THERMAL AND HIGH PRESSURE EFFECTS ON THE
STRUCTURAL PROPERTIES
OF CONDENSED DAIRY BASED SYSTEMS

A thesis submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy

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2012
DECLARATION

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

Paul George
11th December 2012
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1) George, P., Lundin, L., & Kasapis, S. (2012). Fundamental studies on the structural functionality of whey protein isolate in the presence of small polyhydroxyl compounds as co-solute. Food Chemistry, Accepted for publication


Presentations


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<td>AR-G2</td>
<td>Advanced Rheometer – Generation 2</td>
</tr>
<tr>
<td>ω</td>
<td>Angular frequency</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>Ca^{2+}</td>
<td>Calcium ions</td>
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<td>cP</td>
<td>Centipoise</td>
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<td>DSC</td>
<td>Differential Scanning Calorimeter</td>
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<td>FTIR</td>
<td>Fourier transform infrared spectroscopy</td>
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<tr>
<td>T_g</td>
<td>Glass transition temperature</td>
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<tr>
<td>HPP</td>
<td>High pressure processing</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
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<tr>
<td>LVR</td>
<td>Linear viscoelastic region</td>
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<tr>
<td>G''</td>
<td>Loss Modulus</td>
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<tr>
<td>μm</td>
<td>Micrometer</td>
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<tr>
<td>mM</td>
<td>Millimolar</td>
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<tr>
<td>MDSC</td>
<td>Modulated differential scanning calorimeter</td>
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<tr>
<td>M</td>
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<td>Pa</td>
<td>Pascal</td>
</tr>
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<td>rad</td>
<td>Radian</td>
</tr>
<tr>
<td>σ</td>
<td>Shear stress</td>
</tr>
<tr>
<td>G'</td>
<td>Storage modulus</td>
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<td>TTS</td>
<td>Time temperature superposition</td>
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w/w  Weight by weight
WPI  Whey protein isolate
WLF  William, Landel and Ferry
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Table 6.1: Thermal properties of 60 – 80% (w/w) immunoglobulin systems detected by differential scanning calorimetry
Food science in this modern era focuses on several critical aspects to improve human health and wellbeing, of which one among the key objectives is to improve the quality of processed food by using ingredients of better nutritional value, and also by developing novel processing conditions that preserve its nutritional quality. Globular dairy proteins have been identified as a multifaceted ingredient that has both bioactive properties and structural functionalities. The stability of protein molecules against thermal denaturation and their functional attributes such as surface activity, thickening and gelling characteristics, emulsion stabilisation etc. are subjected to alter with the presence of other ingredients such as sugars and polyols. Whey protein isolate and bovine serum albumin in presence of glucose syrup were investigated for their conformational stability and structuring capabilities. Investigation was extended to high solid protein/sugar systems through thermo-mechanical observations to understand their network and molecular vitrification behaviour. Apart from thermal processing conditions, effect of high hydrostatic pressure on the globular conformation and structural functionality of protein systems were studied. Techniques of small deformation dynamic oscillation in shear and differential scanning calorimetry acts as the primary tools to investigate the transitions associated with protein denaturation, aggregation and structural relaxation.

The first system to be investigated was whey protein isolate which is predominantly composed of β-lactoglobulin, in the presence of glucose syrup as co-solute. Presence of co-solute enhanced the thermal stability of protein molecules against denaturation by increasing the surface free energy of the globular conformation. Elasticity of whey protein aggregates marginally increased with the addition of glucose syrup following thermal treatment. Imaging using confocal laser scanning microscopy reveals that presence of glucose syrup reduces the aggregate size of protein, thus facilitating the formation a uniformly arranged polymeric network, rather than random thick aggregates. Presence of polymeric structures in high solid system induces early network vitrification which was extensively studied using the theories of polymeric free volume. In an effort to understand the effect of co-solute on protein molecules with high content of α-helix, the second system for investigation was formulated with bovine serum albumin (BSA) and glucose syrup. Thermal and mechanical properties of samples were analysed with calorimetry and small deformation dynamic oscillation. Apparently, BSA molecules are stabilised with presence of glucose syrup for systems from low to high solids exhibiting higher denaturation temperature and better network formation.
Third experimental chapter of thesis attempts to explain the effect of thermal denaturation and subsequent aggregation on the structural relaxation of globular proteins in presence of co-solute. Technique of small deformation dynamic oscillation has been widely used to explore the mechanical properties during vitrification process of condensed whey protein isolate and bovine serum albumin in presence of sugar (80% total solids, w/w) which has aggregates of different density. The principle of time-temperature superposition was adapted to understand the free volume and reaction rate theories involved during the vitrification process through Williams, Landel and Ferry (WLF) and Arrhenius mathematical models. From this module of investigation it is evident that, conformational freedom of molecules plays a critical role in the network glass transition temperature of the system.

To investigate the effect of high hydrostatic pressure on globular conformation of protein, pressure treated immunoglobulin samples was examined in comparison to thermal effects at atmospheric pressure. Immunoglobulins exhibit pressure stability throughout the experimental concentration range by conserving native conformation, which results in cohesive structure formation observed by small-deformation dynamic oscillation on shear, modulated differential scanning calorimetry and infrared spectroscopy. Application of the combined WLF/free volume theoretical framework demonstrates that pressurized immunoglobulin preparations are able to form glassy systems upon cooling at subzero temperatures. This has been attributed to a reduction in polymeric free volume under pressure and the development of an efficient friction coefficient amongst tightly packed particles that link to form a three-dimensional matrix. Pressure treated assemblies of condensed immunoglobulins demonstrate viscoelastic behaviour matching that of the thermally treated counterparts, but retain bioactivity, which is largely lost with thermal treatment.
INTRODUCTION

1.1 BIOPOLYMERS

Functionalities of biopolymers in food and pharmaceutical industry has been a subject of interest for past few decades as they provide desired stability, performance and consistency for the products. Several proteins and polysaccharides with repeating monomeric units of amino acids or glucose have been extracted from natural sources which exhibit characteristic functionalities of stabilisers, emulsifiers, viscosity enhancers, gelling agents etc. (Fonkwe, et al., 2003; Gabriele et al., 2009). Adapting the classical approach of synthetic polymer science to the field of biopolymers or hydrocolloids have led to the characterisation and modelling of polymer networks, based on their interaction, structure, functionality and assembly mechanism.

Microstructures of polymer networks are dependent on factors such as functional groups, conformational ordering, molecular weight, solvent quality, salt content, pH etc. Flory (1974) classified protein and polysaccharide network into four different categories based on their conformation: a) Ordered lamellar structure b) Disordered covalent network c) Physically aggregated network (ordered and disordered) d) Disordered structures. Extensive investigation has led to an understanding of the gelation mechanism and network properties of biopolymers which enabled to distinguish the gelling behaviour of polysaccharides as cold setting and globular proteins as heat setting systems.

Polysaccharide based network is a well investigated topic, where its gelation mechanism, micro-structure, effect of solvent quality, pH, salt concentration, interaction within polymeric mixtures etc. has been thoroughly investigated. However, polymeric peptide chains and aggregated globular protein networks in high solid systems were least investigated unlike polysaccharides, therefore our current study focuses on understanding mechanical and thermodynamic characteristics of dairy based globular proteins namely, whey protein isolate (WPI), bovine serum albumin (BSA) and bovine immunoglobulins (Ig) in presence of cosolutes from low to high solid systems.

Proteins are complex polymeric systems formed by the condensation of 21 different amino acids and they play a vital role within biological systems. Unlike glycosidic linkages within polysaccharide systems, proteins are formed of complex amide linkages between various amino acids which reflect the structural complexity of protein based polymers. Based on the structural arrangement of amino acids, proteins can be classified as either globular or fibrous. Globular proteins in their native state possess spherical compact structures but with a
rough surface topology. Regardless of its compact nature, conformations of globular proteins are highly dynamic where the peptide chains and reactive amino groups arrange themselves to adapt with the environmental conditions. Fibrous proteins on the other hand are rod shaped molecules containing linear polypeptide chain which function as structural agent.

1.2 STRUCTURE OF PROTEINS

Linderstrom-Lang in 1952 introduced the concept of structural hierarchy for proteins. Four levels of protein structures have been identified resulting from the arrangement of amino acids and from the folding of polypeptide chains by itself. The four levels of protein structures are: primary, secondary, tertiary and quaternary.

1.2.1 PRIMARY STRUCTURE

The primary structure ($1^\circ$) of protein represents the sequence in which amino acids are arranged in a linear fashion through amide or peptide bonds. Peptide bonds are formed by the condensation of the $\alpha$-carboxyl group and the amino group from the adjacent amino acid with the removal of a water molecule. Generally, the length of protein sequence ranges from 150 – 650 amino acids.

![Peptide bond formation](image)

Figure 1.1 Primary structure of protein (Damodaran, 2008).

Three dimensional structure of protein is determined by the polymeric sequence of amino acids within the peptide chain. Therefore concepts from the synthetic polymer chemistry can help in understand the properties of proteins from a different perspective (Tanford, 1961).
1.2.2 SECONDARY STRUCTURE

Secondary structure (2°) refers to the spatial arrangement of amino acids within the peptide sequence. Amino acid residues when arranged with a definite pattern by the repeating sequence of dihedral angles (φ and ψ) produces periodic structures. Generally, there are two forms of secondary state based on the arrangement, helical and sheet like conformation.

Helical conformation

Pauling and colleagues first described the helical conformation of proteins on the basis of model building studies. Helical structures are composed of 3.6 amino acid residues per turn and is generated by repeating the sequence at a definite torsion angle (φ ≈ -60° and ψ ≈ -50°) thus aligning NH groups into N-terminus and C=O towards the C terminus of the helix. α-helix is stabilised by hydrogen bonding. Within the protein matrix, three forms of helical conformation can be seen which includes α-, 3₁₀- and β-helix of which α conformation is the most predominant and stable. Most of the helical structure found in protein is amphiphilic in nature with one half of the helical structure formed of hydrophilic and other half with hydrophobic residues (Pauling, Carey, & Bronson, 1951).

Sheet conformation

The β-strands of a β-pleated sheet have a helical structure with dihedral angles (φ ≈ -120° and ψ ≈ +140°) that align NH and C=O bonds nearly perpendicular to the long axis of the polypeptide chain. Hydrogen bonding between the β-strands creates β-pleated sheets (Pauling, Carey, & Bronson, 1951). These sheets can either be parallel or anti-parallel. When the β-strands align in a single direction within the sheet, we consider them as parallel, and anti-parallel for the vise-versa arrangement of strands. This difference in the arrangement of the strands produces variation in the geometry of hydrogen bonding, where the anti-parallel β sheet produces hydrogen bonds with zero angle but within the parallel sheet structure the hydrogen bonds lie with an angle. This difference in the architectural pattern makes anti-parallel β sheet more stable than parallel conformation.
1.2.3 TERTIARY STRUCTURE

Secondary structure upon folding to a compact three dimensional form creates tertiary structure ($3^\circ$) through optimisation of various interactions such as hydrophobic, electrostatic, van der walls and hydrogen bonding between the amino acid residues so as to create minimum entropy within the system. The vital mechanism behind this peptide folding phenomenon includes reshuffling of hydrophobic and hydrophilic groups, where the hydrophobic moieties are preserved in the core whereas hydrophilic residues rest at the protein-water interface. Through the process of forming three dimensional structures, the protein molecules reduce the interfacial surface area with the solvent molecules (water). In other words protein molecules undergo this process to minimize the surface contact with the outside environment.

Figure 1.2. Tertiary structure of $\beta$ – lactoglobulin where arrows indicate $\beta$ sheet strands
(Papiz et al., 1986)

1.2.4 QUATERNARY STRUCTURE

Proteins with multiple peptide chains create a complex structure known as quaternary state which includes dimers, trimmers or any higher degree of association. Formation of such oligomeric structures is due to the protein-protein interaction which is a net result of hydrogen
bonding, hydrophobic and electrostatic interactions. As an example, $\beta$-lactoglobulin forms dimers, octomer and even exists as a monomer by the influence of pH.

![Diagram of quaternary structure formation](image)

Figure 1.3 Mechanism of quaternary structure formation (Damodaran, 2008)

From a thermodynamic perspective, native conformation of protein is the most stable, where the system has minimum surface area with the solvent with all the hydrophobic groups being buried in the protein core. The forces that contribute to the folding of protein structure are intra and inter molecular which stabilises the overall protein structure.

