteins (at low ionic strength < 0.1M), far from the isoelectric point of proteins. Under conditions of reduced electrostatic repulsion (at high ionic strength and close to the isoelectric point of the proteins) turbid, milk-white gels are formed with particulate structure (Clark et al., 1981; Shimada and Matsushita, 1981; Stading et al., 1993; Foegeding et al., 1995; Croguennec et al., 2002). Other studies have reported that divalent cations such as calcium chloride compared with monovalent sodium chloride, produced similar effects in gel strength for β-lactoglobulin and bovine serum albumin gels at low concentration (Hegg et al., 1979; Mulvihill and Kinsella, 1988; Kuhn and Foegeding, 1991). Recently, studies by Iametti et al., (1998) reported that when ovalbumin was high pressure treated (at 600 and 800 MPa) in the presence of 10% sodium chloride, structural modifications were reduced suggesting that sodium chloride increased both ionic strength and decreased water activity, thereby preventing protein denaturation. Although, many of these studies have focused on the effects of salt on gel strength and microstructure, no studies to date have used Raman spectroscopy to study effects of salts on protein conformation and interactions. This study will investigate the role of NaCl on ovalbumin, β-lactoglobulin, whey and egg albumen proteins and their mixtures using Raman spectroscopy and rheology.

Most food systems contain proteins, water and salts; thus the interactions of these components have many practical implications. Hardy and Steinberg (1984) reported that as salt concentration increased the amount of interacted salt also increased. Salt tended to partition itself between protein and water, at low water content, salt interacted with the protein and at high water content, it tended to bind water and interacted less with the protein. These interactions are important; for example, in cheese production, the binding of salts influence the growth of micro-organisms, which in turn, influence the hydrolysis...
Chapter 7. Effects of salt on the rheological properties and molecular interactions of unheated, heated and high pressure treated ovalbumin and β-lactoglobulin

of proteins during ripening. This hydrolysis determines many of the rheological and textural properties of the final product. In meat products, salts are used to alter solubility of meat proteins and play an important role in water holding capacity (Hamm, 1975; Comfort and Howell, 2003).
Chapter 7. Effects of salt on the rheological properties and molecular interactions of unheated, heated and high pressure treated ovalbumin and β-lactoglobulin

7.2 Materials
Materials used were the same as in section 2.1

7.3 Methods

7.3.1. FT Raman spectroscopy

7.3.1.1. Sample preparation

Solutions of individual whey and egg albumen proteins 15% (w/w) were prepared in D₂O. A mixture of 7.5% (w/w) whey protein and 7.5% (w/w) egg albumen protein, in D₂O was also prepared. 1% (w/w) NaCl was added to the above protein solutions. Heated samples of the above protein solutions were made as follows. Protein solution (7 ml) was poured into stainless steel tubes 50 mm long and 15 mm diameter and heated at 90°C for 30 minutes to form gels (Howell and Lawrie, 1984). The gels were stored overnight at 4°C. Unheated and heated samples were placed in NMR tubes (5 mm diameter, Precision grade, Aldrich Chemical Company Milwaukee USA) and analysed by FT-Raman spectroscopy. Similarly, solutions of ovalbumin and β-lactoglobulin protein 15% (w/w) were prepared in D₂O and mixtures of 7.5% (w/w) ovalbumin and 7.5% (w/w) β-lactoglobulin protein, in D₂O were also prepared in 1% NaCl solution for Raman spectroscopic studies only.

7.2.1.2. FT Raman spectroscopy, data collection and analysis

Raman spectra were recorded at 4°C on a Perkin Elmer 2000 FT-Raman spectrometer (Beaconsfield, Buckinghamshire, UK). Spectral resolution was set at 4 cm⁻¹, and laser power 1600 mW; the data presented were based on 128 co-added spectra. Frequency calibration of the instrument was undertaken using the sulphur line at 217 cm⁻¹. The spectra were analysed using Grams 32 software (Galactic Industries Corp, Salem NH).

The protein spectra obtained were baselined and the intensity was normalised using the phenylalanine peak at 1004 cm⁻¹ after subtraction of the sucrose spectra from the protein spectra (Tu 1986, Howell and Li-Chan 1996). The major bands in the spectra, related to
vibrational motions of various side chains or polypeptide backbone, were assigned by comparison with Raman spectra of proteins which have been reported in the literature (Howell and Li-Chan, 1996; Howell et al., 2001). The interaction of proteins was investigated by the analysis of the difference spectra calculated from the average of the normalised spectra of individual proteins minus the experimental spectra of the protein mixtures. For the difference spectra the intensity values are recorded on the y axis.

7.3.2. Large deformation testing

7.3.2.1. Heat treated samples

Egg albumen and whey protein solutions (15% w/w) in distilled water were poured into stainless steel tubes, 50 mm long and 15 mm in diameter and heated in a water bath at 90°C for 30 minutes to promote network formation (Howell and Lawrie, 1984). The above samples were made in 1% (w/w) NaCl solutions. The heated samples were stored at 4°C overnight to age. The gelled samples were cut into 15 mm long cylinders and were tested on TA-XT2 texture analyser (Stable Microsystems, Godalming, Surrey, UK). The force at maximum compression (12 mm) and the Young’s modulus (the gradient of the stress-strain curve) were measured. The test speed was set at 0.2 mm/sec, and the samples were compressed by a distance of 12 mm to a final height of 3 mm. The interaction between the proteins was calculated using the interaction index devised by (Howell and Lawrie, 1984; Ngarize et al., 2003) as follows:

\[
\text{Interaction index} = \frac{\text{Actual value} - \text{additive value}}{\text{Additive value}} \times 100
\]

* the value derived from summing the contributions of the component proteins measured in isolation, in proportion to the concentration of each in the mixture.
7.3.2.3. Pressure treated samples

The solutions for high pressure treated gels were prepared by the same method as heat treated gels except the samples were placed in visking tubing tied at both ends, heat sealed in a high pressure polythene bag (Crayovac W.R Grace Limited Cromwell Road, Cambridge, United Kingdom). The samples were introduced into the working chamber (300 ml capacity) of a Stansted Food Lab high pressure rig (Stansted Fluid power, Ltd, 70 Bentfield road, Stansted, CM24 8HT, Essex, UK.) containing a low compressibility fluid (2:8 mixture of castor oil:ethanol). The equipment was installed in an air conditioned laboratory at 20°C. The temperature in the pressurisation chamber increased during compression reaching a maximum after 2 min (the maximum temperature achieved in all treatments was 30°C, corresponding to the treatment at 400 MPa, 500 MPa, 600 MPa), then decreased to 20°C in 0.5 min and remained stable at 20°C until the end of the cycle. The compression and decompression times were about 1-2 min and 0.5 min respectively. The samples were stored at 4°C, similar to the heat-treated ones. For samples less than 10 ml, the Stansted Micro Food-Lab, Model S-FL-085-9W high pressure rig was used.
Chapter 7. Effects of salt on the rheological properties and molecular interactions of unheated, heated and high pressure treated ovalbumin and β-lactoglobulin

7.3 Results and discussion
7.4.1 Rheological studies

7.4.1.1. Large deformation testing
The addition of 1% sodium chloride to egg albumen and whey isolate proteins caused variable changes in the hardness and appearance for both heated and high pressure induced gels. The results for large deformation testing are shown in Table 7.1. Whey protein gels appeared opaque with a more brittle consistency when contrasted with transparent gels formed without salt. For egg albumen, the gels had a creamy white appearance similar to the unsalted gels in chapter 3 (Ngarize et al., 2003); these results are consistent with work by other researchers, which support the view that for egg white and ovalbumin, turbid gels are favoured under conditions close to the isoelectric point and high ionic strength, when electrostatic repulsive forces are minimised and random agglomerates of large aggregates are formed (Tani et al., 1995, Kitabake and Kinekawa, 1995). The difference in appearance for whey proteins has been shown to be due to an alteration from the more usual “string of beads” network structure of whey protein isolate gels into a more randomly aggregated structure (Langton and Hermansson, 1992; Barbut, 1995; Comfort and Howell, 2003). The force at maximum (12 mm) compression for the heated samples was highest in whey protein gels at 1448 (± 100) compared with 778 (± 30) for the egg albumen gels (p<0.05). The Young’s modulus followed a similar pattern at 20 Pa (± 1.3) and 10.0 Pa (± 0.40) for whey and egg albumen respectively (p<0.05).
Chapter 7. Effects of salt on the rheological properties and molecular interactions of unheated, heated and high pressure treated ovalbumin and β-lactoglobulin

Table 7.1: Large deformation analysis of egg albumen (EA) and whey protein (W) (15% w/w) and their mixtures (W/EA) heated at 90° C for 30 mins and high pressure treated at 600 MPa for 20 min in the presence of 1% NaCl

<table>
<thead>
<tr>
<th>Protein gel (15% w/w)</th>
<th>Force (g) at maximum compression</th>
<th>Young’s modulus (Pa)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Heated at 90° C</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egg albumen</td>
<td>778 (±30)</td>
<td>10.0 (±0.40)</td>
</tr>
<tr>
<td>Whey</td>
<td>1448 (±100)</td>
<td>20 (±1.80)</td>
</tr>
<tr>
<td>10:5 W/EA</td>
<td>1267 (±80)</td>
<td>16 (±0.50)</td>
</tr>
<tr>
<td>Interaction index</td>
<td>+ 4.0</td>
<td></td>
</tr>
<tr>
<td>7.5:7.5 W/EA</td>
<td>1090 (± 50)</td>
<td>13 (±1.00)</td>
</tr>
<tr>
<td>Interaction index</td>
<td>- 2.0</td>
<td></td>
</tr>
<tr>
<td>5:10 W/EA</td>
<td>993 (± 30)</td>
<td>11 (±2.00)</td>
</tr>
<tr>
<td>Interaction index</td>
<td>-1.3</td>
<td></td>
</tr>
<tr>
<td><strong>High pressure</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egg albumen</td>
<td>422 (±20)</td>
<td>4.2 (±0.2)</td>
</tr>
<tr>
<td>Whey</td>
<td>1350 (±40)</td>
<td>18 (± 1.20)</td>
</tr>
<tr>
<td>10:5 W/EA</td>
<td>560 (±15)</td>
<td>6.0 (± 0.50)</td>
</tr>
<tr>
<td>Interaction index</td>
<td>-46</td>
<td></td>
</tr>
<tr>
<td>7.5:7.5 W/EA</td>
<td>421 (±20)</td>
<td>4.0 (±0.4)</td>
</tr>
<tr>
<td>Interaction index</td>
<td>-42</td>
<td></td>
</tr>
<tr>
<td>5:10 W/EA</td>
<td>348 (±20)</td>
<td>3.0 (±0.4)</td>
</tr>
<tr>
<td>Interaction index</td>
<td>-60</td>
<td></td>
</tr>
</tbody>
</table>

Figures in parenthesis are standard deviation values based on five replicates. Interaction index = (Actual value-additive value)/additive value x100. The value derived from the contributions of the component proteins measured in isolation, in proportion to concentration of each in the mixture (Howell and Lawrie, 1984).
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In the mixed gels, the highest Young's modulus was obtained for the 10:5 whey/egg albumen protein mixture 16.0 Pa (± 0.50) compared with 13.0 Pa (± 1.00) and 11.0 Pa (± 2.00) for the 7.5:7.5 and 5:10 whey and egg albumen mixtures respectively. Similarly, the force at maximum compression was highest for the 10:5 whey/egg albumen mixture at 1267 (± 80) compared with 1090 (± 50) and 993 (± 30) for the 7.5:7.5 and 5:10 whey/egg albumen mixtures. Mixtures of whey and egg albumen particularly in the ratio 10:5 whey/egg albumen protein showed no synergistic interactions observed in our previous study in the absence of salt (Ngarize 2003). In this study in the presence of salt, the corresponding interaction index was highest in the 10:5 whey/egg albumen protein mixture at +4. In contrast, for the 7.5:7.5 and 5:10 whey/egg albumen mixtures a negative interaction index was obtained, -2.0 and -1.3 respectively. The low interaction index for the 10:5 whey/egg albumen protein mixture may be explained by the fact that the addition of 1% sodium chloride might have resulted in the aggregation of the individual proteins resulting in less interaction than expected, while the negative interaction index in the 7.5:7.5 and 5:10 whey/egg albumen protein mixture meant that the proteins might have aggregated completely thus reducing interactions. Overall, these results suggest that a certain mixture of proteins which results in optimum partial unfolding and interaction on heating or under pressure is required to achieve synergistic interactions. The transformation of one type of structure to another by the addition of salts or change in ionic strength is of importance to the food industry (Langton et al., 1996). Knowledge about the relationship between texture and ionic strength can be used to optimise food production processes as well as to develop new products with the desired sensory quality.

For the high pressure treated samples (600 MPa, 20 min), (Table 7.1) whey isolate protein had the highest force at maximum compression 1350 (± 40) compared with egg albumen protein at 422 (± 20) (P<0.05). The Young's modulus followed a similar pattern with a corresponding Young's modulus of 18 Pa (± 1.20) and 4.2 Pa (± 0.20) for whey and egg albumen respectively (p<0.05). In the mixtures, synergistic interaction was
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reported for the 10:5 whey/egg albumen mixture, with force at maximum compression at 560 (± 15) but values were significantly lower than for the heated sample (90°C, 30 min) (p<0.05). Negative interaction indices were observed in all the three mixtures. Sodium chloride has been reported to act as a baroprotectant during high pressure processing of ovalbumin by preventing protein denaturation or maximum unfolding of the protein (Iametti et al., 1998). This might explain both the low values of force at maximum compression and Young’s modulus values as well as negative interaction in high pressure treated samples. In order to elucidate the mechanism of gelation and protein interactions the gels were analysed by FT Raman spectroscopy described below. Results for egg albumen and whey isolate proteins were similar to the individual ovalbumin and β-lactoglobulin proteins; therefore only the latter are discussed.
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7.4.2. FT Raman spectroscopy

7.4.2.1. Comparison of unheated samples of ovalbumin and β-lactoglobulin protein (15% w/w) in 1% sodium chloride

The FT Raman spectra for unheated ovalbumin and β-lactoglobulin in 1% sodium chloride (pD 7.0) are shown in Fig. 7.1, 7.2 and data for selected peaks are shown in Table 7.2. There were some general similarities observed in the spectra of these two proteins. In unheated ovalbumin the band observed at 522 cm\(^{-1}\) has been assigned to disulphide bonds in the gauche-gauche -trans conformation by other workers who reported this band at 525 cm\(^{-1}\) (Li-Chan and Qi, 1998; Ngarize et al., 2003). It appears that the addition of 1% sodium chloride to ovalbumin shifted the band at 525 cm\(^{-1}\) to 522 cm\(^{-1}\) suggesting a change in conformation around this region. Similarly, for β-lactoglobulin bands were observed at 504 cm\(^{-1}\) and 515 cm\(^{-1}\) for the all gauche conformation, this is consistent with reports from other workers (Li-Chan et al., 1994) and results reported in chapter 3 (Ngarize et al., 2003). The addition of sodium chloride to both ovalbumin and β-lactoglobulin did not cause major changes in conformation in this region. NaCl is known to be a neutral Hofmeister salt (Somers, 1997) and appears to have had minimal influence on protein conformation in the native state of these proteins.

One striking feature observed in both ovalbumin and β-lactoglobulin in 1% sodium chloride was the broadening of the peak at 1207-1210 cm\(^{-1}\) attributed to the tyrosine residue (Li-Chan et al., 1994; Howell and Li-Chan, 1996). This band often plays an important role in hydrogen bond formation (Tu, 1986). There were also shoulder peaks at 1180 cm\(^{-1}\) and 1230-40 cm\(^{-1}\) attributed to the tryptophan residues and β-sheet respectively. In ovalbumin there was an additional shoulder peak at 1252 cm\(^{-1}\) assigned to the α-helical structures in globular proteins (Carew et al., 1975 and Bouraoui et al., 1997).
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Table 7.2: Normalised intensity values at selected regions of the Raman spectra of unheated (U) and heated at 90°C for 30 min (H) ovalbumin and β-lactoglobulin (15% w/w) in 1% NaCl solution

<table>
<thead>
<tr>
<th>Peak</th>
<th>Normalised intensity*</th>
<th>Ovalbumin</th>
<th>β-lactoglobulin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>U</td>
<td>H</td>
</tr>
<tr>
<td>Cystine S-S</td>
<td>0.14±0.01</td>
<td>0.34±0.01</td>
<td>0.13±0.01</td>
</tr>
<tr>
<td></td>
<td>(491)</td>
<td>(492)</td>
<td>(504)</td>
</tr>
<tr>
<td></td>
<td>0.11±0.01</td>
<td>0.26±0.02</td>
<td>0.12±0.01</td>
</tr>
<tr>
<td></td>
<td>(522)</td>
<td>(517)</td>
<td>(513)</td>
</tr>
<tr>
<td>Trpophan</td>
<td>0.17±0.01</td>
<td>0.14±0.01</td>
<td>0.25±0.02</td>
</tr>
<tr>
<td></td>
<td>(753)</td>
<td>(752)</td>
<td>(759)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.27±0.02</td>
<td>0.25±0.03</td>
<td>0.24±0.01</td>
</tr>
<tr>
<td></td>
<td>(829)</td>
<td>(831)</td>
<td>(828)</td>
</tr>
<tr>
<td></td>
<td>0.16±0.01</td>
<td>0.20±0.04</td>
<td>0.21±0.01</td>
</tr>
<tr>
<td></td>
<td>(855)</td>
<td>(849)</td>
<td>(853)</td>
</tr>
<tr>
<td>CH bend/Trp</td>
<td>0.29±0.01</td>
<td>0.32±0.02</td>
<td>0.38±0.01</td>
</tr>
<tr>
<td></td>
<td>(1345)</td>
<td>(1345)</td>
<td>(1342)</td>
</tr>
<tr>
<td>CH2</td>
<td>0.84±0.01</td>
<td>1.11±0.02</td>
<td>1.35±0.06</td>
</tr>
<tr>
<td></td>
<td>(1458)</td>
<td>(1451)</td>
<td>(1451)</td>
</tr>
<tr>
<td>COO-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>C-N/C-Cstretch</td>
<td>0.04±0.06</td>
<td>0.11±0.01</td>
<td>0.03±0.06</td>
</tr>
<tr>
<td></td>
<td>(1057)</td>
<td>(1047)</td>
<td>(1054)</td>
</tr>
<tr>
<td>Amide 111'</td>
<td>0.34±0.01</td>
<td>0.38±0.06</td>
<td>0.29±0.06</td>
</tr>
<tr>
<td></td>
<td>(940)</td>
<td>(946)</td>
<td>(935)</td>
</tr>
<tr>
<td></td>
<td>0.27±0.02</td>
<td>0.38±0.02</td>
<td>0.30±0.02</td>
</tr>
<tr>
<td></td>
<td>(957)</td>
<td>(965)</td>
<td>(958)</td>
</tr>
<tr>
<td></td>
<td>0.25±0.01</td>
<td>0.54±0.01</td>
<td>0.45±0.01</td>
</tr>
<tr>
<td></td>
<td>(991)</td>
<td>(984)</td>
<td>(990)</td>
</tr>
</tbody>
</table>

* intensity at the wavenumber (cm⁻¹) shown in parenthesis, normalised to the intensity of the phenylalanine band at 1004 cm⁻¹ for spectral data which were average of 128 scans and baseline corrected. ND means not determined because there was no peak.
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Figure 7.1: FT Raman spectra (400-1800 cm\(^{-1}\)) for unheated ovalbumin 15% (w/w) dissolved in D\(_2\)O with 1% NaCl. The spectra were baselined and normalised to the phenylalanine peak at 1004 cm\(^{-1}\).

Figure 7.2: FT Raman spectra (400-1800 cm\(^{-1}\)) for unheated β-lactoglobulin 15% (w/w) dissolved in D\(_2\)O with 1% NaCl. The spectra were baselined and normalised to the phenylalanine peak at 1004 cm\(^{-1}\).
However, there were some differences noted, for example a higher intensity was observed for the tryptophan bands at 753 cm\(^{-1}\), 1180 cm\(^{-1}\), and a well resolved peak at 1033 cm\(^{-1}\) (phenylalanine) in ovalbumin compared with β-lactoglobulin (p<0.05). These results indicate unfolding of ovalbumin molecule, accompanied by exposure of the formerly buried hydrophobic residues within the ovalbumin molecules compared with β-lactoglobulin. Further changes in hydrophobic interactions were observed in β-lactoglobulin, there were higher peak intensities for bands at 1450-1460 cm\(^{-1}\) assigned to the CH\(_2\) methylene asymmetric bending or deformations of CH\(_2\) and CH\(_3\) groups. In addition, increased peak intensity was noted in CH bending or CH\(_3\) (1350 cm\(^{-1}\)) symmetric vibrations and Trp vibrational modes for β-lactoglobulin compared with ovalbumin (p<0.05).

The high peak intensities observed in β-lactoglobulin suggest that the addition of salt enhanced hydrophobic interactions in both β-lactoglobulin and ovalbumin. Sodium and other salts have been reported to cause charge neutralization and enhanced protein-protein interactions leading to formation of more stable gel network structures (Damodaran and Kinsella, 1982). In contrast, preferential exclusion data on protein stability and solubility offers an alternative explanation; in the native state of the proteins, sodium chloride acts as an additive that tends to increase protein solubility and promote unfolding, and interacts preferentially with the protein surface, thus appearing to be bound (MacClements, 2002).

It appears, that in terms of protein stability in the unfolded state, proteins have a much greater surface area exposed to the solvent than do folded proteins. Consequently, an additive that interacts more favourably with the protein surface, especially those non-polar surfaces that tend to frequent the folded interior, than with the bulk solvent tend to be a denaturant whereas an additive excluded from the protein surface is a stabiliser (McClements, 2002). The addition of sodium chloride, at low concentration, to both ovalbumin and β-lactoglobulin protein solutions increased solubility of both proteins and
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subsequent unfolding and exposure of hydrophobic groups. Arakawa and Timasheff (1982) established that the extent to which salts are excluded from the protein surface correlates well with the Hofmeister series.

It is well established from previous studies that the bands located near 830 cm\(^{-1}\) and 850 cm\(^{-1}\) can be useful for monitoring the micro-environment around tyrosine residues (Li-Chan \textit{et al.}, 1994; Howell and Li-Chan, 1996; Ngarize \textit{et al.}, 2003). The tyrosine doublet is assigned to vibrations of the para-substituted benzene ring of tyrosine residues, which are affected, by the environment and involvement of the phenolic hydroxyl group in hydrogen bonding. In the case of tyrosine residues which are exposed to the aqueous or polar environment or which act as simultaneous acceptors and donor of moderate to weak hydrogen bonds, the tyrosine ratio value of the doublet bands (850/830) usually ranges from 0.90–1.45 and can be as high as 2.5. On the other hand, tyrosine ratio values for tyrosine residues which are buried in a hydrophobic environment and which tend to act as hydrogen donors usually range between 0.7 and 1.0 and can be as low as 0.3 in the case of extremely strong hydrogen bonding to a negative acceptor (Tu, 1986, Bouraoui \textit{et al.}, 1997).

The tyrosine doublet ratio values for unheated ovalbumin and β-lactoglobulin were 0.59 and 0.89 respectively, suggesting that the tyrosine residues were buried in a more hydrophobic environment and were involved as strong hydrogen donors after the addition of sodium chloride. The addition of salts has been reported to cause changes in protein conformation by other workers (Caille \textit{et al.}, 1983; Przybycien and Bailey, 1989; Bouraoui \textit{et al.}, 1997).

There were differences in the Amide III' region at 980-990 cm\(^{-1}\) (anti-parallel β-sheet); a higher peak intensity was observed for β-lactoglobulin compared with ovalbumin (p<0.05). Bands at 930-960 cm\(^{-1}\) typical of helical structures, had a lower peak intensity for β-lactoglobulin compared with ovalbumin, suggesting a dominance of more helical structures in ovalbumin. These findings are in agreement those reported in chapter 3 on
whey and egg albumen which indicated that secondary structure of $\beta$-lactoglobulin is dominated by $\beta$-sheet structures while ovalbumin is a more helical protein (Ngarize et al., Susi and Byler, 1988; Howell and Li-Chan, 1996). It appears that salt did not induce changes in conformation in this region since most of the bands are similar to those reported on whey and egg albumen protein in the absence of salt in chapter 3 (Ngarize et al., 2003).

There were no significant differences in the band near 1050-1080 cm$^{-1}$ assigned to C-C C-N skeletal stretching vibrations typical of intermolecular sheet interactions (Parker, 1983) for both ovalbumin and $\beta$-lactoglobulin. A peak at 1410 cm$^{-1}$ (assigned to side chain vibrations of the imidazole ring of His or the C=O stretch of Asp or Glu carboxylate groups) was not observed for either protein. This may suggest a change in ionisation of the imidazole ring group.
7.4.2.2. Comparison of heated samples of ovalbumin and β-lactoglobulin in 1% sodium chloride

FT Raman spectra for heated ovalbumin and β-lactoglobulin in 1% sodium chloride (pD 7.0) are shown in Fig. 7.3, 7.4 and data for selected peaks are shown in Table 7.3. Raman spectra of heated ovalbumin and β-lactoglobulin exhibited similar increase in peak intensity of the disulphide region, around 500-520 cm$^{-1}$, which is indicative of the all gauche conformation. Further changes were observed for the gauche–gauche trans conformation at 530-540 cm$^{-1}$, the increased intensity of these bands after heating may be attributed to formation of disulphide bonds during gel strengthening at high temperatures. This is consistent with previous reports (Li-Chan, 1994; Howell and Li-Chan, 1996 and Ngarize et al., 2003). However, some reports have attributed the Raman band at 530 cm$^{-1}$ to vibrations such as C-C-C deformation of rc-alkyl groups (Li-Vien et al., 1991).