1.3 WHEY PROTEINS

Whey proteins are generally considered as the bi-product of cheese manufacture after the precipitation of casein at pH 4.6. The major constituents of whey proteins are $\beta$-lactoglobulin, $\alpha$-lactalbumin, bovine serum albumin, immunoglobulins and glycomacropeptides (Webb, & Whittier, 1970).

$\beta$-Lactoglobulin

$\beta$-lactoglobulin is a globular protein with 162 amino acid residues with a molecular weight of 18.4 kDa. Each monomer of $\beta$-lactoglobulin has one free sulfhydryl group and two disulphide bonds. At natural pH of whey, $\beta$-lactoglobulin tends to exist as a dimer with a molecular weight of 36 kDa (deWit, 1981). The secondary structure of this fraction has approximately 6.8% of $\alpha$-helix, 51.2% of $\beta$-sheets, 10.5% of $\beta$-turns and 31.5% of random
coils (Damodaran, 2008). Upon heating, \( \beta \)-lactoglobulin tends to denature by losing its secondary structure and undergoes aggregation to form a polymeric network.

\textit{\( \alpha \)-Lactalbumin}

\( \alpha \)-lactalbumin is the second major fraction of globular protein in whey. The sequence of peptide comprises of 123 amino acids with 14.2 kDa of molecular weight. It has double the amount of disulphide bonds than \( \beta \)-lactoglobulin making it temperature resistant. However the sulfhydryl-disulphide interchange reaction between \( \alpha \)-lactalbumin and \( \beta \)-lactoglobulin increases the heat sensitivity of the overall whey protein composition (deWit, 1981). The isoelectric point of \( \alpha \)-lactalbumin is around 5.1 and exists as a monomer.

1.4 BOVINE SERUM ALBUMIN

Bovine serum albumin is a water soluble protein present in milk (deWit, 1981). It possesses an oblate shape containing three domains which are stabilised by disulphide bonds. It has a molecular weight of 66.5 kDa comprising of 583 amino acid residues. The stabilised secondary conformation of BSA contains 67% helix, 10% beta turns, and 23% extended chains. Bovine serum albumin contains no \( \beta \)-sheets (Peters, 1996; Brown, 1977). Various environmental stresses like temperature, pressure, salt and solvent quality are believed to alter the above conformational composition.

1.5 BOVINE IMMUNOGLOBULINS

Bovine immunoglobulins accounts to 1.9 – 3.3% of the total proteins in milk. These are classified as glycoproteins which bind to other molecules with high specificity (Horowitz & Pigman, 1977). The major classes of immunoglobulins found in milk are IgM, IgA and IgG. IgG constitutes the major fraction of immunoglobulins in whey with approximately 80% of total immunoglobulins. Monomer of IgG has a molecular weight of 160 kDa which consist of two heavy polypeptide chains with a constant domain of 310-500 residues and a variable region with 107-115 residues. Heavy chains are bridged to light chains with di-sulphide bonds with light chain composing of constant domain with 107-110 residues and variable region with 107-115 residues (Whitney et al., 1976).
1.6 PROTEIN DENATURATION

Native conformation of proteins is sensitive to external factors such as temperature, pressure, pH, salt, reagents etc. In extreme cases of these environmental conditions, proteins tends to lose their three dimensional secondary conformation irreversibly, leading to aggregation of molecules. The three dimensional conformation of molecules plays a vital role in executing the biofunctionality of the protein. As they lose their native secondary structure during denaturation, their functionality within the biological systems are compromised. But on the other hand, the polymeric network of the protein has wide application in food and pharmaceutical industry based on their structure functionality. Whey proteins are typical example of dairy proteins which are widely used in food formulations to replace polysaccharides as a gelling agent.

The concept of protein denaturation has been debated over the years among scientists, as the chemistry of protein denaturation has not been well understood. According to Kauzmann (1959) protein denaturation “denote a process (or sequence of processes) in which the spatial arrangement of polypeptide chains within the molecule is changed from that typical of the native protein to a more disordered arrangement (the terms configuration, conformation and state of folding can be substituted for spatial arrangement in this definition)”. Denaturation is an endothermic process which requires energy input either through heat, light, sound, pressure etc. or combined effect of the above factors. Apart from the physical agents, chemical denaturing agents such urea, acids etc. triggers changes in the conformational alignment.

*Temperature*

Heat is a very common denaturing agent of protein molecules especially in food industry, as much of the foods are thermally processed to increase stability and shelf life. This affects the functional properties of food and can swing either in favour of industry or *vice-versa*. Upon heating above a critical temperature, protein molecules undergo a sharp transition where they lose their secondary conformation. This critical temperature of transition can be termed as denaturing temperature and varies from protein to protein. The mechanism of thermal denaturation involves the stability of non-covalent interactions. As we increase the temperature of the system from the optimum range, the hydrogen bonding and electrostatic interactions become unstable whereas the hydrophobic force within the protein core stabilises until a critical temperature (70 - 80°C). Three dimensional state of protein molecules at
ambient temperature has minimum conformational entropy and are quite stable, whereas on heating the entropy of the system increases and favours an unfolded state. Therefore the stability of protein at a given temperature can be considered as the net effect of all these factors (Creighton, 1993).

Denaturation of β-lactoglobulin is a widely investigated topic over the years. Using the technique of optical rotatory dispersion, it has been found that β-lactoglobulin, dissociates from the quaternary conformation (dimer) at temperatures between 20 and 45°C. Upon heating until 85°C a reversible change in the optical rotation was observed but at temperatures above 85°C irreversible changes was recorded (Sawyer, 1968; McKenzie, 1971). Similar observations were made for other globular proteins where irreversible reaction occurs beyond a critical temperature.

**Hydrostatic Pressure**

Like temperature, hydrostatic pressure is a medium for the denaturation of protein. Most proteins undergo denaturation at pressures between 1-12 kbar. Denaturation pressure for each class of protein depends on several factors such as molecular weight, number of di-sulphide bonds, level of total solids (w/w) etc. The peptide chains with protein molecules are flexible and compressible. Upon application of high pressure the void spaces within the arrangement of amino acids gets compressed. The average partial specific volume ($\nu^0$) for globular proteins is 0.74 mL/g which can be expressed as the sum of three components.

\[ \nu^0 = Vc + Vcav + \Delta Vsol \]  

(1.1)

where $Vc$ denotes the sum of atomic volume, $Vcav$ represent the sum of the volumes of the void spaces in the interior of the protein and $\Delta Vsol$ represent change in volume during hydration (Gekko, & Hasegawa, 1986; Damodaran, 2008).

Application of high hydrostatic pressure is an emerging non-thermal technology in industries for the inactivation of microorganisms and also for the gelation of proteins (Lado, & Yousef, 2002). Exposure of beef muscles to 1- 3 kbar hydrostatic pressure induces breakdown of myofibrils which finds application in tenderising meat (Suzuki, Watanabe, Iwamura, Ikechi, & Saito, 1990) and gelation of myofibrillar proteins (Angsupanich, Edde, & Ledward, 1999).
Shear

Mechanical force generated through high shear can lead to protein denaturation. Extreme agitation results in the formation of air bubbles which adsorb protein on air-liquid interface. Energy of the air-liquid interface is higher than the bulk phase leading to the conformational changes in protein molecules. Flexibility of the peptide chain is one of the governing factors for the denaturation where flexible chains are highly prone to denaturation than rigid ones (Nishioka, & Ross, 1981).

pH

Protein molecules are amphoteric thereby possessing both repulsive and attractive forces. Net repulsive forces generally don’t support stabilised state of protein. If the system has pH less than the isoelectric point the net charge of the protein will be positive but if the pH is above the isoelectric point the net charge will be negative. Apart from the number of charged groups, the location of them also plays a vital role in the stabilisation of protein as a function of pH. Extreme alkaline pH induces more of unfolding than extreme acidic pH and this result from the ionisation of partially buried carboxyl, phenolic and sulfhydryl groups. This makes the polypeptide chain to unwind thus exposing to the aqueous environment (Nakai, & Modler, 1996; Damodaran, 2008).

Small molecular weight additives

Addition of small molecular weight co-solute has an effect on the conformational stability of proteins. Compounds like urea, guanidine chloride (GuHCl) and detergents destabilise the native folding of peptide bonds resulting in denaturation of proteins (Dyer, Nelson, & Murai, 1992). Stabilising agents like sugar promotes the preferential hydration of the protein surface but by weakly interacting with the protein whereas the destabilising agents binds to the surface of the protein molecule causing dehydration (Grandori, Matecko, Mayr, & Muller, 2001). Protein when present in the mixture of stabilising and destabilising agents follows additivity rule. Concentration of GuHCl to destabilise protein increases in the presence of stabilising agents such as sucrose (Taylor et al., 1995).
1.7 GLOBULAR PROTEIN AGGREGATION

Denatured protein molecules undergo aggregation to form a polymeric network. It is believed that, at ambient temperature the hydrophobic groups are buried within the protein core. Upon heating above the denaturation temperature, free energy of the system increases within the protein molecule leading to unfolding of the structure, thus exposing the hydrophobic groups. For the system to attain thermodynamic stability, molecules forms network to bury the hydrophobic regions. Aggregates resemble either as a fine stranded network or amorphous particular structures (Ross-Murphy, 1998). Within the secondary state, there is a decrease in the percentage of $\alpha$-helix and an increase in the $\beta$-sheet. Morphology of the heat set gels are dependent on environmental conditions such as pH. For pH values below isoelectric point, fibrillar networks are formed rather than particular stranded gels (Kavanagh, Clark, & Ross-Murphy, 2000). Rod structures form side to side aggregation and its length depends on ionic strength (Aymard, Nicolai, Durand, & Clark, 1999). These are of particular interest in medical science as they resemble the mechanism of $\beta$-amyloid fibrils. Fibrillar protein deposits in dense and insoluble form damaging organs, especially the brain. These are generally referred to as prion disease like bovine spongy encephalopathy in cattle and dementias such as Alzheimer’s and Huntington’s chorea (Takahashi, Ueno, & Mihara, 1999).

Interactions within the protein molecules can be covalent such as disulphide or iso-amide or non-covalent which includes hydrogen bonding, electrostatic interactions or hydrophobic interactions. In most occasions it is a combination of covalent and non-covalent interactions. Gelation mechanism of $\beta$-lactoglobulin has been widely investigated in the past to have a general idea about the aggregation pattern of globular proteins (Bromley, Kerbs, & Donald, 2006).

Nature of globular protein gel is also dependent on the concentration of the protein in the system and also the temperature of denaturation. For all the globular protein, a critical gelation concentration $C_0$, is required to form strong networks. If the system has protein concentration below the critical limit, the end products remains in the solution state but with increased viscosity. Figure 1.5 describes the influence of protein concentration and temperature of the system to form a strong gel.
Figure 1.4 Aggregation pattern for heat set globular protein gels. For pH values below isoelectric point fibrils are formed by the aggregating monomers (A). Under other conditions, a pre-aggregate is formed which forms the particulate gels (B) (Gosal & Ross-Murphy, 2000).
Figure 1.5 Phase diagram depicting the critical concentration and temperature to form globular protein gel where $T_u$ represents the minimum denaturation temperature and $C_0$, the critical gelation concentration of protein (Gosal, & Ross-Murphy, 2000).

1.8 EFFECT OF CO-SOLUTE ON PROTEIN CONFORMATION

Much of the food and pharmaceutical formulation with globular proteins contains other function ingredients such as sugars or polyols (McClements, 2002). These co-solutes alter the properties of globular proteins from their native state. Addition of neutral solvents to the system of globular proteins affects its thermal stability, (Baier, Decker, & McClements, 2004; Baier, & McClements, 2003; Dierckx, & Huyghebaert, 2002), gelation (Baier, & McClemments, 2001, 2005; Kulmyrzaev, Bryant, & McClemments, 2000) and foaming properties (Davis, & Foegeding, 2007; Lau, & Dickinson, 2005; Murray, & Liang, 1999). The molecular properties of co-solutes such as size, charge, shape etc. plays a vital role in altering the functional properties of globular proteins. Therefore determining the influence of co-solutes based on their physicochemical properties can lead to the utilisation of globular proteins to a better extent by increasing the conformation stability against environmental stress such as temperature or pressure (McClements, 2002). Many studies have shown that the conformational stability of protein in the presence of co-solutes is because of preferential
interactions between the protein surface and co-solute molecules within the system (McClements, 2002; Saunders et al., 2000; Timasheff, 1998).