Amide III' bands at 980-990 cm$^{-1}$ indicated significant increase in β-sheet structures for both proteins gels, 0.54 ± 0.01 for ovalbumin and 0.89 ±0.01 for β-lactoglobulin after heating, compared with 0.25 ± 0.01 and 0.45 ± 0.01 for unheated ovalbumin and β-lactoglobulin respectively (p< 0.05). However, there was no significant decrease in α-helical structures at 930-960 cm$^{-1}$ (p> 0.05) for either protein; this is in contrast to previous findings reported in chapter 3 (Ngarize et al., 2003) where there was a decrease in α-helical structures at 930-960 cm$^{-1}$ following heating. Increase in β-sheet structures is a common feature of heated globular proteins and plays a significant role in gelation (Wang and Damodaran, 1991; Howell and Li-Chan, 1996). The addition of 1% sodium chloride did not appear to have changed these bands substantially. Both proteins showed a significant increase in band intensity at 1450-1460 cm$^{-1}$ assigned to the CH$_2$ methylene asymmetric bending of deformations of CH$_2$ and CH$_3$ groups compared with the unheated samples (p<0.05), suggesting changes in the environment around the aliphatic or hydrocarbon side chains after heating. In addition, the band (1350 cm$^{-1}$) attributed to CH bending or CH$_3$ symmetric vibrations and Trp vibrational modes showed a marked increase in ovalbumin with no significant change for β-lactoglobulin, indicating further involvement of aliphatic residues in hydrophobic interactions.
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Table 7.3: Normalised intensity values at selected regions of the Raman spectra of heated at 90°C for 30 min (H) and high pressure treated (HP) (600 MPa for 20 mins) ovalbumin and β-lactoglobulin (15% w/w) in 1% NaCl solution

<table>
<thead>
<tr>
<th>Peak Assignment</th>
<th>Normalised intensity*</th>
<th>Ovalbumin</th>
<th>β-lactoglobulin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H</td>
<td>HP</td>
<td>H</td>
</tr>
<tr>
<td>Cystine S-S</td>
<td>0.26±0.01 (517)</td>
<td>0.11±0.02 (515)</td>
<td>0.20±0.02 (504)</td>
</tr>
<tr>
<td>Trpophan</td>
<td>0.14±0.01 (831)</td>
<td>0.13±0.02 (828)</td>
<td>0.23±0.01 (757)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.25±0.03 (849)</td>
<td>0.19±0.04 (854)</td>
<td>0.34±0.02 (827)</td>
</tr>
<tr>
<td>CH bend/Trp</td>
<td>0.32±0.02 (1345)</td>
<td>0.29±0.01 (1341)</td>
<td>0.39±0.03 (1343)</td>
</tr>
<tr>
<td>CH₂</td>
<td>1.11±0.02 (1451)</td>
<td>1.11±0.01 (1450)</td>
<td>1.49±0.05 (1453)</td>
</tr>
<tr>
<td>COO-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>C-N/C-Cstetch</td>
<td>0.11±0.01 (1047)</td>
<td>0.10±0.06 (1050)</td>
<td>0.06±0.02 (1053)</td>
</tr>
<tr>
<td>Amide 111’</td>
<td>0.38±0.06 (946)</td>
<td>0.40±0.06 (939)</td>
<td>0.45±0.02 (936)</td>
</tr>
<tr>
<td></td>
<td>0.38±0.02 (965)</td>
<td>0.39±0.06 (960)</td>
<td>0.55±0.04 (956)</td>
</tr>
<tr>
<td></td>
<td>0.54±0.01 (984)</td>
<td>0.37±0.01 (987)</td>
<td>0.85±0.01 (986)</td>
</tr>
</tbody>
</table>

* intensity at the wavenumber (cm⁻¹) shown in parenthesis, normalised to the intensity of the phenylalanine band at 100 4 cm⁻¹ for spectral data which were average of 128 scans and baseline corrected. ND means not determined because there was no peak.
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Figure 7.3: FT Raman spectra (400-1800 cm\(^{-1}\)) for heated (90°C, 30min) ovalbumin 15% (w/w) dissolved in D\(_2\)O with 1% NaCl. The spectra were baselined and normalised to the phenylalanine peak at 1004 cm\(^{-1}\).

Figure 7.4: FT Raman spectra (400-1800 cm\(^{-1}\)) for heated (90°C, 30min) β-lactoglobulin 15% (w/w) dissolved in D\(_2\)O with 1% NaCl. The spectra were baselined and normalised to the phenylalanine peak at 1004 cm\(^{-1}\).
Chapter 7. Effects of salt on the rheological properties and molecular interactions of unheated, heated and high pressure treated ovalbumin and β-lactoglobulin

The involvement of tyrosine residues in the presence of 1% sodium chloride during gel formation exhibited marked differences for unheated and heated samples of ovalbumin and β-lactoglobulin. The tyrosine doublet ratio 850/830 cm\(^{-1}\) for ovalbumin increased from 0.59 in the unheated sample to 0.80 (p< 0.05) for the heated sample whereas for β-lactoglobulin there was no significant difference, a slight decrease from 0.89 to 0.80 was observed. Despite the increase in intensity for ovalbumin the tyrosine doublet ratio remained less than 1 for both ovalbumin and β-lactoglobulin suggesting buriedness or participation of tyrosine phenolic groups as hydrogen bond donors.

Further changes were observed in the hydrophobic groups, the broadening of the peak at 1207-1210 cm\(^{-1}\) attributed to the tyrosine residue, for both proteins with β-lactoglobulin showing one shoulder peak at 1173 cm\(^{-1}\) for the tryptophan residue and ovalbumin showing two shoulder peaks at 1176 cm\(^{-1}\) and 1223 cm\(^{-1}\), the peak intensities for these groups were significantly lower compared with the unheated samples (p<0.05). In addition, peak intensities of tryptophan residue at 760 cm\(^{-1}\) decreased for both heated and high pressure treated ovalbumin and β-lactoglobulin, suggesting increased exposure of tryptophan residues and changes in the tertiary structure of proteins in the absence of salt (Li-Chan and Qi 1998).

7.4.2.3. Comparison of high pressure treated (600 MPa for 20 min) samples of ovalbumin and β-lactoglobulin in 1% sodium chloride
FT Raman spectra of high pressure treated ovalbumin and β-lactoglobulin are shown in Fig. 7.5, 7.6 and Table 7.3. Similar changes in the conformation of the disulphide region were indicated by a significant decrease in band intensity of the high pressure treated ovalbumin and β-lactoglobulin at around 500-520 cm\(^{-1}\), compared with the heated samples, namely 0.11 ± 0.02 for high pressure treated ovalbumin and 0.14 ± 0.01 for high pressure treated β-lactoglobulin compared with 0.26 ± 0.01 and 0.20 ± 0.02 for heated ovalbumin and β-lactoglobulin respectively. Previous results in chapter 3 on whey and egg albumen in the absence of salt showed a similar decrease in disulphide band intensity.
in high pressure treated samples compared with heated samples (Ngarize *et al.*, 2003). Other workers reported similar involvement of disulphide bonds during heat and pressure induced gelation (Li-Chan, 1996 Iametti *et al.*, 1997, 1998). It appears that the presence of 1% sodium chloride did not cause notable changes in the conformation of the disulphide bonds.

Notable changes were observed in the Amide III’ band at 980-990 cm\(^{-1}\) (β-sheet structure region); Amide III’ results primarily from the C-N stretching and N-H in plane bending vibrations of the peptide bond (Li –Chan, 1996). There was a significant decrease in the β-sheet structure at 980-990 cm\(^{-1}\) for both proteins in the high pressure treated samples, 0.37 ± 0.01 for ovalbumin and 0.74 ± 0.01 for β-lactoglobulin compared with heated samples 0.54 ± 0.01 for ovalbumin and 0.85 ± 0.01 for β-lactoglobulin (p<0.05). The low levels of β-sheet structure is a common feature in all the high pressure treated samples in all the studies regardless of the addition of sugars and salts. No significant changes were observed for both high pressure treated proteins in the 900-960 cm\(^{-1}\) Amide III’ region representing the α-helical structures.
Chapter 7. Effects of salt on the rheological properties and molecular interactions of unheated, heated and high pressure treated ovalbumin and β-lactoglobulin

Figure 7.5: FT Raman spectra (400-1800 cm\(^{-1}\)) for high pressure treated ovalbumin 15\% (w/w) dissolved in D\(_2\)O with 1\% NaCl. The spectra were baselined and normalised to the phenylalanine peak at 1004 cm\(^{-1}\).

Figure 7.6: FT Raman spectra (400-1800 cm\(^{-1}\)) for high pressure treated β-lactoglobulin 15\% (w/w) dissolved in D\(_2\)O with 1\% NaCl. The spectra were baselined and normalised to the phenylalanine peak at 1004 cm\(^{-1}\).
Bands at 1450-1460 cm\(^{-1}\) exhibited differences in the two proteins. For ovalbumin no difference peak intensity was observed between the heated and high pressure treated samples both had bands at 1.11 ± 0.01 suggesting minor change in conformation during pressure treatment around these residues. However, for β-lactoglobulin a higher peak intensity of 1.88 ± 0.01 was observed for the high pressure treated sample compared with 1.49 ± 0.05 for the heated sample (p<0.05). This difference in behaviour has been attributed to effects of ionic strength on β-lactoglobulin. Previous workers have reported that an increase in ionic strength due to addition of salts, masks the charged groups that have become newly accessible through heat heat-induced conformational rearrangements, thus enhancing hydrophobic groups (Sawyer and Puckeridge, 1973; Thompson and Brower, 1988).

The tyrosine doublet ratio 850/830 cm\(^{-1}\) for high pressure treated ovalbumin and β-lactoglobulin was 0.70 and 0.67 respectively. These results suggests an increase in buriedness or involvement of tyrosine residues as strong hydrogen bond donors! No peaks were observed for the band at 1420 cm\(^{-1}\) typical for COO\(^{-}\) (of Asp and Glu amino acids); thus similar results were obtained for both unheated and heated in the above sections.
Chapter 7. Effects of salt on the rheological properties and molecular interactions of unheated, heated and high pressure treated ovalbumin and β-lactoglobulin

7.4.2.4. Comparison of unheated mixtures of ovalbumin and β-lactoglobulin in 1% sodium chloride

FT Raman spectra for unheated mixtures of ovalbumin and β-lactoglobulin are shown in Fig. 7.7 and Table 7.4. The binary mixtures of the two proteins formed pale milky white solution. Differences between the two proteins are clearly demonstrated by the difference between calculated and experimental spectra. Both unheated mixtures of ovalbumin and β-lactoglobulin had slightly lower intensity values of disulphide bands at 500-520 cm\(^{-1}\) in the experimental spectrum (0.13 ± 0.01) than the calculated spectrum (0.14 ± 0.02). These results suggest that disulphide bonds were altered by interactions between the two proteins. Howell and Li-Chan (1996) observed similar findings in unheated mixtures of α-lactalbumin and β-lactoglobulin.

The intensity of 1450-1460 cm\(^{-1}\) band assigned to the CH\(_2\) methylene asymmetric bending of deformations of CH\(_2\) and CH\(_3\) and for the CH bend /trp at 1350 cm\(^{-1}\) were higher for the calculated spectra compared with the experimental spectra. These results suggest less involvement of the aliphatic residues in hydrophobic interactions in these mixtures than expected in the presence of salt. These peaks showed a marked prominence in the difference spectra. Unheated solutions of the mixture showed a much lower tyrosine doublet ratio 850/830 cm\(^{-1}\) in the experimental spectrum (0.69 ± 0.01) than the calculated spectrum (0.86 ± 0.02) (p<0.05), suggesting either increasing buriedness or involvement of tyrosine residues as strong hydrogen bond donors in the mixture. Howell and Li-Chan (1996) observed similar findings on protein mixtures of α-lactalbumin, β-lactoglobulin, and lysozyme. Further changes were also noted in the aromatic residues at 757 cm\(^{-1}\) and 1555 cm\(^{-1}\) indicating involvement of hydrophobic interactions. Changes were also observed between experimental and calculated values for Raman bands assigned to α-helical (900-960 cm\(^{-1}\)) and β-sheet cm\(^{-1}\) (980-990 cm\(^{-1}\)) structures. The β-sheet cm\(^{-1}\) content was higher for the calculated spectra than the experimental spectra suggesting involvement of these structures while the helical region showed little change.
Chapter 7. Effects of salt on the rheological properties and molecular interactions of unheated, heated and high pressure treated ovalbumin and β-lactoglobulin

Table 7.4: Normalised intensity values at selected regions of the experimental (bold font) and calculated (italics) spectra of unheated (U) and heated at 90°C for 30 min (H) binary mixtures of ovalbumin and beta-lactoglobulin (15% w/w) in 1% NaCl

<table>
<thead>
<tr>
<th>Peak</th>
<th>Assignment</th>
<th>Normalised Intensity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>U (cm⁻¹)</td>
</tr>
<tr>
<td>Cysteine S-S</td>
<td>0.013±0.01</td>
<td>0.22±0.02</td>
</tr>
<tr>
<td></td>
<td>(502)</td>
<td>(504)</td>
</tr>
<tr>
<td></td>
<td>0.11±0.01</td>
<td>0.15±0.01</td>
</tr>
<tr>
<td></td>
<td>(515)</td>
<td>(516)</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.13±0.01</td>
<td>0.15±0.02</td>
</tr>
<tr>
<td></td>
<td>(760)</td>
<td>(759)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.27±0.03</td>
<td>0.28±0.03</td>
</tr>
<tr>
<td></td>
<td>(828)</td>
<td>(828)</td>
</tr>
<tr>
<td></td>
<td>0.19±0.02</td>
<td>0.21±0.02</td>
</tr>
<tr>
<td></td>
<td>(851)</td>
<td>(852)</td>
</tr>
<tr>
<td>CH bend/Trp</td>
<td>0.37±0.02</td>
<td>0.29±0.03</td>
</tr>
<tr>
<td></td>
<td>(1342)</td>
<td>(1341)</td>
</tr>
<tr>
<td>CH₂ bend</td>
<td>1.13±0.03</td>
<td>1.19±0.01</td>
</tr>
<tr>
<td></td>
<td>(1450)</td>
<td>(1450)</td>
</tr>
<tr>
<td>C-Nor C-Cstretch</td>
<td>0.01±0.01</td>
<td>0.02±0.01</td>
</tr>
<tr>
<td></td>
<td>(1047)</td>
<td>(1052)</td>
</tr>
<tr>
<td>COO'</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Amide 111'</td>
<td>0.36±0.04</td>
<td>0.38±0.02</td>
</tr>
<tr>
<td></td>
<td>(937)</td>
<td>(939)</td>
</tr>
<tr>
<td></td>
<td>0.37±0.02</td>
<td>0.41±0.02</td>
</tr>
<tr>
<td></td>
<td>(959)</td>
<td>(961)</td>
</tr>
<tr>
<td></td>
<td>0.22±0.02</td>
<td>0.64±0.02</td>
</tr>
<tr>
<td></td>
<td>(982)</td>
<td>(985)</td>
</tr>
</tbody>
</table>

Intensity at the wavenumber (cm⁻¹) shown in parenthesis, normalised to the Phenylalanine band at 1006cm⁻¹ for spectral data which were the average of 128 scans and baseline corrected. ND means not determined because there was no peak.
Chapter 7. Effects of salt on the rheological properties and molecular interactions of unheated, heated and high pressure treated ovalbumin and β-lactoglobulin

Figure: 7.7. Experimental and difference FT Raman spectra (400-1800 cm\(^{-1}\)) for unheated ovalbumin and β-lactoglobulin mixture (7.5:7.5 w/w) dissolved in D\(_2\)O with 1 % NaCl. Spectra were baselined and normalised to the phenylalanine peak at 1004 cm\(^{-1}\)

Figure: 7.8. Experimental and difference FT Raman spectra (400-1800 cm\(^{-1}\)) for heated (90°C, 30min) ovalbumin and β-lactoglobulin mixture (7.5:7.5 w/w) dissolved in D\(_2\)O with 1 % NaCl. Spectra were baselined and normalised to the phenylalanine peak at 1004 cm\(^{-1}\)
7.4.2.5. Comparison of heated mixtures of ovalbumin and β-lactoglobulin in 1% sodium chloride

FT Raman spectra for heated mixtures of ovalbumin and β-lactoglobulin are shown in Fig. 7.8 and Table 7.5. Participation of the disulphide bonds at 500-520 cm\(^{-1}\) was evident, a higher intensity was noted in the experimental spectra at 0.22 ± 0.02 compared with 0.18 ± 0.02 in the calculated spectra (p<0.05). These results suggest disulphide bonds are significantly altered by interactions between the two proteins following heating. The involvement of disulphide bonds in heat gelation has been previously documented (Howell and Lawrie, 1985; Shimada and Cheftel, 1988).

An increased exposure of aromatic residues suggesting involvement of hydrophobic interactions was observed in the heated mixture of ovalbumin and β-lactoglobulin. The bands assigned to tryptophan at 759 cm\(^{-1}\), 1180 cm\(^{-1}\) and 1555 cm\(^{-1}\), 1580 cm\(^{-1}\) were less intense in the experimental than in the calculated average spectrum indicating more exposed Trp residues in the heated samples. Interactions involving the aliphatic hydrophobic groups were indicated by lower values of CH\(_2\) methylene asymmetric bending of deformations of CH\(_2\) and CH\(_3\) groups and for the CH bend/Trp at 1350 cm\(^{-1}\) band in the experimental compared with the calculated. On the whole interactions for hydrophobic residues were lower than expected in the mixture in the presence of salt which supports the weaker gels obtained in rheological studies.

The experimentally observed values for heated mixtures showed a lower tyrosine doublet ratio 850/830 of 0.76 compared with the calculated value of 0.81 suggesting that tyrosine residues were involved as H-bond donors or were in a more buried environment than expected in the absence of interactions. Changes in the Amide III region showed lower intensity values in the experimental than calculated spectrum for bands at 900-961 cm\(^{-1}\) and β-sheet structures indicating a decrease in α-helical and β-sheet structures respectively following heat unfolding and denaturation; however, these values are significantly higher than those of unheated samples (p<0.05). Howell and Li-Chan (1996) observed similar findings in mixtures of β-lactoglobulin and α-lactalbumin in the absence of salt.
Chapter 7. Effects of salt on the rheological properties and molecular interactions of unheated, heated and high pressure treated ovalbumin and β-lactoglobulin

Table 7.5: Normalised intensity values at selected regions of the experimental (bold font) and calculated (italics) spectra of 90°C heated (H) and high pressure treated (600 MPa for 20 mins) binary mixtures of ovalbumin and beta-lactoglobulin in 1% NaCl

<table>
<thead>
<tr>
<th>Peak</th>
<th>Normalised Intensity*</th>
<th>ovalbumin/β-lactoglobulin</th>
<th>ovalbumin/β-lactoglobulin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H</td>
<td>HP</td>
<td>H</td>
</tr>
<tr>
<td>Cysteine S-S</td>
<td>0.22±0.02</td>
<td>0.11±0.01</td>
<td>0.18±0.02</td>
</tr>
<tr>
<td></td>
<td>(501)</td>
<td>(504)</td>
<td>(506)</td>
</tr>
<tr>
<td></td>
<td>0.15±0.02</td>
<td>0.12±0.01</td>
<td>0.16±0.01</td>
</tr>
<tr>
<td></td>
<td>(516)</td>
<td>(515)</td>
<td>(516)</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.15±0.02</td>
<td>0.14±0.01</td>
<td>0.23±0.02</td>
</tr>
<tr>
<td></td>
<td>(759)</td>
<td>(761)</td>
<td>(756)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.28±0.03</td>
<td>0.31±0.04</td>
<td>0.34±0.01</td>
</tr>
<tr>
<td></td>
<td>(828)</td>
<td>(825)</td>
<td>(826)</td>
</tr>
<tr>
<td></td>
<td>0.21±0.02</td>
<td>0.21±0.02</td>
<td>0.28±0.02</td>
</tr>
<tr>
<td></td>
<td>(852)</td>
<td>(851)</td>
<td>(853)</td>
</tr>
<tr>
<td>CH bend/Trp</td>
<td>0.29±0.03</td>
<td>0.37±0.01</td>
<td>0.39±0.04</td>
</tr>
<tr>
<td></td>
<td>(1341)</td>
<td>(1340)</td>
<td>(1340)</td>
</tr>
<tr>
<td>CH₂ bend</td>
<td>1.19±0.01</td>
<td>1.41±0.06</td>
<td>1.49±0.05</td>
</tr>
<tr>
<td></td>
<td>(1450)</td>
<td>(1450)</td>
<td>(1451)</td>
</tr>
<tr>
<td>C-Nor C-Cstretch</td>
<td>0.02±0.01</td>
<td>0.03±0.06</td>
<td>0.05±0.01</td>
</tr>
<tr>
<td></td>
<td>(1052)</td>
<td>1061</td>
<td>(1050)</td>
</tr>
<tr>
<td>COO⁻</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Amide 111'</td>
<td>0.38±0.02</td>
<td>0.38±0.03</td>
<td>0.42±0.02</td>
</tr>
<tr>
<td></td>
<td>(939)</td>
<td>(940)</td>
<td>(936)</td>
</tr>
<tr>
<td></td>
<td>0.41±0.02</td>
<td>0.39±0.01</td>
<td>0.55±0.04</td>
</tr>
<tr>
<td></td>
<td>(961)</td>
<td>(958)</td>
<td>(959)</td>
</tr>
<tr>
<td></td>
<td>0.64±0.02</td>
<td>0.45±0.01</td>
<td>0.86±0.06</td>
</tr>
<tr>
<td></td>
<td>(985)</td>
<td>(987)</td>
<td>(988)</td>
</tr>
</tbody>
</table>

Intensity at the wavenumber (cm⁻¹) shown in parenthesis, normalised to the Phenylalanine band at 1006 cm⁻¹ for spectral data which were the average of 128 scans and baseline corrected. ND means not determined because there was no peak.
Figure: 7.9 Experimental and difference FT Raman spectra (400-1800 cm\(^{-1}\)) high pressure treated (600 MPa for 20 min) ovalbumin and \(\beta\)-lactoglobulin mixture (7.5:7.5 w/w) dissolved in D\(_2\)O with 1 % NaCl. Spectra were baselined and normalised to the phenylalanine peak at 1004 cm\(^{-1}\).
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7.4.2.6. Comparison of high pressure treated (600 MPa for 20 min) mixtures of ovalbumin and β-lactoglobulin in 1% sodium chloride

FT Raman spectra for unheated mixtures of ovalbumin and β-lactoglobulin are shown in Fig. 7.9 and Table 7.5. The use of high pressure has consistently resulted in lower intensity of the disulphide bonds (500-520 cm\(^{-1}\)) in previous results (Ngarize et al., 2003) and chapter 4-6 compared with heat treatment. In the present study, results indicated lower intensity of the disulphide bonds in the experimental than in the calculated spectra, suggesting disulphide bonds were significantly altered by interactions between ovalbumin and β-lactoglobulin during pressure treatment than in presence of NACl. The use of high pressure induced gelation reported above (Table 7.1) resulted in weaker gel strength which may be due to lower disulphide bond involvement.

High pressure treatment resulted in lower band intensity of the Amide III' bands representing the β-sheet structures at 980-990 cm\(^{-1}\) compared with the heated samples, though the experimental spectrum showed lower intensity than the calculated spectrum. The involvement of β-sheet structures in pressure-induced gelation is known to be much weaker than in heat induced gelation (Li-Chan et al., 1994; Mine et al., 1990). Similarly, the experimental spectrum showed lower intensity values than the calculated for the bands at 900-960 cm\(^{-1}\) indicating a decrease in α-helical structures following pressure unfolding and denaturation.

The tyrosine doublet ratio 850/830 cm\(^{-1}\) was similar for both experimental and calculated spectra and gave a value of 0.70 suggesting a more buried or hydrophobic environment of tryptophan residues. However, peak intensities for aliphatic hydrophobic groups were lower for CH\(_2\) methylene asymmetric bending of deformations of CH\(_2\) and CH\(_3\) groups at 1450 cm\(^{-1}\) and for the CH bend/tryptophan at 1350 cm\(^{-1}\) bands in experimental spectra compared with the calculated values. An increased exposure of aromatic residue groups at 760 cm\(^{-1}\) and 1180 cm\(^{-1}\) indicated further involvement of hydrophobic interactions in the mixtures.
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No peaks were observed for the band at 1420 cm$^{-1}$ typical for COO$^-$ (of Asp and Glu amino acids).