Timasheff (1998, 2002) have identified the preferential interaction between protein molecule and co-solute as two different kinds: steric exclusion and differential interaction. The principle of steric exclusion relates to the dimension and size of co-solute and solvent molecules. It is believed that, if the size of the co-solute molecules is either smaller or larger than solvent molecules, the co-solutes gets preferentially excluded or accumulated on the protein surfaces. In the scenario of differential interaction, the factor that contributes is the strength of the molecular interactions between co-solute or solvent with the protein molecule. If the co-solute exhibit variable attraction to the protein molecule either less or more than the solvent, then they get preferentially excluded or accumulated. The net result of steric exclusion and differential interaction can be considered as the overall preferential interaction for a given protein - co-solute combination (McClements, 2002). Protein molecules prefer to have small surface contact area with the co-solute, for which the best conformation is the folded state. Thereby addition of co-solute like polyols or sugars can stabilise the folded native conformation of protein molecules. This can be witnessed by the elevation in denaturation temperature of protein.

1.9 EFFECT OF CO-SOLUTE ON PROTEIN FUNCTIONALITY

Water Solubility

Damodaran (1998) defined water solubility of a given protein as the relative strength of interaction with other protein molecules compared to interactions with other constituents within the medium. If the magnitude of protein-solvent interaction is higher than the protein-protein interaction then the protein molecules prefers to be surrounded with the solvent molecules. But if the protein-protein interactions are more favourable than the solvent, the protein molecules prefer to stand together, leading to precipitation. Water solubility of protein molecules depends on the strength of hydrophobic groups on the surface where high hydrophobicity leads to poor water solubility. Addition of small interacting co-solutes can alter the conformation of the protein molecules leading to the variation in protein solubility (Arakawa, & Timasheff, 1985).
**Protection against Temperature**

Thermal processing like sterilisation, pasteurisation, cooking, spray drying etc. are commonly found techniques in food industry to increase the shelf life of food products by reducing the microbial load. This leads to biochemical modification of the molecules through processes like denaturation, gelatinisation, gelation etc. Globular proteins are one of the ingredients that get adversely affected by heat where it loses its native conformation. This leads to altered functionality of the peptide chains where they tend to aggregate. It has been found that addition of small molecules like sugar or polyols, can stabilise or protect globular proteins from temperature (MacDonald, Lanier, & Giesbrecht, 1996).

**Protection against Pressure**

High pressure processing is now considered as an alternative to thermal processing in food industry. However as like thermal processing, pressure can induce physicochemical changes to protein molecules. Globular proteins under high pressure unfold to form aggregates, thus changing its functional property. Protein systems under high pressure have been extensively studied in the past where presence of co-solutes, especially sugar has provided a baroprotection for the protein molecules from being denatured. The conformational changes of \(\beta\)-lactoglobulin solutions under high pressure have been investigated in presence and absence of sucrose. Presence of sugar (5%) has reduced the denaturation and aggregation of protein molecules during pressure processing (Iametti et al., 1998, 1999). Co-solutes are expected to be preferentially excluded more from the unfolded state than from the folded state of the protein molecules. This is hypothesised to be the reason for the pressure stabilisation of the protein molecules in the presence of co-solutes. (McClements, 2002).

**Effect on Gelation of Globular Proteins**

Structure functionality of globular proteins has been well established as they form three-dimensional networks that can entrap water and other water soluble components. Functionality of this viscoelastic material depends on the spatial organisation and molecular interactions, of the protein within the gel network which depends on protein type, protein structure, aqueous phase composition and mechanical stress (Zeigler, & Foegeding, 1991; Doi, 1993).
Globular proteins in native state do not form gels where the folded state remains in the aqueous phase of the system, as the repulsive forces dominate the attractive forces. During the process of heating, the protein molecules unfold and expose the non-polar and sulfhydryl group from the core to the crust which then interacts with each other and leads to protein aggregation (Mulvihill, & Donovan, 1987). Addition of co-solutes can alter the gelation mechanisms of globular proteins. Following outcomes are expected on the addition of co-solutes to the system of globular proteins. The denaturation or aggregation temperature of protein is altered to higher or lower temperature. Co-solute changes the magnitude of intermolecular forces (attractive and repulsive) between the protein molecules which reflects the structural organisation and intermolecular molecular bonds of the network. By the addition of co-solutes, we increase the level of total solids within the system, which leads to increased viscosity. This decreases the rate of protein interaction thus the duration for protein aggregation increases (McClements, 2002).

1.10 HIGH SOLID BIOPOLYMER SYSTEM

High solid systems or systems with low moisture finds wide application in food and pharmaceutical industry as like systems of low and intermediate levels of solids. Over the time, the interest for understanding the physicochemical and functional characteristics of high solid material has been increasing in both academic and industrial arena. Many state diagrams and mathematical models have been developed so far which finds application in dehydrated and partially frozen foods, and pharmaceutical compounds (Kasapis, 2004). Efforts have been made in the past to understand the functionality of globular proteins in the presence of co-solutes until intermediate levels of solids (> 50%, w/w). But for the high levels of co-solutes (>60%, w/w), there has been no research done to date. Therefore the current work will focus on understanding the physicochemical properties from low to high levels of co-solute within the system of globular proteins. Understanding the true amorphous character of these high solid systems requires knowledge on glass transition and glassy state. Glass transition and glassy state of polysaccharide/co-solute system has been widely investigated in the past where the network property of polysaccharides and gelatin has been investigated. The technique of small deformation dynamic oscillation on shear was used to observe nature of network variation using the master curve of viscoelasticity which covers different regimes of rubbery and glassy state as a function of temperature and time (Kasapis, & Sablani, 2005).
Low levels of co-solutes such as sucrose or glucose syrup up to 40% solids on systems of κ-carrageenan, agarose, deacetylated gellan and gelatin creates stronger network whereas replacing sugar with hydrogen bond disrupters such as urea reduces the network strength. This also emphasise the importance of hydrogen bonds for the polymer interaction (Kasapis et al., 2003). As we approach intermediate levels of co-solute, a noticeable drop in the strength of network for polysaccharide systems can be observed, whereas gelatin on the other hand shows a better network strength (Al-Amri et al., 2005). It is believed that polysaccharides at the intermediate levels of co-solute transform from a highly enthalpic network to an entropic system whereas gelatin on the other hand gets phase separated and retains strong structural integrity. Effect of co-solutes at different concentration, especially at levels of solids above 40% (w/w) on globular proteins such as whey protein isolate and bovine serum albumin can help us determine the functionalities such as altered denaturation temperature, gels with better viscoelastic properties etc.

1.11 GLASS TRANSITION

Amorphous systems undergo a phenomenon of glass transition where disordered supercooled liquids switch between liquid and solid states. This transition occurs over a temperature range or more often referred to a definite temperature called glass transition temperature ($T_g$). There occurs a complete rearrangement of the molecules during this transition process. The changes mainly occur in the translational mobility of molecules and the thermodynamic character of the system matches with the second order phase transition (Sperling, 1992; White, & Cakebread, 1996). In 1960’s the phenomenon of glass transition was identified within the amorphous system with not much of understanding of the molecular processes involved. By the development of modern analytical tools, the true picture of glass transition was obtained. Several variables such as enthalpy, entropy, volume changes, changes in heat capacity and thermal expansion were used to define glass transition (Roos, 1995). Most food particles upon removal of water forms amorphous or partially amorphous structures as a result of different processing steps like dehydration, baking, spray drying, extrusion etc. (Slade, & Levine 1991). With the development in the field of amorphous systems, many mathematical models and state diagrams were developed which helped to understand the factors affecting relaxation time which is the rate at which the changes occurs by the influence of an external disturbance on the material (Levine, & Slade, 1986; Roos, & Karel, 1990; Roos, 2008).
Water being a universal solvent plays a major role in all the food compounds. In relation to the glass transition, water acts as a plasticizer, or softening agent. This has great practical significance during food processing and storage. Hydration of amorphous organic solid dramatically influences the vitrification by reducing the glass transition temperature. In past, there have been many models developed to predict the glass transition temperature of systems based on the concentration of residual water such as Gordon-Taylor equation. But with the development of modern analytical tools and by looking in depth the molecular process involved during the glass transition, it is believed that $T_g$ of a system depends on several factors like the chemical composition, molecular weight etc. and based on a universal factor such as concentration of water within the system (Kasapis, 2008).

### 1.12 ANALYSING GLASS TRANSITION

Changes in parameters such as dielectric, mechanical and thermodynamic properties of the material help to record the glass transition of a material. Differential scanning calorimetry is the most preferred method to analyse glass transition which detects change in heat capacity of the material during the transition period. Conventionally, the change in the slope of heat flow signal resulting from the variation in the heat capacity helps to identify this transition process (Roos, 1995).

Mechanically, there is a dramatic change in the relaxation period for the rubbery and glassy material. Thermal analysis and mechanical spectroscopy helps to understand the changing relaxation period of the material along with the associated thermodynamic properties during the transition period. Techniques like, electron spin resonance spectroscopy and nuclear magnetic resonance helps to understand glass transition from an atomic level where as Fourier Transform infrared spectroscopy and other vibrational spectroscopic techniques help to understand the changing molecular packing of the material during this process. Various theories have been put forward to describe the molecular processes involved during the glass transition period. From a thermodynamic point of view, glass transition can be defined as the point at which the configurational entropy of the system reaches zero. Experimentally determined glass transition temperature, involves the kinetic factor where the time scale of observation and heating and cooling rates influence the glass transition temperature (Arridge, 1975). Therefore the time related relaxation in the glassy state could be given by noting the temperature at which the sample attains equilibrium, but fails to give an explanation based on the molecular packing of the glassy state (Kasapis, 2008). For the mechanical understanding of rubber to glass transition, synthetic polymer scientists widely
use the concept of free volume. Ferry (1980a) described free volume as the vacant space between the packing irregularities of long chain segments, or as the space required for the free rotation and vibration of the molecules. In polymer melts the free volume constitutes around 30% of the total volume and it reduces to 3% within the glassy consistency (Cangialosi, Schut, van Veen, & Picken, 2003).

Small deformation dynamic oscillation on shear can help us differentiate different viscoelastic state of the material including the glassy state as shown in Figure 1.6. with parameters such as storage modulus ($G'$) describing elastic behaviour and loss modulus ($G''$) representing viscous property. Typically in a gelling polysaccharide system at high temperatures, sample tent to be in a liquid state with viscous moduli over elastic moduli. As we lower the temperature, gelling polysaccharides undergo a coil to helix transition where systems attain a rubbery plateau with elastic moduli over viscous moduli. On further cooling there will be a considerable increase in the values of storage and loss moduli known as glass transition, marked as third phase in Figure 1.6 which ultimately will lead to a glassy consistency (phase four). Similar trend can be observed as we go from low frequency, molecular weight or concentration to higher order.

Figure 1.6. Variation of $G'$ (■), $G''$ (□) and tan δ (○) as function of temperature, frequency, molecular weight and concentration of polymers (Ong, Whitehouse, Abeysekera, Al-Ruqaie, & Kasapis, 1998).
Viscoelastic properties of a material are influenced by both temperature and time. In order to fully grasp the viscoelastic property of the material we need to analyse the sample over a wide range of temperature and time. With the current technology, attaining wide range of temperature is feasible whereas range of time is limited. Therefore we introduce the concept of reduced variables or time temperature superposition. The viscoelastic response of a material for a given temperature and time is interchangeable i.e. effect of increasing frequency resembles viscoelasticity at lower temperature (Ferry, 1980b). This principle has been used in time-temperature superposition where inaccessible frequency range can be obtained by performing frequency sweeps at different temperature. In time temperature superposition, a series of frequency sweeps are obtained at regular intervals of temperature. One of the temperatures is selected as the reference temperature ($T_0$) and using the curve at this temperature as reference, we horizontally shift rest of the curves along the time scale. Successful shifting of the curve will result in the development of a mastercurve of viscoelasticity as a function of time or frequency as shown in figure 1.6. Shifting of the curves reproduces a set of shift factors called $a_T$, which represents the ratio of any relaxation time at temperature $T$, to its value at temperature $T_0$.