7.4 Conclusion

This study has shown, by large deformation rheology, that no synergistic interactions were evident in whey and egg albumen mixtures in the presence of 1% sodium chloride, as negative interaction indices were obtained in most cases. It appears the addition of sodium chloride enhanced protein aggregation and presented the formation of strong gel networks. Several important changes were observed in the Raman spectra of ovalbumin, β-lactoglobulin and their binary mixtures. On the whole, changes in all bands were much lower than for values obtained in the absence of salt (chapter 3,4) (Ngarize et al., 2003). The changes in the Raman bands near 500-540 cm$^{-1}$ provided direct evidence of involvement of disulphide bonds in gel formation during heat and pressure induced gelation even in the presence of 1% sodium chloride. Changes in the tyrosine doublet ratio 850/830 cm$^{-1}$ indicated increased hydrogen bonding in a non-polar environment during both heat and pressure treatment. Heating produced a higher β-sheet structure content compared with the high pressure treated samples which contributed to the high gel strength in the heated samples; however, the overall values were lower for egg albumen proteins and higher for whey proteins compared with samples without sodium chloride. There was a marked involvement of hydrophobic groups in unheated, heated and high pressure treated samples, including their binary mixtures in the presence of salt.
8.1 Introduction

To date, effects of pressure have been reported on four main areas; micro-organisms, enzyme activity, proteins and phase changes. The inactivation of micro-organisms is fundamental to food preservation. Heating food to temperatures of up 100°C can destroy vegetative cells and spores, however, the destruction of bacteria using temperature depends on varying resilience of different organisms, time and temperature used. High pressure of up to 1000 MPa can destroy vegetative cells but does not kill spores (Heinz and Knorr, 2002). The inability of high pressure to inactivate bacterial spores makes the case for combined use of high pressure and temperature more favourable. In recent years, researchers have focused mainly on use of combined heat and high pressure treatment to inactivate micro-organisms. The resistance of vegetative cells of bacteria to high pressure appears to be greatest at approximately 20 to 30°C; at higher or lower temperatures, micro-organisms are more sensitive to high pressure treatment. However, the resistance to high pressure by vegetative cells of bacteria decreases even when pressure is combined with heat at non-lethal temperatures (Raso et al., 2003). In general, the inactivation of most vegetative cells by high pressure at low temperatures shows an initial exponential rate, followed by pronounced tailing (Smelt, 1998). The tail tends to disappear when high pressure is combined with heat (Patterson and Kilpatrick, 1998; Kalchayanand et al., 1998). In contrast to vegetative cells, inactivation of spores by high pressure follows two steps, initial germination followed by inactivation of germinated spores (Gould and Sale, 1971; Takahashi et al., 1991). This finding has led to the investigation of the combined use of pressure with raised temperature, and more recently with radiation and chemical agents because of the tendency of some spores to be pressure resistant (Gould, 2001, Raso et al., 2003).

The combined use of heat and pressure has also been widely used due to the resistance of enzymes to pressure treatment. In general, combinations of pressure with moderate temperatures increase the level of enzyme inactivation; however, in some cases an increase in enzyme activity has been reported (Hendrickx et al., 1998). Knorr et al., (1992) commented on the importance of food composition, and the pressure medium with
Chapter 8. Combined use of high pressure and heat treatment during gelation of egg albumen and whey proteins and their binary mixtures

regard to enzyme inhibition. Gome and Ledward (1996) found that increasing both temperature and holding time decreased the activities of both purified commercial and crude extract of polyphenoloxidase due to some aggregation of the enzyme. Complete inactivation of the enzyme was found at 800 MPa for 5 min at 20°C and inactivation was irreversible. However, pressure treatment at 200 MPa for 10 min led to significant inhibition of polyphenoloxidase activity in a mushroom extract, but treatment at 400 MPa increased enzyme activity; this was not found with the pure enzyme. The authors suggested that the reason for this finding was due to either interactions with other components in the extract or release of membrane-bound enzyme under pressure.

Combining high pressure and heat is already of great practical interest, especially in preserving low acid foods (Ogawa et al., 1990). Combination of mild temperatures and pressure allows the pasteurisation/sterilisation of foods at lower temperatures. However, studies on the combined effects of pressure and heat on protein-protein interactions have not been studied before. Recent studies by Harte et al., (2003) investigated the combined use of high pressure (300-676 MPa for 5 min) and thermal treatment (85°C for 30 min) in low-fat set yoghurt made from milk and reported improved yogurt yield stress and reduction in syneresis. However, application of thermal treatment followed high pressure treatment. In contrast, Tedford et al., (1998) reported the combined use of pressure and heat treatment on the protein structure of heat sensitive proteins of egg white, ovalbumin and lysozyme and the milk protein β-lactoglobulin in their study. However, the pressures used were as low as 100 MPa with temperatures of up to 75°C. The objective of the present study was to use a combination pressure (600 MPa) and heat treatment at fixed temperatures of 50°C and 60°C to study the gelation of egg albumen and whey proteins in isolation and in mixtures.
Chapter 8. Combined use of high pressure and heat treatment during gelation of egg albumen and whey proteins and their binary mixtures

8.2 Materials

Materials were the same as in section 2.1

8.3 Methods

8.3.1. Large deformation testing

8.3.1.1. Heat treated samples

Protein gels were prepared as in section 4.3.2.1 and 4.3.2.2

8.3.1.2. Sample preparation for combined heat and pressure at 50°C and 60°C (600 MPa for 20 min)

The solutions for high pressure treated gels were prepared by the same method as heat treated gels except the samples were placed in visking tubing tied at both ends, heat sealed in a high pressure polythene bag (Crayovac W.R Grace Limited Cromwell Road, Cambridge, United Kingdom). The samples were introduced into the working chamber (300 ml capacity) of a Stansted Food Lab high pressure rig (Stansted Fluid Power, Ltd, 70 Bentfield road, Stansted, CM24 8HT, Essex, UK.) containing the low compressibility fluid (2:8 of a mixture of castor oil:ethanol). The temperature of the working chamber was set at 50°C and 60°C respectively. The equipment was installed in an air conditioned laboratory at 20°C. The temperature in the pressurisation chamber increased during compression reaching a maximum after 2 minutes (the maximum temperature achieved in all treatments was 30°C, corresponding to the treatment at 400 MPa, 500 MPa, 600 MPa), then decreased to 20°C in 0.5 min and remained stable at 20°C until the end of the cycle. The compression and decompression times were about 1-2 minutes and 0.5 min respectively. The samples were stored at 4°C similar to the heat-treated ones. For samples less than 10 ml, the Stansted Micro Food-Lab, Model S-FL-085-9W high pressure rig was used. Temperatures of 50°C and 60°C were chosen, since due to technical limitations of the machine, we could not use higher temperatures. Secondly the proteins studied did not gel below these temperatures.

8.3.2. DCS sample preparation

Samples were prepared according to method in 2.2.2.3.
Chapter 8. Combined use of high pressure and heat treatment during gelation of egg albumen and whey proteins and their binary mixtures

8.4 Results and Discussion

8.4.1. Rheological studies

8.4.1.1. Large deformation studies

Table 8.1: Large deformation analysis of gels of egg albumen (EA) and whey protein (W) (15% w/w) in distilled water and their mixtures (W/EA), high pressure treated at 600 MPa for 20 min at 50°C

<table>
<thead>
<tr>
<th>Heat treatment</th>
<th>Force (g) at maximum compression</th>
<th>Young's modulus (Pa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg Albumen</td>
<td>1159 (± 90)</td>
<td>17.2 (± 1.3)</td>
</tr>
<tr>
<td>Whey</td>
<td>905 (± 50)</td>
<td>12.1 (± 0.1)</td>
</tr>
<tr>
<td>Combined high pressure and heat treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egg albumen</td>
<td>528 (±10)</td>
<td>5.8 (± 0.1)</td>
</tr>
<tr>
<td>Whey</td>
<td>728 (± 20)</td>
<td>9.2 (±0.9)</td>
</tr>
<tr>
<td>10:5 W/EA</td>
<td>468 (± 30)</td>
<td>5.1 (± 0.5)</td>
</tr>
<tr>
<td>Interaction index</td>
<td>-29</td>
<td></td>
</tr>
<tr>
<td>7.5:7.5 W/EA</td>
<td>242 (± 15)</td>
<td>2.7 (± 0.6)</td>
</tr>
<tr>
<td>Interaction index</td>
<td>-60</td>
<td></td>
</tr>
<tr>
<td>5:10 W/EA</td>
<td>143 (± 10)</td>
<td>1.7 (±0.2)</td>
</tr>
<tr>
<td>Interaction index</td>
<td>-76</td>
<td></td>
</tr>
</tbody>
</table>

Figures in parenthesis are standard deviation based on five replicates. Interaction index = (Actual value-additive value*)/Additive value x 100 (*). The value derived from
Chapter 8. Combined use of high pressure and heat treatment during gelation of egg albumen and whey proteins and their binary mixtures

summing the contributions of the component proteins measured in isolation, in proportion to the concentration of each in the mixture (Howell and Lawrie, 1984).

Table 8.2: Large deformation analysis of gels of egg albumen (EA) and whey protein (W) (15% w/w) in distilled and their mixtures (W/EA) high pressure treated at 600 MPa for 20 min at 60°C

<table>
<thead>
<tr>
<th>Protein gel (15% w/w)</th>
<th>Force (g) at maximum compression</th>
<th>Young’s modulus (Pa)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Heat treatment</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egg Albumen</td>
<td>1159 (±90)</td>
<td>17.2 (±1.30)</td>
</tr>
<tr>
<td>Whey</td>
<td>905 (±50)</td>
<td>12.1 (±0.10)</td>
</tr>
<tr>
<td><strong>Combined high pressure and heat treatment</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egg albumen</td>
<td>815 (± 20)</td>
<td>10.0 (± 0.30)</td>
</tr>
<tr>
<td>Whey</td>
<td>742 (± 20)</td>
<td>8.00 (± 0.50)</td>
</tr>
<tr>
<td>10:5 W/EA</td>
<td>637 (± 20)</td>
<td>7.6 (± 0.70)</td>
</tr>
<tr>
<td>Interaction index</td>
<td>-17</td>
<td></td>
</tr>
<tr>
<td>7.5:7.5 W/EA</td>
<td>390 (± 40)</td>
<td>4.4 (± 0.40)</td>
</tr>
<tr>
<td>Interaction index</td>
<td>-50</td>
<td></td>
</tr>
<tr>
<td>5:10 W/EA</td>
<td>366 (± 30)</td>
<td>3.8 (±0.20)</td>
</tr>
<tr>
<td>Interaction index</td>
<td>-54</td>
<td></td>
</tr>
</tbody>
</table>

Figures in parenthesis are standard deviation based on five replicates. Interaction index = (Actual value-additive value*)/Additive value x 100 (*). The value derived from summing the contributions of the component proteins measured in isolation, in proportion to the concentration of each in the mixture (Howell and Lawrie, 1984).
The results for the combined effect of pressure and heat treatment (at 50°C and 60°C) are shown in Tables 8.1 and 8.2 respectively. The combined use of pressure at 600 MPa and 50°C indicated significantly lower values for force at maximum compression, 528 (± 10) and Young’s modulus 5.8 Pa (± 0.10) for egg albumen protein gels compared with pressure 633 (± 36) and heat 1159 (± 90) treatment alone (p<0.05). The Young’s modulus values were 8.5 (± 0.8) and 17.2 (± 1.30) for the high pressure and heat treated samples respectively. For whey protein gels the values for force at maximum compression were 728 (± 20) and 702 (± 30) for the combined heat and pressure and for pressure alone, the value for the force at maximum compression for heated whey protein gels was highest at 905 (± 50). The values for the Young’s modulus showed a similar pattern at 9.2 Pa (± 0.90), 8.5 Pa (± 0.80) and 12.1 Pa (± 0.10) for combined treatment, pressure and heated samples of whey protein.

Egg albumen and whey proteins differed in the way they responded to combination treatment. For egg albumen, the low value for force at maximum compression compared with both heat and pressure alone suggests that at 50°C the protein had not completely unfolded. This was probably because ovalbumin which is responsible for the gelling behaviour of egg albumen and accounts for 54% of egg albumen has a denaturation temperature at around 75-85°C (Donovan et al., 1975; Li-Chan and Nakai, 1989). It appears that proposals put forward by Ledward (1998) might explain this phenomenon; the unfolding of proteins by heat or pressure give rise to an elliptic phase diagram (section 1.5.3) in the temperature-pressure plane which describes the conditions under which the protein is in the native or unfolded state at a given temperature or pressure. It is seen that up to a certain temperature, pressure stabilises the protein to heat denaturation. On the removal of the denaturing agent the proteins will refold to their native like structure, hence at 50°C at a pressure of 600 MPa, pressure protects egg albumen and therefore the protein is not completely unfolded so the gels are weaker than those produced by heat and pressure alone.
Chapter 8. Combined use of high pressure and heat treatment during gelation of egg albumen and whey proteins and their binary mixtures

The unfolding and gelation pattern for whey protein isolate during the combination treatment gave mixed results. The highest force at maximum compression was obtained for the heated sample compared with pressure alone or a combination of heat and pressure suggesting that the combined treatments did not allow the protein to unfold completely. Similarly, β-lactoglobulin is responsible for most of the gelling behaviour of whey protein isolate and also accounts for almost 60% (w/w) of protein present in the isolate (Bottomley et al., 1990). The denaturation temperature of whey isolate is around 80-90°C, and that of β-lactoglobulin ranges between 76 °C and 82 °C (Aguilera, 1995), so the heated sample reflects the denaturation and unfolding pattern of β-lactoglobulin.

In the mixed gels, the highest Young's modulus value was obtained for the 10:5 whey/egg albumen mixture, 5.1 Pa (± 0.50) compared with 2.7 Pa (± 0.60) and 1.7 Pa (± 0.20) for the 7.5:7.5 and 5:10 whey/egg albumen mixtures respectively. The high Young's modulus value was also reflected in the force at maximum compression 468 (± 30) for 10:5 whey egg albumen mixture compared with 242 (± 15) and 143 (± 10) for the 7.5:7.5 and 5:10 whey/egg albumen mixtures. This finding confirmed that the highest gelation values were obtained for the 10:5 whey/egg albumen mixture as observed in previous studies reported in chapter 3-6 (Ngarize et al., 2003). However, the interaction indices were all negative for the three mixtures, suggesting that the use of combined heat and pressure treatment led to less than the expected values for the two proteins. This has implications for processing and product formulation, and should be further investigated to ascertain if use of higher temperatures would produce a different outcome.

The results for the combined heat and pressure treatment at 60°C are shown in table 8.2. The gel strength values at 60°C were significantly higher than those obtained at 50°C (600 MPa, 20 min, Table 8.1). Also for egg albumen gels, the combined use of pressure at 600 MPa and 60°C for 20 min indicated significantly higher values for force at maximum compression, 815 (± 20) and Young's modulus 10 Pa (± 0.30) compared with
pressure treatment 633 (± 36) but significantly lower values than heat treatment alone 1159 (± 90) (p<0.05). Results from DSC studies may confirm the importance of the role of optimum unfolding for interaction proposed by Howell and Lawrie (1985). DSC results indicated that the denaturation of egg albumen proteins had four endothermic peaks (Tm), 57°C, 63°C, 73°C and 79°C. The thermal denaturation of egg albumen proteins has been reported using DSC and three endothermic peaks were identified at pH 7, (Tm), 61°C, 75°C and 84°C which corresponded to denaturation of ovotransferrin, lysozyme and ovalbumin respectively (Donovan et al., 1975; Li-Chan and Nakai (1989). Since, the unfolding of egg albumen proteins starts at approximately 60°C, the treatment at 600 MPa and 60 °C for 20 min gave increased force at maximum compression compared with the combination treatment at 50°C.

For whey protein isolates a significant increase was also observed, with the combined use of pressure at 600 MPa and 60°C compare to 50 °C. A significant increase in the value for force at maximum compression, 742 (± 20) for whey protein isolate compared with the pressure 702 (± 30) but significantly less than heat 905 (± 50) treatment alone (p<0.05). Similarly, Young’s modulus values were 8.0 Pa (± 0.50) for combined heat and pressure, 12.1 Pa (± 0.10) and 10.2 Pa (± 0.10) for heat and pressure alone respectively. The gradual increase in the force at maximum compression suggests that whey proteins gradually unfold as the temperature was increased above their denaturation temperature. DSC results for whey protein isolate were found to produce a flat line indicating denaturation of the protein during processing. The DSC results for β-lactoglobulin are discussed overleaf.

In the mixed gels, the highest Young’s modulus value was obtained for 10:5 whey/egg albumen mixture 7.6 Pa (± 0.70) compared with 4.4 Pa (± 0.40) and 3.8 Pa (± 0.20) for the 7.5:7.5 and 5:10 whey/egg albumen mixtures respectively. The high Young’s modulus values is also reflected in the force at maximum compression 637 (± 20) for 10:5 whey egg albumen mixture compared with 390 (± 40) and 366 (± 30) for the 7.5:7.5 and 5:10 whey/egg albumen mixtures. Like all the mixtures at combination of 600 MPa
and 50°C for 20 min, the mixtures treated at 600 MPa and 60 °C for 20 min, also indicated negative interaction indices, although the values at 60 °C were higher than at 50 °C. Both combination treatment mixtures were lower than heat treatment alone, suggesting that use of combination treatment might not be as effective as heat alone and reinforces the view that optimum unfolding of proteins is required for maximum interaction.

8.4.2. DSC analysis

8.4.2.1. Whey Proteins

There were no conformational transitions observed on the DSC trace of Bipro whey isolate (15 % w/w) in distilled water. It was concluded that the isolate had been denatured during manufacture.

8.4.2.2. Egg albumen proteins

There were four endothermic peaks (Tₘ), 57°C, 63°C, 73°C and 79°C. The thermal denaturation of egg albumen proteins has been reported using DSC by Donovan et al., (1975) and Li-Chan and Nakai (1989) and three endothermic peaks were identified at pH 7, at 61°C, 75°C and 84°C which corresponded to the denaturation of ovotransferrin, lysozyme and ovalbumin respectively.

8.3.2.3. DSC studies on ovalbumin and β-lactoglobulin and their binary mixtures

The DSC thermogram for ovalbumin 15 % (w/w) in distilled water is shown in Figure. 8.1 and has one main endothermic transition (Tₘ), 75°C (ΔH: 0.83 J/g).
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Figure 8.1: DSC thermogram for ovalbumin 15% (w/w) in distilled water (pH 7)

The DSC thermogram for β-lactoglobulin 15% (w/w) in distilled water is shown in Figure 8.2 indicating a co-operative endothermic transition ($T_m$), 76°C ($\Delta H$: 0.50 J/g). Reported values show a range from 60-85°C due to variation in heating rate, sample preparation and environmental conditions (Mulvihill and Donovan, 1987; Boye and Alli 2000).

Figure 8.2: DSC thermogram for β-lactoglobulin 15% (w/w) in distilled water (pH 7)
The DSC thermogram for the 10:5 β-lactoglobulin/ovalbumin mixture 15% (w/w) in distilled water (pH 7.7) showed two endothermic transitions ($T_m$), 70°C ($\Delta H$: 0.73 J/g) and ($T_m$), 74°C ($\Delta H$: 0.01 J/g) as a shoulder, corresponding to the denaturation of ovalbumin and β-lactoglobulin. These results represent a significant decrease in transition temperatures of both proteins compared with values obtained at 75 and 76°C for ovalbumin and β-lactoglobulin respectively ($p<0.05$). It appears that the broad endothermic peak of ovalbumin is overlapping that of β-lactoglobulin so that the $T_m$ of β-lactoglobulin is not clearly defined and appears as a shoulder at 74°C. Although, both ovalbumin (pI 4.5) and β-lactoglobulin (pI 5.2) are negatively charged there appears to be interaction between the two proteins. Howell and Li-Chan (1996) and Howell *et al.*, (1995) reported aggregation due to mainly electrostatic interactions of basic lysozyme (pI 10) with β-lactoglobulin (pI 5.2).
One might suggest that when the two proteins are heated together each protein had an effect on the thermal denaturation of the other, it is possible that ovalbumin made β-lactoglobulin less thermally stable, therefore the early unfolding and denaturation of ovalbumin, initiated cross-linking interactions with the exposed sites on the β-lactoglobulin which speeded up the unfolding of the β-lactoglobulin molecule resulting in a lower T_m of β-lactoglobulin. These findings are in agreement with previous reports which have shown that β-lactoglobulin undergoes conformational changes above 40°C resulting in unfolding of the protein into a molten-globule-like structure with increased exposure of previously buried inner hydrophobic and thiol groups (Iametti et al., 1996; Boye and Alii 2000; Comfort and Howell, 2002). In addition, there was an exothermic transition, T_m 77°C ((ΔH: -0.002 J/g) which has been reported to be due to exposure of hydrophobic groups and thiol-disulphide inter-change reactions during protein aggregation and gelation (Ma and Harwalkar, 1990; Hoffmann and van Mil, 1997; Sun and Hayakawa, 2002). In the rheological studies the 10:5 whey/egg albumen mixture indicated synergistic protein-protein interactions (chapter 3)(Ngarize et al., 2003).

![DSC thermogram for the 5:10 β-lactoglobulin/ovalbumin mixture 15% (w/w) in distilled water (pH.7)](image)

Figure 8.4: DSC thermogram for the 5:10 β-lactoglobulin/ovalbumin mixture 15% (w/w) in distilled water (pH.7)
Chapter 8. Combined use of high pressure and heat treatment during gelation of egg albumen and whey proteins and their binary mixtures

The DSC thermogram for the 5:10 $\beta$-lactoglobulin/ovalbumin mixture (Figure. 8.4) showed the main endothermic transition, $(T_m)$ 73°C ($\Delta H$: 0.99J/g) corresponding to the denaturation of ovalbumin and $(T_m)$ 77°C (exothermic) as a shoulder ($\Delta H$: -0.002 J/g) due to aggregation and protein gelation. These results show that in this mixture, denaturation of ovalbumin masked the presence of $\beta$-lactoglobulin, however, the endothermic peak was broader than that observed ovalbumin alone.

The DSC thermogram for the 7.5:7.5 $\beta$-lactoglobulin/ovalbumin mixture (Figure. 8.5) showed two thermal transitions, $T_m$ 70°C ($\Delta H$: 0.92J/g) and $T_m$ 73°C ($\Delta H$: 0.02J/g) as a shoulder, corresponding to the denaturation of ovalbumin and $\beta$-lactoglobulin. A third peak, $(T_m)$ 81°C (exothermic) was due to aggregation and gel formation. These results show that the denaturation of both proteins is affected by the presence of the other, since $(T_m)$ values for ovalbumin and $\beta$-lactoglobulin (70°C and 73°C) in the mixtures are lower than those for each protein in isolation (75°C and 76°C).

Figure 8.5: DSC thermogram for the 7.5:7.5 $\beta$-lactoglobulin/ovalbumin mixture 15% (w/w) in distilled water (pH.7)
8.4 Conclusion

These results show that the combined use of pressure (600 MPa for 20 min) and temperature at 50°C and 60°C resulted in significantly lower values for force at maximum compression and Young's modulus compared with heat alone, but significantly higher values than pressure treatment alone for both whey and egg albumen proteins and their mixtures. However, in the combined heat/pressure treatment for both egg albumen and whey proteins, the force at maximum compression and Young's modulus values increased with increasing temperature that is at 60°C compared with 50°C, suggesting that an increased temperature resulted in greater protein unfolding. For egg albumen, differential scanning calorimetry studies confirmed that the egg albumen transitions ranged from 57-79°C, indicating increased unfolding at 60°C promoting interactions of exposed reactive groups. Interactions were highest in the 10:5 whey/egg albumen mixture, although, for all three combinations 10:5, 7.5:7.5, and 5:10 whey egg albumen mixtures the force at maximum compression and Young's modulus values were lower than expected. DSC studies indicated the denaturation temperatures of ovalbumin and β-lactoglobulin and their mixtures. The two proteins affected the behaviour of the other in the mixtures.

Temperatures above 60°C may result in stronger gels when heat and pressure are used in combination. DSC studies on ovalbumin and β-lactoglobulin mixtures indicated endothermic transitions with lower values compared with the individual proteins. In addition, the presence of an exothermic peak indicated protein aggregation. DSC, therefore confirmed interactions, albeit more negative than were observed in the gelation studies. The present findings indicated that the nature of protein-protein interactions is very important in the design, formulation and processing of products.
9.0 General discussion and conclusions

Protein denaturation and gel formation induced by heat and pressure were studied by several experimental approaches. Rheological techniques including both small deformation and large deformation analysis, were used to determine the visco-elastic properties of networks formed; microscopy was used to study appearance and type of gel structure. Elucidation of the mechanism of gel formation was supported by thermodynamic properties of the proteins as well as structure by FT-Raman spectroscopy. The combination of these methods provided evidence of differences in structure, the nature of protein-protein interactions of individual and combined whey, egg albumen, ovalbumin, β-lactoglobulin and their mixtures during heating and high pressure processing.

9.1 Interactions of unheated whey protein isolate (Bipro), egg albumen, ovalbumin and β-lactoglobulin proteins by Raman spectroscopy

Unheated whey and egg albumen proteins had some common principal features, both proteins showed well-resolved peaks in the Amide III' region between 900-960 cm\(^{-1}\) (α-helix region) accompanied by weaker and lower values for peak intensities between 980-990 cm\(^{-1}\) (Amide IIF, anti-parallel β-sheet). In whey protein isolate, an additional band was observed at 1239 cm\(^{-1}\) (Amide DT, anti-parallel β-sheet) which was absent in egg albumen protein, confirming that even in the native state whey protein is dominated by β-sheet structure. Most unheated proteins, except β-lactoglobulin, have been reported to be dominated by α-helix structures (Clark and Suggett, 1981; Howell and Li-Chan, 1996; Ngarize et al., 2003).

For whey protein two disulphide bonds were observed at 500 and 510 cm\(^{-1}\) attributed to the all-gauche conformation and for egg albumen the location of the disulphide bond at 524 cm\(^{-1}\) indicated the gauche-gauche-trans conformation both findings have already been observed by other workers. Disulphide bonds are known to take part in protein gelation via disulphide-sulphydryl interchange as discussed in section 3.4.2.2
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The band at 1418 cm\(^{-1}\) corresponding to the C=O stretching of dissociated carboxyl (COO\(^-\)) groups of Asp or Glu and side chain vibrations of the imidazole ring of histidine, showed a double peak next to the 1450 cm\(^{-1}\) in whey protein but was absent in egg albumen protein (pH, 7). The presence of the peak at 1418 cm\(^{-1}\) in whey might be explained by a more complete ionisation of whey protein at this pH compared with egg albumen proteins. Similar observations were made for ovalbumin and $\beta$-lactoglobulin.