1.13 WILLIAMS, LANDEL AND FERRY EQUATION

William, Landel and Ferry in 1955 coined the empirical expression which describes the relation between temperature and shift factors $a_T$ during glass transition (William, Landel, & Ferry, 1955).

$$\log a_T = -\frac{(B/2.303f_0)(T - T_0)}{(f_0/\alpha_f) + T - T_0}$$

where $B/2.303f_0$ and $f_0/\alpha_f$ are considered to be the WLF parameters and $T_0$ is the reference temperature. WLF equation can give information on the free volume of the matter. As per the free volume theory, the total volume of a system $v$, comprises of free volume $v_f$ and the volume occupied by the matter $v_0$.

By theory, the shift factor $a_T$ represents the ratio of specific relaxation at temperature $T$, to the reference temperature $T_0$ where shift factor can be described as

$$a_T = \frac{\tau_T}{\tau_{T_0}}$$
where \( \tau \) is the relaxation time. Relaxation time \( \tau (\eta / T \rho) \) is proportional to viscosity (\( \eta \)), temperature (\( T \)) and density (\( \rho \)). Therefore the expression for shift factor \( a_T \), can be written as (Ferry, 1980c)

\[
a_T = \frac{\eta T \rho_o}{\eta_o T \rho}
\]  

1.4

where \( \eta_0 \) and \( \rho_0 \) is the viscosity and density of the material at the reference temperature \( T_0 \). According to Doolittle (1951) free volume is the deciding factor for molecular relaxation and viscosity as described by the free space equation.

\[
\ln \eta = \ln A + B \nu_f / \nu f
\]  

1.5

where \( A \) and \( B \) represents the empirical constants depending on the matter. Combining the equation 1.5 and 1.4 we can redefine \( a_T \) as.

\[
\log a_T = \frac{B}{2.303} \left( \frac{1}{f} - \frac{1}{f_o} \right) + \log \left( \frac{T_o \rho_o}{T \rho} \right)
\]  

1.6

where \( \nu = \nu_0 + \nu_f \) and \( f = \nu_f / \nu \) and \( f \) and \( f_o \) represents the fractional free volume at temperature \( T \) and \( T_0 \) respectively. For simplicity, we ignore the second term at the right hand side of the equation as it doesn’t contribute much therefore the equation for \( a_T \) transform to

\[
\log a_T = \frac{B}{2.303} \left( \frac{1}{f} - \frac{1}{f_o} \right)
\]  

1.7

As we know that, free volume of the matter is dependent on temperature where free volume increases with increase in temperature and \textit{vise versa} as identified by Ferry (1980c). Therefore

\[
f = f_o + af (T - T_0)
\]  

1.8
where $\alpha_f$ is the thermal expansion coefficient. Substituting equation 1.8 in 1.7 gives information about the variables such as shift factor ($a_T$), thermal expansion coefficient ($\alpha_f$) and factorial free volume ($f_0$) providing the following expression.

$$\log a_T = -\left(\frac{B}{2.303 f_0}\right)\frac{(T - T_o)}{f_0 + T - T_o}$$

Comparing the equations, 1.2 with 1.9, we obtain information about the WLF constants $C^o_1$ and $C^o_2$ in terms of fractional free volume and thermal expansion coefficients as.

$$C^o_1 = \frac{B}{2.303 f_o}$$

$$C^o_2 = \frac{f_o}{\alpha_f}$$

The phenomenon of reducing free volume as a function of lowering the temperature is applicable only until the glassy state, as the total free volume of the system gets reduced to 3% of the total volume. As a result the above function is applicable for system in rubbery state and not for material in glassy state.

### 1.14 ARRHENIUS THEORY

Stefan (2001) reported that, to determine the shift factors in glassy state modified Arrhenius equation (Peleg, 1992) will be appropriate.

$$\log a_T = \frac{E_a}{2.303 R} \left(\frac{1}{T} - \frac{1}{T_0}\right)$$
This helps to identify the activation energy \( (E_a) \) for an elementary flow process in the glassy state which is independent of temperature. Upon plotting the shift factors as a function of temperature, samples in the rubbery state obeys the WLF equation based on the free volume and thermal expansion coefficient, whereas from a particular temperature the shifting based on WLF model becomes invalid and follows the Arrhenius concept. This discontinuity can be defined as the transformation from the free volume derived effects to the process of energy barrier for the molecular rotation in the glassy state. This particular temperature of discontinuity can be defined as the mechanical glass transition temperature (Kasapis, 2008).
1.15 REFERENCES


CHAPTER 2
MATERIALS AND METHODS

2.1 RHEOLOGY

Rheology is the field of science widely applied today which deals with the viscoelastic properties of materials. The word rheology is derived from the Greek *rheo* meaning flow. Field of rheology has been applied to different fields such as synthetic and biopolymer science, colloidal science, fluid dynamics, food science, emulsion technology etc. (Mckenna, 2012). Being so diverse, many definitions have been put forward to define this field. Perhaps the simplest of them is, rheology is the study of the relationship between stress and strain within a material as a function of different parameters such as time, temperature etc. Stress can be defined as the force per unit area (N.m\(^{-2}\) or Pa) and strain is the resulting fractional deformation (dimensionless).

Based on the mechanical behaviour, materials are divided into two by the laws of physics: solids and liquids. Ideal solids as defined by Hooke’s law, stress (\(\sigma\)) is directly proportional to strain (\(\gamma\)) and for ideal liquids stress (\(\sigma\)) is proportional to rate of strain (\(d\gamma/dt\)). The proportionality constants for solids and liquids are termed as modulus (\(G\)) and viscosity (\(\eta\)) respectively, where \(G = \sigma/\gamma\) and \(\eta = \sigma/(d\gamma/dt)\). This response from material represents extremes conditions of an ideal solid or liquid. In reality, most of the materials demonstrate combined solid like (elastic) and liquid like (viscous) behaviour, giving rise to ‘viscoelasticity’.

2.1.1 SMALL DEFORMATION DYNAMIC OSCILLATION

It is relevant to obtain information about the viscoelastic property of a system as it undergoes molecular and structural changes like gelation, melting, glass transition etc., for which the timescale of measurement should be shorter than the actual physical process within the sample and without disturbing the natural mechanisms involved.

Sample undergoing a time-dependent strain wave can be modelled as,

\[ \gamma = \gamma_0 \sin \omega t \]  \hspace{1cm} 2.1

where \(\gamma\) represents the time dependent strain, \(\gamma_0\) is the maximum strain and \(\omega\) is the angular frequency (Figure 2.1a). Shear stress wave will also be a sine wave function with different amplitude and phase.
where \( \sigma \) represents the time dependent stress, \( \sigma_0 \) is the maximum stress amplitude and \( \delta \) is the phase angle between the two waves.

The stress for a pure elastic sample is proportional to strain, therefore the strain and stress waves will be in phase to each other (Figure 2.1b). For an ideal liquid, the stress will be proportional to the strain rate for a given time, and the maximum stress will occur when the slope of the strain wave is at maximum (Figure 2.1c). Viscoelastic sample will therefore have stress wave amplitude proportional to strain amplitude, but will have contributions from both in-phase and out-of-phase components (Figure 2.1d). Through the combination of viscoelastic function, stress wave can be represented as,

\[
\sigma = \gamma_0 (G' \sin \omega t + G'' \cos \omega t)
\]

where \( G' \) is the in-phase storage modulus and \( G'' \) is the out-of-phase loss modulus.

Rearranging equation 2.2, stress can be expressed as

\[
\sigma = \sigma_0 (\cos \delta \sin \omega t + \sin \delta \cos \omega t)
\]

From equation 2.3 and 2.4, storage and loss modulus can be defined as

\[
G' = \left(\frac{\sigma_0}{\gamma_0}\right) \cos \delta
\]

\[
G'' = \left(\frac{\sigma_0}{\gamma_0}\right) \sin \delta
\]

Ratio of the above equation represent the loss tangent (\( \tan \delta \)), which aids in detecting structural changes during broad transitions such as gelation, melting or glass formation.

\[
\frac{G''}{G'} = \tan \delta
\]
2.1.2 CLASSIFICATION OF NETWORK BASED ON OSCILLATORY MEASUREMENTS

Mechanical spectrum of a material based on storage modulus ($G'$), loss modulus ($G''$) and dynamic viscosity ($\eta^*$) as a function of oscillatory frequency ($\omega$) gives an indication of the structural type. For a dilute solution, as the frequency increases the viscosity remains constant but the loss modulus representing viscous factor increases proportionally with frequency, with similar trend observed for elastic component (Figure 2.2a). These spectra for the dilute systems were the first successful model on molecular basis using bead-spring arrangement (Rouse, 1953; Zimm, 1956). As we increase the concentration of polymer, there is a high probability that the direct polymer-polymer interaction enhances. Above this coil overlap concentration ($C^*$), a system is said to be in semi-dilute state. By increasing the concentration of the polymer within the system, polymer-polymer interaction becomes more dominant and a uniform density of polymer is achieved within the system. The polymeric chains begin to form continuous network through entangled couplings. Frequency sweep on such systems shows $G'$ and $G''$ becoming less dependent on frequency and during higher order of frequency, $G'$ exceeds $G''$ (Figure 2.2b). Similar observation can be made when true entangled coupling occurs. Within the mechanical spectra for a strong gel, storage and loss
moduli are independent of the angular frequency (Figure 2.2c). For category of materials with weak gels like texture, the spectrum resembles a solid like material with frequency dependence on storage and loss modulus with a higher average value of loss tangent (Figure 2.2d). The relationship between time dependent properties and the level of internal structure is very evident from the following representative mechanical spectra.

![Mechanical Spectra](image)

Figure 2.2 Mechanical spectra representing (a) dilute solution, (b) entangled solution, (c) strong gel and (d) weak gel (Richardson & Kasapis, 1998).

Based on the above principles of rheology, we perform different kinds of oscillatory measurements as a function of temperature, time, frequency and strain to identify the changing functional properties of globular proteins by the addition of co-solutes (Rao, 2007).

**Strain Sweep**

Strain sweep helps to determine the extent of linear viscoelastic range of a network. The logic of small deformation oscillatory measurements in shear is to conduct rheological measurements within the linear viscoelastic region without disturbing the network. Within the region of viscoelasticity, the magnitudes of stress and strain are linear, producing constant
values of storage and loss modulus for a range of strain. Linear viscoelastic region is dependent not only on the network but also on temperature and frequency of observation.

Temperature Ramp

For heat set gels like globular protein aggregates, storage modulus takes over loss modulus upon heating at a particular temperature. At high temperature, protein molecule denatures and form aggregates. This is considered as sol-gel transition at high temperature (65 – 80°C) as opposed to sol-gel transition of polysaccharide in low temperature (20 – 35°C). These experiments are carried out at fixed frequency and strain.

Time Sweep

Time sweep represents the isothermal step where the temperature, frequency and strain are kept constant. During this experiment, the gels get matured through the complete denaturation of residual protein molecules. This is observed by increase in the value of storage modulus as a function of time.

Frequency Sweep

Mechanical spectra as a function of frequency sweep helps to understand the network as explained in the preceding section. It relates mechanical properties of a network to the time scale of observation. In addition to the classification of the network, the data from frequency sweeps at subzero temperature will be used to understand the mechanical glass transition through time temperature superposition.

2.2 DIFFERENTIAL SCANNING CALORIMETRY

Calorimetry is the field of science dealing with the measurement of heat or energy transfer involved during various chemical and physical reactions. (Rahman, Machado-Velasco, Sosa-Morales, & Velez-Ruiz, 2009) For a typical calorimetric experiment, a chemical reaction is made to happen in a sealed container which is insulated from outside environment (Coleman, & Craig, 1996). By knowing the heat capacity \( (C_p) \) of the container and sample, and also by measuring the change in temperature \( (\Delta T) \) of the system, the heat
content of the chemical reaction or the change in enthalpy involved with the reaction ($\Delta H$) can be calculated:

$$\Delta H = \Delta T \, Cp$$  \hspace{1cm} 2.8

In most cases the change in system temperature is kept small; thereby heat capacity is kept constant. From such experiments evolved the definition of ‘calorie’ which is the unit of measurement for heat content or energy. Calorie can be defined as the amount of heat necessary to raise the temperature of 1 g of water at 15 °C by 1°C at atmospheric pressure.

The above concept has been developed commercially to bring forward the modern differential scanning calorimeters. International Confederation for Thermal Analysis (ICTAC) in August 1977 defined differential scanning calorimetry as a technique in which the difference in energy inputs into a substance and a reference material is measured as a function of temperature, while the substance and the reference material are subjected to a controlled temperature program. As this technique measures the thermodynamic properties of thermally induced transitions, it has been applied to various fields of science. Within the biological science, it has been used to study the conformational state of proteins, DNA binding to protein, transitions of biopolymers and the interaction between several biological molecules.

2.2.1 HEAT FLUX AND MODULATED DIFFERENTIAL SCANNING CALORIMETRY

Conventional DSC can be described as an analytical technique where the difference in heat flow between the sample and an inert reference is measured as a function of time and temperature, as both sample and reference is subjected to controlled environment of time, temperature, atmosphere and pressure. The cross section of the conventional DSC is as shown in Figure 2.3
Metallic disc made of constantan alloy serves as the platform for the heat transfer to and from the sample and reference pan. As in pictorial representation, the sample and reference sits on a raised platform and heat is transferred where the differential heat flow to the sample and the reference is measured by a thermocouple. Preheated gas is purged into the chamber to ensure uniform and stable thermal environment so as to obtain good baseline and sensitivity. Through the mechanism of direct heating within conventional DSC, ramping rates up to 200°C/minute can be achieved (TA Instruments, n.d.-c).

Modulated DSC (MDSC) has the same capability of measuring heat flow between sample and reference material. Whereas MDSC uses a different heating and cooling pattern which involves a sinusoidal modulation on the top of the conventional temperature ramp where on an average the sample temperature can be ramped through heating or cooling waves, but not in a linear fashion as like conventional DSC. Therefore the critical parameters to be considered while performing a modulated DSC experiment are: 1) heating ramp rate, 2) period of modulation and 3) temperature amplitude of modulation. In regard to the temperature amplitude of modulation, larger the amplitude, the larger the heat flow response.

\[ \frac{dT}{dt} = \beta + A_T \omega \cos(\omega t) \]  \hspace{1cm} 2.9

where, \( \frac{dT}{dt} \) represents instantaneous heating rate (°C/min), \( \beta \) is the underlying heating rate (°C/min), \( A_T \) denotes modulation amplitude, \( \omega \) is the angular frequency which is \( 2\pi / \) modulation period (min\(^{-1}\)) and \( t \) denotes time (minutes). Equation 2.9 indicates that amplitude
of modulation is directly proportional to sensitivity of transitions therefore experiments with high amplitude of modulation helps to resolve low response transitions.

2.2.2 HEAT FLOW COMPONENTS IN DSC

Equation 2.10 describes the heat flow components for both DSC and MDSC (Thomas, 2005).

\[
\frac{dQ}{dt} = C_p \beta + f(T,t)
\]

where \( \frac{dQ}{dt} \) represents the heat flow, \( C_p \) is the heat capacity, \( \beta \) denotes heating rate and \( f(T,t) \) is the heat flow from kinetic processes. Therefore total heat flow (\( \frac{dQ}{dt} \)) measured by a conventional DSC is composed of two parts where one part denotes heat capacity and rate of temperature change, whereas other part is a function of absolute temperature and time.

Modulated DSC signal helps to determine the total and two individual components thus leading to a better understanding of the complex and overlapping transitions in materials. This is possible in modulated DSC as it uses two heating rates: average heating rate providing information on total heat flow and sinusoidal heating rate giving information on heat capacity. Heat capacity component (\( C_p \beta \), reversing heat flow) and kinetic component (\( f(T,t) \), non-reversing heat flow) helps to resolve complex transitions associated with material. Reversing heat flow exhibits second order phase transition such as glass transition whereas non-reversing heat flow represents first order phase transitions like melting, gelation etc. (TA instruments).

2.3 FOURIER TRANSFORM INFRARED SPECTROSCOPY

Absorption of electromagnetic radiation in the infrared region by a molecule results in changes in vibrational and rotational status. Vibrational frequency of a molecule plays a vital role in determining the absorption frequency whereas the efficiency in the transfer of infrared photon energy from the source to the target molecules represents absorption intensity which is dependent on the change in the dipole moment as a result of molecular vibration. Because of this reason, a molecule will absorb infrared light only if the absorption causes a change in the dipole moment. Within complex systems wavelength normally ranges within the mid-infrared region (600-4000 cm\(^{-1}\)) which is recorded either using dispersive infrared analysis or Fourier
Transform Infrared Spectroscopy (FTIR). In most cases FTIR is preferred as it is faster and has a better signal to noise ratio (Yang, & Zhang, 2011).

Development of FTIR was a great leap forward for the infrared analytical capabilities which offers a number of advantages over conventional infrared systems. Within the mechanical setup, the infrared source emits infrared radiation which passes through an interferometer which modules the infrared beam by performing an optical inverse Fourier Transform on the entering IR radiation. The modulated IR beam passes through the sample where it is absorbed to various extents at different wavelength by various molecules present in the system. The intensity of the IR beam is finally detected by a detector. The critical part of the FTIR is the interferometer.

2.4 CONFOCAL LASER SCANNING MICROSCOPY

Confocal laser scanning microscopy and fluorescence microscopy has become an essential tool in the field of biological, medical and material science due to its functionalities that are not available using the normal contrast modes of optical microscopy (Loren, Langton, & Hermansson, 2007). The availability of wide range of fluorochromes has made it possible to identify various components with a very high degree of specificity. Confocal microscopy proves to be more effective than conventional optical microscopy with features like ability to control depth of field, eliminating the background information away from the focal plane and also provides information on serial optical sections along with spatial filtering that helps in eliminating out of focus light.

Beam of laser emitted from the excitation source passes through a pinhole aperture which is fixed in a confocal plane with a scanning point on the specimen and a second pinhole aperture positioned in front of the detector. The dichromatic mirror reflects the laser and scans the specimen in a defined focal plane. This then excites the florescent dye on the specimen which emits secondary fluorescence that passes back to the dichromatic mirror and are focused at the confocal point of the detector pinhole aperture. The emitted fluorescence gets filtered through the detector pinhole aperture which eliminates out of focus light rays. Refocusing the objective lens within the confocal microscope helps to alter the plane of specimen by changing emission and excitation points which helps to identify the microstructure within the sample up to an extent.
2.5 MATERIALS

2.5.1 WHEY PROTEIN ISOLATE

Whey protein isolate (WPI) was purchased from Murray Goulburn Nutritionals Cooperative Ltd., Victoria, Australia. As documented by the supplier in the certificate of analysis, the composition of whey protein isolate (MG2460) was 91.3 % protein comprising of (β-lactoglobulin, α-lactalbumin, bovine serum albumin, immunoglobulins), 0.7 % fat, 3.5 % moisture, 3.8 % ash and 0.44 % sugar (lactose). pH of 10 % whey protein isolate (w/w) was 6.3 and the bulk density of the powder was 0.45 g/ml. The standard microbial plate count for whey protein analysed was 9,900 cfu/g.

2.5.2 BOVINE SERUM ALBUMIN

Bovine serum albumin was purchased from Sigma Aldrich, USA. According to the certificate of analysis (A-7906), the product was 96 % in concentration determined by agarose gel electrophoresis with a moisture content of 3 % and 1 % bovine serum albumin in 0.15M NaCl possessed a natural pH of 6.9. The molecular weight of albumin from bovine serum is approximately 66 kDa.

2.5.3 IMMUNOGLOBULINS

Immunoglobulins were extracted from whey protein isolate (Murray Goulbourn Nutritionals) by the method of selective salt precipitation using ammonium sulphate. Saturated ammonium sulphate (SAS) solution was prepared by dissolving the salt in distilled water. Saturated ammonium sulphate was diluted to 40 % using distilled water and mixed with whey protein (20 % total solids, w/w) in equal proportion. The formed precipitate was separated through centrifugation at 4000 rpm for 15 minutes at 4 °C. The pellet was redissolved in distilled water and dialysed for 24 hours at 4°C for complete removal of salt (ammonium sulphate). Solution containing immunoglobulins was then freeze dried and stored at -20 °C. All three fractions of immunoglobulins present in the bovine milk are expected to be present in the extract, with IgG dominating the major fraction. This was later confirmed using SDS PAGE analysis.
2.5.4 GLUCOSE SYRUP

Glucose syrup was purchased from Manildra Group (N27/07/08). Certificate of analysis confirms total solids of the material as 81.2% (w/w) with a dextrose equivalent (DE) of 43.40. According to the supplier it’s a wheat starch derivative and has a sulphur dioxide content of 102 mg/kg. pH value of 10% glucose syrup in distilled water was 5.2.

2.5.5 CALCIUM CHLORIDE

Calcium chloride (CaCl₂) was supplied by Sigma Aldrich (C1016) as amorphous white granules with a purity of 95.6% as determined by EDTA titration by the supplier. It has a molecular weight of 110.98 g/mol and form colourless solution at a concentration of 100mg/ml of water.

2.6 INSTRUMENTS

2.6.1 AR-G2

AR-G2 (TA instruments, USA) is a controlled stress rheometer with combined motor and transducer technology. The assembly of this model is in such a way that, the lower component of the measuring system is fixed and the upper component is attached to a shaft which is controlled by an induction motor. Induction motor helps in reducing the friction between rotating and stationary components. The rotating shaft needs to be supported by a bearing. In normal CMT (Combined motor and transducer) rheometers, an air bearing of jet or diffusion type is used, whereas in an attempt to develop the rheometers with minimum friction, AR-G2 is assembled with a magnetic bearing. This helps to obtain the low torque measurements accurately. To investigate properties of systems in glassy state, AR-G2 uses an environmental test chamber (ETC) which allows the purging of liquid nitrogen. Thereby the mechanical properties of systems even at -90 °C can be analysed using small deformation oscillatory measurements in shear. We have extensively used this provision of AR-G2 to analyse the glass transition phenomenon of protein/sugar systems. Alternatively to measure the transitions of materials within the normal temperatures (0-100 °C) such as gelation, melting, denaturation etc., the rheometer was equipped with a peltier plate with a temperature range of -20 – 200 °C. All the measurements on AR-G2 was carried out using parallel plate
geometry and probes with different dimensions were used ranging from 5 – 40 mm in diameter based on the sample and experimental protocol (TA Instruments, n.d.).

2.6.2 PHYSICA, MCR 301

MCR 301 (Anton Paar, Austria), is an advanced controlled stress rheometer to analyse the rheological properties of wide range of materials. It uses an electrically commutated (EC) synchronous motor technology, low friction air bearings and normal force sensors to accurately perform both rotational and oscillatory mode of measurements on liquids and solids. Patented technologies and software interfaces such as TruGap™ (for measuring the gap), TruRate™ (controller in rotation and step strain), TruStrain™ (for accurate strain control) helps to measure and understand the network property of systems more efficiently.

Protein-sugar systems with low and intermediate level of solids were measured using bob and cup measuring geometry (13.33mm and 14.33mm in radius) with peltier control to observe the denaturation and aggregation of globular protein within the temperature range of 0 – 100 °C using oscillatory mode of measurements (Anton Paar, n.d).

2.6.3 Q-2000

Q-2000 (TA instruments, USA) is a modulated differential scanning calorimeter which helps in understanding the thermodynamic properties of a material. It is a modern DSC with superior features like baseline flatness, precision, sensitivity and resolution. It’s been equipped with modern techniques such as 50-position auto-sampler, RCS 90 refrigerated cooling system with a temperature range of -90 – 540 °C, Tzero cell technology and an efficient sinusoidal oscillation which is overlaid over the normal temperature ramping (modulation). Samples are loaded in hermetic aluminium pans sealed using a Tzero press. An empty hermetic aluminium pan serves as the reference (TA instruments, n.d).

2.6.4 microDSC VII

microDSC (Seteram, France) is a highly sensitive calorimeter used to analyse various materials such as pharmaceutical compounds, polymers, food etc. The main advantage of microDSC over conventional DSC is that the sample pan can be loaded with 850mg of material as compared to 15-20mg of sample in conventional DSC; this enables detection of minor transitions within the material. Measurements happen within the calorimetric block
made of double aluminium chamber which can hold two measurement cells (sample and reference cell). Temperature is controlled using two stages of Peltier-effect thermo-elements. Atmospheric and pressurised immunoglobulin samples were examined for their denaturation profiles using microDSC VII (Setaram, n.d).