Changes in the hydrophobic groups were monitored by following changes in the conformation of Trp and Tyr and aliphatic groups CH\(_2\) and CH\(_3\) (1450 cm\(^{-1}\)), CH bending or CH\(_3\) symmetric vibrations (1350 cm\(^{-1}\)) and the tyrosine doublet ratio at 850/830 cm\(^{-1}\). Whey protein isolate showed a significantly higher peak intensity compared with egg albumen for bands at 1450 cm\(^{-1}\) and 1350 cm\(^{-1}\) (p<0.05). In 20% (w/w) sucrose solution, major changes were observed for the tyrosine doublet at 850/830 cm\(^{-1}\) for both unheated samples of ovalbumin and $\beta$-lactoglobulin; the doublet was replaced by one broad peak at 830 cm\(^{-1}\) with the shifting of the band at 850 cm\(^{-1}\). The peak intensity of the band at 830 cm\(^{-1}\) was 1.00 ±0.01 and 1.57 ±0.20 for ovalbumin and $\beta$-lactoglobulin respectively. These findings show a major difference from proteins without sugar (Ngarize et al., 2003). These changes in conformation of proteins suggest that sucrose may modify protein conformation, via a mechanism that involves hydrogen bonding with tyrosine residues.

Addition of NaCl had no significant effect on the tyrosine doublet but produced one striking feature for both proteins, a major broadening of the hydrophobic tyrosine residue at 1207-1210 cm\(^{-1}\), which was in contrast to findings in whey and egg albumen proteins in the absence of salt (Ngarize et al., 2003). Addition of sugars and salt caused a marked peak intensity in the aliphatic groups CH\(_2\) and CH\(_3\) (1450 cm\(^{-1}\)), CH bending or CH\(_3\) symmetric vibrations (1350 cm\(^{-1}\)) for both ovalbumin and $\beta$-lactoglobulin but peak intensity was more pronounced for $\beta$-lactoglobulin; these data suggest that both sugars and salts enhanced hydrophobic interactions but via a different mechanism for each (chapter 4 and 5).
9.2 Interactions of heated and high pressure treated whey and egg albumen proteins

Whey and egg albumen comprise globular proteins which denature and undergo conformational changes leading to aggregation and gelation during heating.

*Large deformation testing.*

*Whey protein isolate:*
There were significant differences in the appearance and textural properties between heat and pressure induced whey protein gels. Heating produced fine stranded gels compared with porous fine stranded gels, which were accompanied by a loss of fluid from the gel network structure in gels produced by high pressure (Section, 4.4.2). Whey protein gels produced by high pressure were glossy and smooth with a rubbery texture compared with heat-treated gels which were less glossy, harder and brittle. High pressure treated (in commonly used range 400-600 MPa for 20 min) whey proteins had significantly lower values for force at maximum compression and Young’s modulus and tended to lose water upon compression, compared with heat treated gels. Moreover, heating at 95°C produced a significantly higher force at maximum compression and Young’s modulus compared with gels heated at 90°C, suggesting that at 95°C whey proteins are more unfolded and therefore have more groups exposed to the surface for further interaction. Transmission electron micrographs confirm these findings (chapter 4) and supported by other workers who reported that high pressure treated whey protein gels formed larger aggregates with irregular pores (Dumay et al., 1994). The experiments in the present study suggests that the unfolding of the protein is less substantial during pressure denaturation than during heating.

Progressive increase in pressure was correlated with an increase in force at maximum compression and Young’s modulus, with whey protein isolate having significantly higher force at maximum compression and Young’s modulus than egg albumen (p< 0.05) for the pressure treatments used in this study (400-800 MPa for 20 min). Use of higher pressures for whey proteins produced gel strength which was similar to
Chapter 9. General discussion and conclusion

Heat-treated gels when obtained at pressures above 650 MPa, suggesting that the amount and duration of pressure determines the gel strength. This increase in gel strength as a function of pressure could have significant technological implications in product development and may be related to the number of cross-links within the gel network structure. Textural attributes of processed foods depend on the type of protein used and how the gel is prepared (Barbut, 1996).

The effect of the addition of sugars on the rheological properties of whey proteins was largely determined by the sugar concentration, temperature and time of heating. In the presence of 10% and 20% (w/w) sucrose concentration, the force at maximum compression and Young’s modulus showed an increasing trend at both 90°C and 95°C; however, surprisingly gels did not form at all in 40% sucrose solution at both temperatures except when the holding time was increased to one hour. The presence of sugar altered the gelation temperature of whey proteins, McClements et al., (2002) suggested that the presence of sugars can alter the gelation mechanism of a protein by changing the temperature at which the protein unfolds which means that the system has to be heated to a higher temperature before gelation occurs. In high pressure treated whey proteins, no gelation was observed in 10% sucrose solution and the lowest values for force at maximum compression and Young’s modulus were obtained. Sucrose has been reported to protect proteins from pressure denaturation and result in the formation of weaker gels (Dumay et al., 1994). In contrast the addition of 10-40% sucrose enhanced the gelation of egg albumen proteins.

Addition of 1% (w/w) sodium chloride resulted in opaque and more brittle gels compared with the clear and transparent unsalted whey protein gels. These gels had a significantly higher force at maximum compression and Young’s modulus values compared with egg albumen (p<0.05) suggesting that salt had a greater influence on the gel strength of whey proteins compared with sugar. Addition of salt has been reported to shift the gel microstructure from fine stranded to particulate structures between pH 4 and 6 and give rise to white opaque gels in which the size of aggregates differed with pH (Langton and Hermansson, 1992).
Combination of heat and pressure (600 MPa for 20 min) at 50 °C and 60 °C produced gels with lower force at maximum compression and Young's modulus compared with heat alone but significantly higher values than pressure alone. Interestingly, gel strength increased with increasing temperature at 60°C compared with 50°C. However, temperatures greater than 60°C could not be tried due to the limitations of the equipment. The combination treatment resulted in lower than expected gel strength values in all mixtures of egg albumen and whey proteins.

9.3. Changes in whey protein isolate and β-lactoglobulin protein conformation following heat and high pressure treatment by DSC and Raman spectroscopy

DSC analysis of whey proteins showed no conformational transitions suggesting that the whey protein isolate had been denatured during manufacture. β-lactoglobulin gels showed one co-operative endothermic peak \( T_m \) at 76 °C (\( \Delta H: 0.50 \, \text{J/g} \)). Upon heating, the Raman spectra of whey and β-lactoglobulin showed similar conformational changes. There was increased peak intensity in the disulphide region (500-540 cm\(^{-1}\)). In contrast, peak intensity for high pressure treated whey protein had significantly lower values for disulphide peak intensity, explaining the corresponding lower gel strength in high pressure treated gels (\( p<0.05 \)). Sulphhydryl-disulphide interchange reactions are a necessary pre-requisite for the formation of gels in globular proteins during both heat and high pressure gelation (Mulvihill and Kinsella, 1988, Tani et al., 1997, Law et al., 1998, Iametti et al., 1998). In this study, the role of disulphide bond in the high pressure treated gels has been unequivocally identified using Raman spectroscopy, the only technique available for direct measurements of disulphide bonds (Howell and Li-Chan, 1996). The addition of salt or sugar did not cause significant changes in this region in either heated and high pressure treated whey protein.

Changes in secondary structure were evident in the Amide III' bands (980-990 cm\(^{-1}\)) which indicated an increase in β-sheet structures and a decrease in α-helical structures in both heated and high pressure treated β-lactoglobulin gels, except that heating
resulted in significantly higher levels of β-sheet structures (p<0.05). Quantitative changes in secondary structure were studied by deconvolution techniques and indicated an increase in β-sheet structures from 54% (native) to 70% for heated β-lactoglobulin. In contrast, high pressure treated β-lactoglobulin had a significantly lower content of β-sheet structures at 37% following high pressure treatment compared with 70% for the heated sample. In addition, for β-lactoglobulin there was an increase in random structure following high pressure treatment, previous reports confirm our finding (Pannick et al., 1999). However, for ovalbumin there was a slight decrease in random coil from 13% (native) to 11% (high pressure treated). There was a significant decrease in α-helix in both high pressure and heated β-lactoglobulin (p <0.05). Further involvement of β-sheet structures was indicated by bands at 1625 cm\(^{-1}\) and 1680 cm\(^{-1}\) for heated gels and 1626 cm\(^{-1}\) and 1684 cm\(^{-1}\) for high pressure treated gels which are associated with aggregated intermolecular β-sheet (Clark et al., 1981; Nagano et al., 1994). However, the absence of the Amide III β-sheet structures at 1239 cm\(^{-1}\) in both high pressure treated β-lactoglobulin and ovalbumin gave further evidence that the involvement of β-sheet structures is less intense in high pressure treated protein gels, and may explain why pressure treated gels were found to be weaker than heat treated gels (Ngarize et al., 2003 submitted and Van Camp and Huyghbaert, 1995). The mechanism for the formation of β-sheet during denaturation is thought to be via hydrogen bonding of adjacent polypeptide chains, between C=O and NH groups (Ngarize et al., 2003). In this study, inter-molecular hydrogen bonding was further confirmed by the appearance of aggregation bands at 1680-1690 cm\(^{-1}\).

Changes in the hydrophobic groups were monitored by following changes in the conformation of Trp and Tyr and aliphatic groups CH\(_2\) and CH\(_3\) (1450 cm\(^{-1}\)), CH bending or CH\(_3\) symmetric vibrations (1350 cm\(^{-1}\)) and the tyrosine doublet ratio at 850/830 cm\(^{-1}\). The bands at 1350 cm\(^{-1}\) and 1450 cm\(^{-1}\) had decreased peak intensity in the heated samples, suggesting the contribution of hydrophobic residues in protein aggregation and gelation. In contrast, the high pressure treated whey protein and β-lactoglobulin had significantly higher peak intensity values for the 1450 cm\(^{-1}\) (1.50 ± 0.07) band compared with heated whey protein (1.43 ± 0.03) (p<0.05) suggesting that protein unfolding during pressure denaturation results in increased exposure of
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hydrophobic groups which participate in protein aggregation. The increased exposure of hydrophobic groups during pressure denaturation of β-lactoglobulin was reported by Swanson et al., (2002) to result in increased intrinsic tryptophan fluorescence and threefold increase in extrinsic 1-anilino-napthalene-8-sulfonate (ANS) fluorescence. In addition, these authors reported, using Near ultraviolet Circular dichroism (CD), that the native tertiary structure essentially disappears following pressure treatment, explaining the increased exposure of hydrophobic groups observed in the present study, since high pressure is known to destabilise hydrophobic groups (section 1.5.3). Thus the free thiol group of cysteine in β-lactoglobulin becomes accessible and forms S-S links, resulting in the non-cooperative unfolding to form an intermediate protein structure known as the molten globule state, stabilized by disulphide bonds.

Data on molecular dimensions of pressure induced denatured states are few, however, these pressure denatured states do not show a well organised tertiary structure, their secondary structure is essentially unaltered and they have the ability to bind hydrophobic probes (Silva and Weber, 1993; Swanson et al., 2002). Indeed, the thermal unfolding of proteins to molten globule states indicates that there are two types of molten globule; the first shows co-operative thermal unfolding that can be approximated by two-state transitions, whereas the second one shows gradual unfolding (Nishii et al., 1995). However, it is now accepted that the molten globule is a kinetic intermediate and also depends on the amount of heat or pressure applied (Masson and Clery, 1996; Hendrickx and Knorr, 2002).

Large deformation testing.

Egg Albumen:
Like whey protein gels, there were also significant differences in appearance and textural properties between heat and pressure induced egg albumen protein gels. Heating produced significantly stronger egg albumen gels compared with whey protein isolate (p<0.05). High pressure treated egg albumen gels were glossy and smooth in appearance with a rubbery texture compared with heat treated gels which were harder and less glossy. In contrast to whey proteins, which formed gels at 400 MPa (20 min), no gelation was observed for egg albumen below 500 MPa (20 min),
the hardness and consistency of the gels increased with increasing pressure, but remained significantly different and gave lower values for force at maximum compression, as well as low Young’s modulus values compared with heat treatment alone. Bonomi et al., (1998) also reported that egg albumen does not form a gel below pressures of 500 MPa.

Electron microscopy studies revealed that the microstructure for heated egg albumen was characterized by a less porous, more compact network structure compared with high pressure treated egg albumen. Hayashi (1989) reported that high pressure processing allows liquid whole egg, albumen, or yolk to retain its natural flavour and nutritional value; therefore this technology is a useful tool for the preservation of egg albumen, since mildest heat treatment can denature or modify most of its functional properties.

The addition of sucrose (10-40% w/w) altered the gelation properties of egg albumen, depending on the heating temperature used. Gels formed at 90°C (30 min), at varying concentrations of sucrose 10%, 20% and 40% resulted in an increase in both force at maximum compression and Youngs’ modulus values, the highest force at maximum compression, with highest values obtained at 40% (w/w) sucrose concentration. In contrast, no gelation was observed for whey proteins in 40% (w/w) sucrose solution suggesting that sugar promotes greater unfolding of egg albumen proteins at high sugar concentrations. However, at 95°C, the optimum gelation figures for egg albumen were obtained for 20% sucrose concentration with significantly lower values for 40% sucrose concentration. These results suggest that at higher concentrations sucrose reduced protein unfolding and consequently protein-protein interactions, thus resulting in lower values for force at maximum compression. In addition, sugar concentration markedly affected gelation properties and was far more effective at 20% sucrose at 95°C than at 10% and 40% sucrose. Previous reports by Donovan et al., (1975) indicated that 10% sucrose increased heat stability of egg albumen proteins due to reduced water activity.