### 2.6.5 FT-IR – SPECTRUM 100

Spectrum 100 (Perkin Elmer, USA) is a modern Fourier Transform infrared spectrophotometer designed to identify the stretching and vibrations of molecular bonds. The principle hardware components include dynascan interferometer, electronically temperature stabilised detector, source doubling mirror, sigma-delta conversion technology, second detector expandability etc. It was equipped with Universal ATR (UATR) accessory which helps in analysis of wide range of materials, ranging from liquids to glassy material. Software Spectrum serves as the user interface for this unit.

### 2.6.6 LEICA TCS SP5

Leica TCS SP5 (Leica Microsystems, USA) provide the most sophisticated machinery for the confocal laser scanning microscopy. Apart from the usual, it has a fixed Peltier plate with a temperature range of 0 – 100 °C. It aids in capturing the microimages of changing network morphology of protein systems as a function of temperature. Protein samples were stained with rhodamine dye and an emission band width of 553-619nm was used (Argon laser). Objective lens with dimension N PLAN L 20.0 X 0.4 DRY was used (Leica, n.d).

### 2.6.7 QFP – 35L - 600 HIGH PRESSURE FOOD PROCESSING SYSTEM

Science and theory behind high pressure processing has been known for several decades, but the technology to build commercial processing unit came into existence very recently. High pressure stands as a strong substitute for thermal processing with added advantages, provided if we can generate pressures up to 600 MPa. Critical processing parameters are pressure, temperature and holding time and this depends on product and processing safety requirements.

QFP 35L-600 (Avure, USA) can process up to 25kg per cycle with a maximum operating pressure of 600 MPa (87,000 psi). During processing, a maximum temperature of
50 °C can be achieved. This has been manufactured and tested in accordance with ASME (American Society of Mechanical Engineers) boiler and pressure vessel code, Section VIII, Division3 (Avure, n.d).

2.7 SAMPLE PREPARATION

2.7.1 WHEY PROTEIN / GLUCOSE SYRUP

Whey protein/glucose syrup samples were formulated using a fixed concentration of whey protein (15 %, w/w) with varying concentration of glucose syrup from 0 – 65 % (w/w). Required amount of glucose syrup was mixed with 10mM CaCl$_2$ using magnetic stirrer. After obtaining a clear solution, required amount of whey protein was added to the system as batches. The system was stirred for two hours to ensure proper dissolution. After mixing they were stored at 4 °C to ensure proper hydration.

2.7.2 BOVINE SERUM ALBUMIN (BSA) / GLUCOSE SYRUP

Samples were prepared with fixed concentration of BSA (15%, w/w) with percentage of glucose syrup varying between 0 – 65% (w/w). Glucose syrup was dissolved in 10mM CaCl$_2$ under constant stirring using a magnetic stirrer. BSA was added as batches under mixing conditions. After the addition of all ingredients, samples were further mixed for two hours to ensure proper dispersion followed by storage at 4 °C overnight for proper hydration.

2.7.3 IMMUNOGLOBULIN

Freeze dried fractions of immunoglobulin from whey proteins were dissolved in distilled water to achieve an initial concentration of 30% (w/w) through gentle mixing using magnetic stirrer. Samples were then introduced to vacuum evaporator to achieve condensed preparations. Samples were concentrated to 60, 70 and 80% (w/w) total solids. Samples were then vacuum sealed and stored at 4 °C for pressure treatment.
2.8 EXPERIMENTAL METHODS

2.8.1 RHEOLOGY

Experimental for WPI / glucose syrup and BSA / glucose syrup samples from 15 – 60% (w/w) total solids

Samples were analysed using a controlled stress rheometer, with a bob and cup geometry. Samples were loaded at 20 °C. Solvent trap was placed to prevent moisture loss from the sample. Systems were heated from 20 °C to 85 °C at 1°C/min under a constant strain of 0.1 % and an angular frequency of 1 rad/s. At 85 °C the system was placed at isothermal for 30 min for whey protein samples and 60 minutes for BSA samples. This was followed by a frequency sweep from 0.1 – 100 rad/s at 85 °C. System was then cooled from 85 °C to 5 °C at 1°C/min under constant strain and angular frequency of 0.1 % and 1 rad/s respectively.

Experimental for immunoglobulin samples for 60 and 70% (w/w) total solids

Concentrated immunoglobulin samples were analysed using parallel plate geometry of 10mm dimension. Samples were loaded on a peltier plate at 20 °C with edges covered in silicone oil (50cS) to prevent moisture loss. Samples were heated to 85 °C at 2 °C/min under constant strain and angular frequency of 0.1 % and 1 rad/s. An isothermal step for 20 minutes was performed at 85 °C to ensure complete denaturation of protein, followed by a frequency sweep ranging from 0.1 – 100 rad/s. Samples were then cooled to 5 °C at 2°C/min under constant strain of 0.1% and frequency of 1 rad/s.

Experimental for high solid preparations

Mechanical behaviour of samples undergoing glass transitions were analysed using rheometer attached with an environmental test chamber (ETC). Analysis was carried out using 5 mm parallel plate geometry under isolated conditions. Liquid nitrogen was purged into the chamber to extend the temperature range of the system. Samples were first heated to 85 °C at 1 °C/min followed by an isothermal at 85 °C for 20 minutes. Systems were cooled to sub-zero temperature at 1 °C/min to reproduce the mastercurve of viscoelasticity, where the sample in rubbery state passes through glass transition and to glassy consistency at sub-zero temperatures. To observe the network relaxation of systems with various degree of denaturation, samples were heated to various temperatures depending on experimental
After attaining a glassy consistency, frequency sweeps (0.1 – 100 rad/s) were taken at regular intervals of 4 °C to mathematically model the system with the concept of time-temperature superposition.

2.8.2 DIFFERENTIAL SCANNING CALORIMETRY

Modulated differential scanning calorimetry and micro differential scanning calorimetry was used to analyse systems of globular proteins (WPI, BSA & immunoglobulins).

Modulated Differential Scanning Calorimetry (MDSC)

WPI/glucose syrup and BSA/glucose syrup samples (~10mg) were placed in a hermetically sealed aluminium pan. An empty pan served as the reference. Samples were heated from 20 °C to 95 °C at 1 °C/min. Modulation amplitude of 0.53 °C at every 40 s were used throughout the experiment. Heat flow (W/g) trace was monitored to study various denaturation patterns of protein systems. For systems of high solids (>70 %, w/w) samples were heated to 95°C and then cooled to -70°C at 1°C/min to observe the molecular glass transition. To observe glass transition of non-denatured system, samples were cooled from 20°C to -70°C at 1°C/min.

Micro Differential Scanning Calorimetry (μDSC)

To observe the transitions of protein systems involving relatively low change in enthalpy, we use highly sensitive microDSC (Seteram VII). Pressurised and atmospheric samples of immunoglobulins were place in microDSC hastelloy pans (~600 mg) and an equal amount of water served as reference. Samples were heated from 20 °C to 95 °C at 1°C/min to observe the endothermic peak representing denaturation of immunoglobulin molecules.

2.8.3 FOURIER TRANSFORM INFRARED SPECTROSCOPY

FT-IR spectroscopy of protein samples were recorded using Perkin Elmer Spectrum 100 attached with ATR crystal. Infrared spectrum was recorded in absorbance mode. For each sample an average of 4 scans were taken at a resolution of 8 cm\(^{-1}\). Spectra were recorded within the range of 600 – 4000 cm\(^{-1}\). Obtained spectra were later deconvoluted within the
range of interest (Amide I, 1600-1700 \text{ cm}^{-1}) to observe the secondary conformation of protein molecules using the software *spectrum*.

### 2.8.4 CONFOCAL LASER SCANNING MICROSCOPY

Confocal laser scanning microscopy (CLSM) helped to reveal the changing morphology of protein system in the presence of sugar as a function of temperature (Verheul & Roefs, 1998). Samples were stained using rhodamine B dye and placed on a concave glass slide so as to obtain fluorescent emission from protein molecules. The specimen was sealed using coverslip which was glued along it’s four edges. Specimens were scanned using N PLAN L 20.0 X 0.4 DRY objective lens at 553 – 619nm and the temperature was raised to 85 °C at 1°C/min. At high temperatures, the network formation of globular protein was observed as well as the variation in the morphology of the network as a function of sugar concentration.

### 2.8.5 HIGH PRESSURE PROCESSING

Bovine immunoglobulin at concentrations of 60, 70 and 80% total solids in distilled water were vacuum sealed. Before processing, the samples were stacked into a cylindrical load basket. The basket was placed into a water filled pressure chamber. For controlling the temperature and pressure, filtered water was pumped at a pre-set temperature. After acquiring the required pressure and after keeping the isobaric conditions for a definite period (holding time), the pressure was released and the load basket was removed. For immunoglobulins, samples were pressurised at 600 MPa for 15 minutes at ambient temperature (~22 °C). Pressurised immunoglobulin samples were retrieved for further analytical measurements to study the extent of denaturation.
2.9 REFERENCES


CHAPTER 3
FUNDAMENTAL STUDIES ON THE STRUCTURAL FUNCTIONALITY OF WHEY PROTEIN ISOLATE IN THE PRESENCE OF SMALL POLYHYDROXYL COMPOUNDS AS CO-SOLUTE

ABSTRACT

The present work deals with the changing network morphology of whey protein isolate (15%, w/w) in the presence of glucose syrup (co-solute) with concentrations ranging from 0 to 65% (w/w) in 10 mM CaCl$_2$ solution thus producing formulations with a total level of solids of up to 80% (w/w). Denaturation behaviour and aggregation of whey protein systems were investigated using small deformation dynamic oscillation on shear, micro and modulated differential scanning calorimetry, and confocal laser scanning microscopy. A progression in the mechanical strength of protein aggregates was observed resulting from enhanced protein-protein interactions in the presence of glucose syrup. Addition of the co-solute resulted in better thermal stability of protein molecules by shifting the process of denaturation to higher temperature, as observed by calorimetry. Observations are supported by micrographs showing coherent networks with reduced size of whey protein aggregates in the presence of high levels of glucose syrup, as opposed to thick and random clusters for systems of whey protein by itself. Glass transition phenomenon was observed for condensed protein/co-solute systems, which were treated with theoretical concepts adapted from synthetic polymer research to pinpoint the mechanical glass transition temperature.

Keywords: Whey protein isolate, Glucose syrup, Thermal stability, Glass transition temperature
3.1 INTRODUCTION

Whey protein is a by-product of cheese manufacture being primarily a mixture of $\beta$-lactoglobulin (50%), $\alpha$-lactalbumin (20%), bovine serum albumin (5%) and immunoglobulins (13%) (de la Fuente, Singh, & Hemar, 2002; Mercade-Prieto, & Gunasekaran, 2009). These globular proteins are widely used in food, pharmaceutical and health care products because of their unique functionalities of foaming capacity, emulsion stabilising, solution thickening and gel formation. The functional characteristics are altered by the influence of solvent quality, mechanical forces, processing temperature, etc. (McClements, 2002).

Within food products, whey proteins are often dispersed in an aqueous phase along with other low molecular weight co-solutes such as sugars, minerals and alcohols (McClements, 2002). These co-solutes can alter the molecular and functional characteristics of the complex system by binding to protein surface groups, but the precise nature of interaction depends on type and concentration of co-solute (Kumosinski, 1990; Timasheff, 1998). Previous studies in low solid systems have shown that sugar induces thermal stability to globular protein by extending their denaturation temperature (Jou, & Harper, 1996; Lee, & Timasheff, 1981). The proposed mechanism for the stability of globular proteins in the presence of sugar is attributed to sugar molecules being preferentially excluded from the protein surface. This is a process that reduces the thermodynamic affinity of the protein molecules for the composite solvent of water with various co-solutes (Arakawa, & Timasheff, 1982; Timasheff, 1993).

To provide functionality in the form of a desirable gelling property, globular protein must be capable of aggregating to produce a three dimensional network that can act as a suspending medium of various ingredients in end products (Mulvihill, & Kinsella, 1987; Zeigler, & Foegeding, 1990). Native whey protein does not form gels when dispersed in water at ambient temperature, as the intermolecular repulsive forces dominate attractive interactions. Upon heating, the globular protein unfolds and exposes the hydrophobic core, leading to increased protein surface hydrophobicity, which is sufficient to promote aggregation and subsequent gel formation. Presence of sugar alters the above physicochemical process by increasing the unfolding temperature of protein molecules and changing the magnitude of the attractive and repulsive forces within the system (Record, Zhang, & Anderson, 1998). This influences the physicochemical properties of the gel including appearance, texture, stability and water holding capacity (Chanasattru, Decker, & McClements, 2008).