For high pressure treated egg albumen, the addition of sucrose resulted in significantly higher values for force at maximum compression and Young’s modulus
for 10% sucrose concentration compared with 20% confirming previous findings by Bonomi et al., (1998) and Iametti et al., (1999) using 10% sucrose. This work is of primary importance from a technological viewpoint, and confirms findings in the present study, because it highlights the observation that egg albumen may be kept in a liquid state after high pressure treatment in the presence of appropriate concentrations of common food ingredients such as sucrose or sodium chloride.

The soluble nature of egg albumen upon high pressure treatment in the presence of either sugar or salt could make the direct use of egg albumen in food more practical provided, the treated material retains the functional properties. The addition of 1% (w/w) sodium chloride resulted in significantly lower values for force at maximum compression and Young’s modulus values for high pressure treated samples compared with heat treated samples with salt, as discussed above.

For egg albumen, the use of combined heat and pressure gave values for force at maximum compression and a corresponding Young’s modulus which were higher at 60°C (600 MPa for 20 min) compared with 50°C (600 MPa, 20 min), suggesting that egg albumen does not completely unfold at 50°C. This was confirmed by DSC results which indicated the denaturation temperature of ovalbumin to be 75°C; previous studies also reported that denaturation of ovalbumin ranges between 75-85°C (Donovan et al., 1975; Li-Chan and Nakai, 1989). If one considers that egg albumen is predominantly 54% ovalbumin, then at 50°C combined with a pressure of 600 MPa for 20 min, egg albumen proteins is partially unfolded and therefore the gel strength is much weaker than that observed at 60°C combined with 600 MPa for 20 min.

9.4. Changes in egg albumen and ovalbumin protein conformation following heat and high pressure treatment by DSC and Raman spectroscopy

DSC analysis of egg albumen showed four endothermic peaks suggesting that this batch of egg albumen (Sigma) was not severely denatured during processing. Ovalbumin showed one main endothermic transition ($T_m$) at 75°C ($\Delta H$: 0.83 J/g). Heating of both egg albumen and ovalbumin produced similar changes in the Raman
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spectra, for this reason only the Raman spectra of ovalbumin will be discussed. In heated egg albumen and ovalbumin, appearance of a strong band at 540 cm\(^{-1}\) following heating indicated a conformational shift around the single cystinyl disulphide bond, providing direct evidence for sulfhydryl-disulphide inter-change, necessary for gelation of proteins; this was reported above for whey proteins (Ngarize et al., 2003) and other workers confirmed these findings (Li-Chan and Qi, 1998).

In the Amide III' $\beta$-sheet structure region (980-990 cm\(^{-1}\)) similar changes to whey protein and $\beta$-lactoglobulin were observed for egg albumen. There was an increase in $\beta$-sheet structures and a decrease in $\alpha$-helical structures in both heated and high pressure treated egg albumen and ovalbumin, with significantly higher levels of $\beta$-sheet structures in heated samples ($p<0.05$). Quantitative changes in secondary structure were studied by deconvolution techniques and indicated an increase in $\beta$-sheet structures from 34 % (native) to 51% for heated ovalbumin ($p< 0.05$), and no change in random coil in the heated samples. In high pressure treated ovalbumin the $\beta$-turns content doubled from 12% to 25%; this was not observed in $\beta$-lactoglobulin. There was a significant decrease in $\alpha$-helix in both heat and high pressure treated ovalbumin with a more pronounced decrease in heated samples. In contrast, to $\beta$-lactoglobulin, a slight decrease in random coil was observed for the high pressure ovalbumin from 13% to 11%.

The increase in random structure in high pressure treated $\beta$-lactoglobulin suggest that high pressure may denature this protein in a manner that increases random structures at the expense of $\beta$-sheet structures and explains the lower content of $\beta$-sheet structures in high pressure treated samples compared with heated samples. Thermal denaturation of globular proteins is usually accompanied by an increase in $\beta$-sheet structures as stated (section 3.4.2.2.). Contrary to thermal denaturation, high pressure treatment also results in the formation of $\beta$-sheet structures but to a lesser extent than heat treatment. Like $\beta$-lactoglobulin there was further involvement of $\beta$-sheet structures which was indicated by Raman bands at 1626 cm\(^{-1}\) and 1682 cm\(^{-1}\) for heated
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Ovalbumin and 1628 cm\(^{-1}\) and 1680 cm\(^{-1}\) for high pressure treated ovalbumin; these are associated with aggregated intermolecular β-sheets. The formation of β-sheets structures for high gel strength values is therefore important.

Changes in the hydrophobic groups were monitored by following changes in the conformation of Trp and Tyr and aliphatic groups CH\(_2\) and CH\(_3\) (1450 cm\(^{-1}\)), CH bending or CH\(_3\) symmetric vibrations (1350 cm\(^{-1}\)) and the tyrosine doublet ratio at 850/830 cm\(^{-1}\). In contrast to whey protein isolate, egg albumen showed no significant differences for the aliphatic groups CH\(_2\) and CH\(_3\) (1450 cm\(^{-1}\)) for both heated and high pressure treated samples. However, for the CH bending or CH\(_3\)/Trp symmetric vibrations (1350 cm\(^{-1}\)) there was a significant increase in peak intensity for the heated gels compared with high pressure treated egg albumen gels (p<0.05). New broad and sharp peaks at 874-880 cm\(^{-1}\) assigned to trp residue was more pronounced for all high pressure treated samples including ovalbumin and egg albumen and whey proteins, indicating further exposure of hydrophobic groups during pressure treatment. The role of hydrophobic groups during protein gelation has already been highlighted in the preceding section on whey proteins.

Further changes in hydrophobic groups were noted in the presence of sugars. The addition of 20% sucrose solution, showed increased peak intensity for the CH bending or CH\(_3\)/Trp symmetric vibrations (1350 cm\(^{-1}\)) for heated ovalbumin and egg albumen compared with high pressure treated protein samples. In contrast, aliphatic groups CH\(_2\) and CH\(_3\) (1450 cm\(^{-1}\)) showed a decrease in peak intensity for the heated samples and an increase in peak intensity for the high pressure treated samples. These data suggest slight differences in involvement of hydrophobic residues during heat and pressure denaturation even in the presence of sugars. Salt caused an increase in peak intensity for ovalbumin and egg albumen in the CH\(_2\) and CH\(_3\) (1450 cm\(^{-1}\)) region and CH bending or CH\(_3\)/Trp symmetric vibrations (1350 cm\(^{-1}\)), suggesting involvement of hydrophobic interactions.
9.5. Interactions between egg albumen and whey protein isolate mixtures.

Mixed gels of whey/egg albumen were made using three different ratios, 10:5, 5:10, and 7.5:7.5 whey/egg albumen. Synergistic interactions were observed for the 10:5 whey/egg albumen protein mixture. The highest force at maximum compression, Young's modulus and interaction index obtained for the 10:5 whey/egg albumen protein mixture compared, to 5:10 and 7.5:7.5 mixtures. In general, the 10:5 whey/egg albumen protein mixture was more cohesive, and exhibited no syneresis upon compression compared with the 5:10 and 7.5:7.5 mixtures. In product formulation, synergistic interactions of whey and egg albumen proteins can be used to produce stronger gels with lower concentrations of proteins. Whey and egg albumen are globular proteins widely used in meat, dairy and bakery products for their functional properties; these proteins not only serve a nutritional role but are used to create structure within the complex systems of mixed gels.

Mixtures of whey and egg albumen proteins formed gels comprising of interpenetrating networks where both networks were continuous throughout the sample (1.5.5.3). There were notable differences in the gel microstructure of high pressure and heat-treated mixtures. Pressure-treated mixtures produced gels with fewer smaller aggregates whereas heat-treated gels contained large dense aggregates; this reflects the stronger heat-set gels compared with the pressure-treated softer gels. The difference in properties has practical implications during processing and implies high pressure may be used to create novel gel structures with properties different from heat-induced gelation.

The addition of varying levels of sucrose to the mixtures produced synergistic interactions for the protein mixed in the ratio of 10:5 whey/egg albumen in 10% sucrose solution at 90°C. In contrast, there was no synergistic interaction in the presence of 10% sucrose in high pressure treated samples, suggesting the previously mentioned baroprotective effect of sucrose. A 10:5 whey/egg albumen protein mixture in 20% sucrose solution heated at 90°C gave a negative interaction index whereas high pressure treated gels gave the highest interaction index albeit negative.
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This result was surprising, considering that the individual proteins had very low force at maximum compression. In contrast, the addition of 1% (w/w) sodium chloride did not result in synergistic interactions in the 10:5 whey/egg albumen protein mixture for both heated and high pressure treated samples, although the highest gel strength were obtained for this mixture. Negative interaction indices were obtained for all the mixtures suggesting less interactions than expected occurred in the presence of salt.

Use of combined heat and pressure showed interactions were highest in the 10:5 whey/egg albumen protein mixture at 60°C (600 MPa, 20mins), compared with 50°C (600 MPa, 20min). However, for all three combinations 10:5 7.5:7.5 and 5:10 whey/egg albumen protein mixtures the force at maximum compression and Young’s modulus values were lower than expected. Overall, the use of combined high pressure and heat to influence protein gelation showed that pressure is a mild denaturant, depending on protein type and duration of pressure. Therefore combination technologies will depend on the type of product to be made and the desired texture.

Changes in conformation, molecular structure and protein-protein interactions in whey protein, egg albumen protein, ovalbumin and β-lactoglobulin (15% w/w in D2O, pD 7.2) and their mixtures (7.5:7.5 protein w/w) were investigated by FT Raman spectroscopy. Differences in the experimental and the theoretical average spectra of the mixtures indicated changes in β-sheet structures, hydrophobic groups, Trp and Tyr and aliphatic groups CH2 and CH3 (1450 cm⁻¹), CH bending or CH3 symmetric vibrations (1350 cm⁻¹) and the tyrosine doublet ratio at 850/830 cm⁻¹ in both heated and high pressure treated proteins, providing evidence of protein-protein interactions, also observed by large deformation rheology. The addition of sugars and salts promoted further changes in hydrophobic groups as well as changes in β-sheet structures.
9.6. Summary of main conclusions

- Gel formed under high pressure (400-600 MPa for 20 min) were weaker with porous gel microstructure and syneresis upon compression compared with gels produced by heat treatment which were strong with a more compact structure.

- Egg albumen proteins did not gel below 500 MPa in contrast to whey proteins which gelled at 400 MPa.

- Mixed gels of whey and egg albumen proteins produced synergistic interactions during both heat and high pressure treatments particularly in the whey/egg albumen (10:5) protein mixture. The gel strength was significantly higher for the heat treated gels compared with pressure treated gels.

- The addition of sugars changed the rheological properties of the gels and each protein displayed different characteristics; sugars enhanced gel strength in egg albumen compared with whey protein. In contrast, salt enhanced gel strength in whey proteins. Their mixtures exhibited negative interaction indices in the presence of salt for both heated and high pressure-treated protein gels. In high pressure-treated protein mixtures the greatest interaction was obtained in 20 % sucrose concentration, while less than expected values were obtained at 10% sucrose concentration.

- Combination of heat and pressure (600 MPa, 20 min) at 50° or 60°C resulted in much weaker gel strength than expected and the mixtures showed negative interaction indices suggesting that proteins used in this study do not completely unfold at these combinations of pressure and temperature.

- Studies using FT Raman spectroscopy showed changes in protein conformation and molecular structure. Changes in the secondary structure indicated that heat denaturation resulted in a loss of α-helix structure and an increase in β-sheet structures, compared with pressure treatment. There was a greater increase in random coil structure for β-lactoglobulin compared with
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ovalbumin under high pressure indicating a higher stability for ovalbumin gels under pressure for both proteins.

- Changes in the disulphide region were more pronounced in heat-treated gels than pressure-treated gels.

- Hydrophobic interactions were evident during both pressure and heat treatment but were more pronounced in high pressure treatment.

- The addition of sugars and salts appeared to enhance hydrophobic interactions by altering the water-protein structure during both heat and high pressure treatment.

- The differences in rheological properties between high pressure-treated and heat-treated gels can be used for the production of novel products with different mixtures of proteins.
9.7. Future work

There are several findings that can be extended and studied in more detail as follows:

- The use of the combination of heat and high pressure with much higher temperatures than used in this study should be undertaken to elucidate if gel strength will resemble heat alone for both individual proteins and mixtures.

- Raman spectroscopy of samples under high pressure can be undertaken using spectrometers fitted with a diamond anvil cell within a pressure vessel.

- The effect of high pressure compared with heat treatment on the nutritional properties of foods can be investigated.
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