Condensed polysaccharide/sugar systems form rubbery gels at ambient temperature, which upon cooling to sub-zero temperatures undergo vitrification to produce materials with a
brittle glassy consistency (Almrhag, George, Bannikova, Katopo, & Kasapis, 2012). Their relaxation processes have been monitored with the theoretical framework of free volume theory that predicts a mechanical glass transition temperature with physical significance (Kasapis, Al-Alawi, Guizani, Khan, & Mitchell, 2000). The approach has been adapted from well established research in amorphous synthetic polymers, which allows determination of viscoelastic properties of materials as a function of an extended range of temperatures or time scale of observation (Dannhauser, Child, & Ferry, 1958).

Our current work focuses on the gelation and vitrification behaviour of whey protein in the presence of glucose syrup as co-solute, within a concentration range that eventually produces condensed systems, allowing treatment of viscoelastic properties on the basis of a glass transition theory. As far as we are aware, there is no corresponding work in the literature on globular protein/sugar systems at high levels of solids, and we aim in this study to compare structural properties with those reported earlier for the polysaccharide/sugar counterparts.
3.2 MATERIALS AND METHODS

Materials

Whey protein isolate: The material used was a product from MG Nutritionals, Murray Goulburn Co-operative Co Ltd., Victoria, Australia. According to the supplier, the composition of whey protein was reported to be 91.3% protein, 0.7% fat, 3.5% moisture, 3.8% ash and 0.44% lactose. The bulk density of the powder was reported to be 0.45 g/ml and produced a microbial standard plate count of 9900 cfu/g. Solution of 10% (w/w) of whey protein in distilled water gave a pH value of 6.3.

Glucose syrup: Glucose syrup used for this investigation was a product of Cerestar (Manchester, UK) with a dextrose equivalent (DE) of 42. The total level of solids was 82% and percentages in formulations of this investigation refer to dry solids. According to the data provided by the supplier, with the aid of gel permeation chromatography, the polydisperse nature of the glucose syrup is established ranging from glucose molecules to high molecular weight fractions.

Methods

Sample preparation: Whey protein/glucose syrup samples were formulated on the basis of a fixed concentration of whey protein (15%, w/w) with varying concentration of glucose syrup from 0 to 65% (w/w). Required amount of glucose syrup was mixed with 10 mM CaCl₂ solution using magnetic stirrer. After obtaining a clear sugar solution, whey protein was added to the system as batches at ambient temperature. Samples were further stirred for two hours to ensure proper dissolution. Following thorough mixing, they were stored stirred for two hours at 4°C to ensure proper hydration. All analyses were performed at the natural pH of whey protein (~6.3).

Rheological characterisation: Measurements of systems from low to intermediate level of total solids (15 – 65%, w/w) were executed on MCR 301 rheometer (Anton Paar, Virginia, USA) using a cup and bob measuring geometry with dimensions of 28.66 and 26.66 mm in diameter, respectively. Under small amplitude oscillatory mode, viscoelastic properties of the samples were analysed at varying conditions of temperature and frequency. They were loaded at 25°C and a solvent trap was placed to prevent moisture loss. Samples were heated to 85°C at 1°C/min, kept at that temperature for 30 min, and then cooled to 5°C at the same scan rate using a constant frequency of 1 rad/s and strain amplitude of 1%.
To investigate the mechanical relaxation of high solid systems (80% total solids, w/w), a control stress rheometer, AR-G2 (TA Instruments, New Castle, DE) attached with an environmental test chamber that controls temperature was employed. Viscoelastic measurements of the sample were made using 5 mm parallel plate geometry, with edges being covered with silicone oil from BDH (50 cS) to prevent moisture loss. Condensed sample was heated to 85°C at 1°C/min, followed by an isothermal run for 30 min at that temperature. System was then cooled to -15°C at the same scan rate using a frequency of 1 rad/s and strain of 0.01%. Upon subsequent heating from subzero temperatures, frequency sweeps were performed within the range of 0.1 – 100 rad/s, at an interval of four degrees centigrade, and results were modelled theoretically for estimation of the mechanical glass transition temperature.

Modulated differential scanning calorimetry: Thermal measurements of samples were performed with Q 2000 (TA instruments, New Castle, DE). The instrument used a refrigerated cooling system to achieve temperatures down to -90°C and a nitrogen DSC cell at 50 mL/min to purge condensation. Samples were loaded in hermetic aluminium pans. Calibration of the heat flow signals, using a traceable indium standard ($\Delta H_f = 28.3$ J/g), and the heat capacity response, with a sapphire standard, enabled accurate measurements. Samples were weighed (~10 mg) and analysed at modulation amplitude of 0.53°C at every period of 40 s. They were equilibrated at 25°C and then heated to 95°C at 1°C/min. Condensed sample with 80% (w/w) total solids was cooled to -90°C at the same scan rate, following the aforementioned thermal treatment, to observe the enthalpic relaxation of the system.

Confocal laser scanning microscopy: This technique assisted with producing tangible evidence of the changing morphology of protein systems in the presence of glucose syrup. Samples were stained with rhodamine B dye and placed on a concave glass slide to obtain fluorescent emission from the protein molecules. A glass coverslip was glued along the four edges covering the sample surface to prevent moisture loss and the temperature was raised to 85°C at scan rate of 1°C/min using a peltier platform. Specimens were scanned with N PLAN L 20.0 X 0.4 DRY objective lens from 553 to 619 nm using argon lasers.
3.3 RESULTS AND DISCUSSION

3.3.1 Observations on the changing network characteristics of whey protein with increasing additions of glucose syrup

In the present investigation, a fixed amount of whey protein isolate was utilised in mixture with an extended range of glucose syrup as the co-solute giving us the opportunity to investigate the denaturation and aggregation behaviour of this material in an aqueous and high solid environment. Small deformation mechanical measurements were carried out first to observe the unfolding and subsequent profile of network formation in these systems ranging from low to intermediate levels of solids.

As shown in Figure 3.1, during the initial heating regime a dramatic increase in the values of storage modulus ($G'$) can be observed as the experimental temperature approaches 70°C. This intensity of thermal treatment leads to the exposure of nonpolar amino acids that were originally located in the interior of the globular molecule dispersed in low viscosity solutions (Mulvihill, & Donovan, 1987). Exposed nonpolar groups create strong and intermolecular interactions of hydrophobic nature leading to aggregation of whey protein molecules and the formation of a three dimensional matrix (Bryant, & McClements, 1998). At the end of the 30 min isothermal regime at 85°C, a sequential increase in the elasticity of the protein network is observed as we substitute water molecules with increasing concentration of glucose syrup. This observation has been attributed to the increased strength of protein-protein interactions in the presence of small polyhydroxyl compounds leading to the formation of cohesive gels (Kulmyrzaev, Bryant, & McClements, 2000).

Controlled decrease in the applied temperature from 85 to 5°C yields an increase in gel rigidity in the order of one-and-a-half decades of storage modulus for all formulations. It appears that the drop in temperature enhances a broad spectrum of attractive interactions between the protein molecules such as van der Waals forces, hydrogen bonds and hydrophobic attractions (Baier, & McClements, 2001). Clearly, the mechanical strength of the globular protein gel has been reinforced by thermal treatment (heating followed by cooling) and increased addition of glucose syrup in preparations.

There are reports in the literature on work that has been carried out using oscillatory rheology to probe the viscoelasticity of gelling polysaccharides (agarose, deacylated gellan and κ-carrageenan) with increasing concentration of sugars (Kasapis, Al-Marhoobi, Deszczynski, Mitchell, & Abeysekera, 2003). Addition of low levels of co-solute (up to 40%, w/w) in mixtures creates a stronger and more thermally stable structure, as compared to their
aqueous counterparts. Further sugar addition in preparations (40 – 60\%, w/w) results in an abrupt drop in the values of storage modulus, as the polysaccharides helices become thermodynamically unstable, with systems transforming from highly aggregated assemblies in the aqueous environment to lightly crosslinked networks in the high sugar matrices (Kasapis, 2008). This phenomenon of polysaccharide “solvation” contrasts strongly with the present observations where globular proteins appear to phase separate in the “unhydrated” sugar based environment leading to firmer structures in these heat set gels.

3.3.2 Calorimetric study of whey protein/glucose syrup systems upon controlled heating

To broaden our understanding on the phase transitions occurring in whey protein in the presence of glucose syrup, thermal events were recorded using the heat flow signals from differential scanning calorimetry. Data are recorded on the right hand side of Figure 3.2 between 50 and 99°C, which give an indication of the process of thermal denaturation in these systems. The midpoint denaturation temperature of the endothermic event acts as an index of convenience for subsequent discussions.

The midpoint denaturation temperature for aqueous whey protein is recorded to be at 69°C from a well resolved thermal peak. Increasing the concentration of glucose syrup displaces this thermal event at higher temperatures in a systematic fashion. Thermograms also become broader indicating reduced cooperativity in protein denaturation in the presence of glucose syrup. At the highest experimental concentration shown in Figure 3.2, i.e. 15% whey protein with 60% co-solute, the midpoint transition temperature is recorded at about 91°C. It has been put forward in the literature that the presence of sugar increases the surface free energy of native protein molecules, which tends to oppose the unfolding of globular architecture thus imparting thermal stability in high solid systems (Timasheff, 1993).

3.3.4 Understanding the vitrification phenomenon within high solid whey protein/co-solute systems

This part of the work deals with the vitrification process observed upon controlled cooling in condensed preparations, following thermal denaturation, and are monitored first here using calorimetry. Dropping the systems well below sub-zero temperatures seizes dramatically the molecular rotations or vibrations of chain segments. This is recorded in the heat flow signals at the left hand side of Figure 3.2 between the temperatures of -30 and -
65°C. Cooling or heating thermograms depict a broad transition in the heat capacity of the material, which is considered to be the calorimetric glass transition. During the cooling process, for example, the onset and midpoint transition temperatures were recorded at -32 and -47°C, respectively.

This is a perfectly thermoreversible process, as heating from the glassy state records an identical midpoint transition temperature. The calorimetric glass transition temperature for 80% glucose syrup preparations has been reported in the literature (Kasapis, & Shrinivas, 2010), with the value (-44°C) being similar to that of whey protein/glucose syrup at 80% total solids. This outcome strongly suggests that the technique of calorimetry records primarily the thermal transition of small polyhydroxyl molecules, as opposed to that of macromolecular additions at relatively small concentrations.

Having developed our understanding of phase transitions in our systems using calorimetry, we now proceed to attain complementary insights based on mechanical measurements. Figure 3.3 reproduces readings for 15% whey protein plus 65% co-solute that has been heated first to induce protein denaturation and then cooled to sub-zero temperatures. A spectacular development of storage and loss modulus values from $10^{4.5}$ to $10^{9.2}$ Pa is observed over a broad temperature range (from 80 to -15°C). This is the master curve of viscoelasticity as the sample passes through the glass transition region, for example at 20 or 30°C, to finally attain glassy consistency at the end of the cooling run. The extreme values of both moduli at sub-zero temperatures correspond to a state where limited motions such as stretching and bending of chemical bonds or β transitions are allowed (Ward, & Hadley, 1993; Mitchell, 2000; Kasapis, & Salblani, 2005).

Fundamental understanding of these observations requires “synchronisation” of the temperature and time effects, which will facilitate identification of the molecular processes responsible for the glass transition region (Ronan, Alshuth, Jerrams, & Murphy, 2007). This is achieved by following the working protocol outlined in Figures 3.4 and 3.5. First, mechanical spectra are obtained from 0.1 to 100 rad/s, at an interval of four degree centigrade, covering the experimental temperature range of 14 to -14°C. Storage and loss modulus values in Figures 3.4(a & b) represent the glass transition region, e.g. near bottom spectrum at 10°C, and the glassy state, e.g. near top spectrum at -10°C.

Then the concept of time-temperature superposition principle is employed to extend the time scale of observation in relation to data obtained at the various experimental temperatures (Farhat, Mousia, & Mitchell, 2003; Tobolsky, 1956). Presently, this is carried out by shifting our frequency sweeps along the horizontal frequency axis at an arbitrarily chosen reference temperature ($T_o = 2°C$) thus producing a master curve of viscoelasticity. This is illustrated in
Figure 3.4c where shear moduli extend over a nine-decade frequency range. Horizontal shifting of the frequency data produces a set of shift factors, $a_T$. Figure 3.5 represents those shift factors plotted as a function of temperature to reveal the progress in viscoelasticity for the whey protein/glucose syrup sample at 80% (w/w) total solids during vitrification.

The theory of free volume has been used extensively by material scientists to achieve mechanistic understanding of glass transition phenomena. It describes the vacant spaces arising from the irregular packing of long chain segments, which during vitrification collapse to a free volume of about 3% thus restricting the molecular chain dynamics (Ferry, 1991). A mathematical expression relating free volume to the thermal expansion coefficient of materials has been postulated (van der Put, 2010). Integrating the dynamic mechanical data into this mathematical model delivers the following equation:

$$\log a_T = \frac{(B/2.303f_o)(T - T_o)}{(f_o/\alpha_f) + T - T_o}$$

where, $f_o$ is the fractional free volume (the ratio of the free to the total volume of the molecule), $\alpha_f$ is the thermal expansion coefficient and $B$ is set to 1. $B/2.303f_o$ and $f_o/\alpha_f$ are considered to be the WLF parameters and termed as respectively.

Applying the WLF equation to condensed whey protein/glucose syrup samples generates WLF parameters $B/2.303f_o$ and $f_o/\alpha_f$ in the order of 12.90 and 52 deg, respectively. Figure 3.5 shows a traceable fit for the factor $a_T$ based on the WLF expression covering the glass transition region at the upper range of experimental temperatures. The sample enters the glassy state at the lower range of experimental temperatures where the modified expression of Andrade is found to provide a more meaningful fit (Gunning, Parker, & Ring, 2000):

$$\log a_T = \frac{E_a}{2.303R} \left( \frac{1}{T} - \frac{1}{T_o} \right)$$

This expression introduces the concept of activation energy ($E_a$) for an elementary flow processes within the glassy state ($R$ is the gas constant). The shift from the kinetics of the free volume theory to the predictions of the reaction rate theory happens at about 1°C. It reflects a change in emphasis from the governing dynamics of free volume to a process that requires an energetic barrier to molecular rotation and has been considered to be the mechanical glass transition temperature ($T_g$).
In addition to data of our mixture, Figure 3.5 reproduces the mechanical glass transition temperature (−44°C) of 80% (w/w) glucose syrup obtained by the same approach (Kasapis, Al-Marhoobi, & Mitchell, 2003; Kasapis et al., 2010). This is congruent with the values (between −47 and −44°C) obtained calorimetrically for glucose syrup and whey protein/glucose syrup at 80% (w/w) solids reported in the preceding sections. It appears, therefore, that estimates of the glass transition temperature from DSC are based primarily on the total level of solids within the system, whereas the mechanical glass transition temperature is a reflection of the network characteristics of the macromolecule added to high solid formulations.

### 3.3.5 Phase morphology of the whey protein/glucose syrup system

Finally, confocal laser scanning microscopy was used to study the network structure of whey protein in a variable amount of sugar. As shown in Figure 3.6a, 15% whey protein isolate after thermal treatment in an aqueous medium forms thick and random aggregates (illustrated as red clusters). Figure 3.6b represents whey protein with a 20% sugar addition where the aggregates can be seen in a more regular arrangement. Further increase in the concentration of the co-solute to 40 and 60% (w/w) reduces the aggregate size considerably thereby creating a coherent network structure, as shown in Figures 3.6(c & d). This type of tangible evidence of phase morphology complements the rheological observations in Figure 1, where the elasticity of the network increased by the addition of glucose syrup as the protein aggregates become coherent and arrange themselves uniformly in a highly functional network.
**Figure 3.1** Trend in the development of storage modulus for 15% whey protein isolate with 0 (■), 10 (□), 20 (♦), 30 (◊), 40 (▲) and 50% (Δ) glucose syrup (scan rate: 1°C/min; frequency: 1 rad/s; strain: 1%).
Figure 3.2 DSC endotherms for 15% whey protein isolate with 0, 10, 20, 30, 40, 50 and 60% glucose syrup arranged successively upwards between 50 and 95°C, and thermograms for 15% whey protein isolate with 65% glucose syrup at subzero temperatures (scan rate: 1°C/min).
Figure 3.3 Cooling profiles of storage (■) and loss (□) modulus for 15% whey protein isolate with 65% glucose syrup scanned at 1°C/min (frequency: 1 rad/s; strain: 0.01%).
Figure 3.4a

Figure 3.4b
Figure 3.4c

Figure 3.4 Frequency variation of (a) $G'$ and (b) $G''$ for 15% whey protein isolate with 65% glucose syrup; bottom curve is taken at 14°C (■), other curves successively upwards 10°C (□), 6°C (♦), 2°C (◇), -2°C (▲), -6°C (Δ), -10°C (●) and -14°C (○), and (c) master curve of reduced shear moduli ($G'_p$ and $G''_p$) as a function of reduced frequency of oscillation ($\omega_a T$) based on the frequency sweeps of the preparations in (a & b) (reference temperature = 2°C).
Figure 3.5 Temperature variation of the factor $a_T$ within the glass transition region (□) and glassy state (■) for 15% whey protein isolate with 65% glucose syrup, and glass transition region (Δ) and glassy state (▲) of 80% glucose syrup (Kasapis & Shrinivas, 2010), with the solid lines reflecting the WLF and modified Arrhenius fits of the shift factors throughout the vitrification regime (dashed line pinpoints the mechanical $T_g$ predictions).
Figure 3.6 Micrographs for 15% whey protein isolate (thermally treated) with 0 (a), 20 (b), 40 (c) & 60% (d) glucose syrup.
3.4 CONCLUSIONS

This work unveils changes in the network characteristics of globular proteins (whey protein isolate) in mixture with glucose syrup from low to intermediate and high levels of solids. Whey protein appears to respond differently to gelling polysaccharides in a high solid environment, with sugar maintaining structure formation rather than inhibiting it at intermediate levels of solids reported earlier for polysaccharides. Thus there are demixed whey protein and sugar rich domains of considerable size in the micrographs obtained presently, as opposed to the homogeneous polysaccharide assemblies at high levels of cosolute found in the literature. Glucose syrup influences the protein system by inducing thermal stability to the globular conformation thereby shifting the process of denaturation to higher temperatures, as observed calorimetrically. Furthermore, condensed preparations of whey protein/glucose syrup were treated with the concepts of theoretical viscoelasticity during the process of vitrification, which aided in the identification of structural relaxations as a function of temperature and time of observation. Utilisation of the concept of glass transition in the structural and textural properties of high solid foodstuffs will assist in evolution from non-science to a subject where amorphous (rubbery or vitreous) products can be engineered on a sound technological basis. Special reference then can be made to sugar glasses in the presence of globular protein that can be used to innovate in the food industry, for example, in replacing gelatin in confections, ice cream, boiled down sweets, etc.
3.6 REFERENCES


UNDERSTANDING THE EFFECT OF CO-SOLUTE ON THERMAL DENATURATION AND SUBSEQUENT AGGREGATION OF BOVINE SERUM ALBUMIN

ABSTRACT

Effect of co-solutes on the gelling characteristics of bovine serum albumin has been addressed from low to high solid systems. 15% (w/w) albumin from bovine serum has been combined with glucose syrup at various concentrations ranging from 0 – 65% (w/w) thus analysing various physicochemical properties involved during denaturation and aggregation through thermal treatment. The thermo-mechanical observations of protein/sugar systems has been carried out using differential scanning calorimetry and small deformation dynamic oscillation in shear. Protein molecules have been stabilised by the addition of glucose syrup which is observed as delayed denaturation. Glucose syrup acts as a plasticizer for bovine serum albumin as they exhibit better elasticity for the heat set gels. In addition, high solid samples (80%, w/w) were studied at sub-zero temperatures to understand the glass transition phenomenon, both thermally and mechanically. Studies on molecular processes during relaxation have been carried out by utilising concepts of free volume through Williams, Landel and Ferry model and reaction rate theories as described by the Arrhenius equation.

Keywords: Bovine serum albumin, glucose syrup, glass transition, thermal denaturation
4.1 INTRODUCTION

Bovine serum albumin (BSA) is a protein fraction present in whey and has important gelling characteristics (Hines & Foegeding, 1993). Even though present in less than 10% in whey protein, along with \( \beta \)-lactoglobulin, it provides diverse functional properties to food products that range from viscous fluids to hard and cohesive material (Hiller & Cheeseman, 1979; Zeigler & Foegeding, 1990). These functional attributes of globular proteins are dependent on molecular structure, chemical environment, mechanical stress, solvent quality etc. Present study attempts to understand the effect of co-solute (glucose syrup) on the functional behaviour of bovine serum albumin from low to high solid systems (15 – 80% total solids, w/w).

In comparison to other globular proteins, bovine serum albumin is a large protein molecule, made up of 580 amino acid residues containing 17 disulphide bonds and a free thiol group at position 34 in the peptide sequence (Kinsella & Whitehead, 1989). According to Kinsella et al. (1989) globular conformation of BSA contains 54% \( \alpha \)-helix and 40% \( \beta \)-structure (\( \beta \)-sheets and \( \beta \)-turns). High percentage of stable helical structure in BSA is expected to deliver high molecular stability to external stresses such as pressure. Mechanism of thermal protein denaturation has been a well-established fact involving two processes. First being the conformational changes of protein by the application of heat resulting in unfolding of some polypeptide segments. During this phase there is a transition from native to progel state where intra-molecular hydrogen bonding and electrostatic interactions in the native state engages in intermolecular bonding. During the subsequent phase, protein-protein interaction intensifies where buried sulphhydryl groups can initiate disulphide-sulphhydryl interchange reactions, thus contributing to network formation (Bernal & Jelen, 1985).

Chapter 3 of the thesis, deals with the effect of co-solute on whey protein isolate (mixture of \( \beta \)-lactoglobulin, \( \alpha \)-lactalbumin, bovine serum albumin & immunoglobulins), which predominantly is an exhibition of \( \beta \)-lactoglobulin in both mechanical and thermal observation as it being the major constituent of WPI. \( \beta \)-lactoglobulin contains approximately 50% \( \beta \)-sheets and 10-15% \( \alpha \)-helix as estimated by infrared spectroscopy and circular dichroism (Creamer, Parry, & Malcolm, 1983). This segment of the thesis concentrates on globular protein with high concentration of \( \alpha \)-helix and low concentration of \( \beta \)-sheets in contrary to \( \beta \)-lactoglobulin having higher concentration of \( \beta \)-sheets than \( \alpha \)-helix. Even though the content of \( \beta \)-sheets is relatively low in native BSA, when the temperature of the system is raised, a decrease in the percentage of \( \alpha \)-helix is observed, followed by an increment in the concentration of \( \beta \)-sheets before aggregation (Clark, Saunderson, Suggett, 1981). This led to
the hypothesis that $\beta$-sheet hydrogen bonding plays a major role in protein aggregation. Wang et al. (1991) demonstrated that addition of 0.5 M NaClO$_4$ and NaCl to BSA enhances the stability of the protein with a sequential increase in $\beta$-sheets, along with a decrease in $\alpha$-helical content. Stability of protein by the addition of kosmotropic agents NaClO$_4$ and NaCl was confirmed by the elevation in denaturation temperature from 64°C to 75 and 70°C respectively.

Sugars have been classified as a kosmotropic agent for protein systems where the molecules get stabilised exhibiting higher denaturation temperature and better elastic network following thermal treatment. This was evident from observations in chapter 3 of the thesis where whey proteins displayed better functional properties and stability in presence of glucose syrup. One among the factors that led to such a transformation of whey protein isolate was its high content of $\beta$-sheets from $\beta$-lactoglobulin and $\alpha$-lactalbumin along with interfacial interactions between protein/sugar complexes. It can also be hypothesised that presence of sugar has increased the percentage of $\beta$-sheets in whey, thus providing excellent gelling characteristics.

Vitrification phenomenon in condensed protein/sugar systems leads to the formation of materials that are highly brittle and glassy in consistency. Mechanical and thermal analysis of such systems provides insights into the molecular processes involved during relaxation. Theories from free volume concept help to broaden our understanding during the network relaxation of the material. Thus glass transition temperature ($T_g$) can act as an index for quality control and product development in high solid systems.
4.1 MATERIALS AND METHODS

Materials

**Bovine serum albumin:** The product was purchased from Sigma Aldrich Private Ltd, New South Wales, Australia. According to the supplier, the molecular weight of the lyophilised powder of bovine serum albumin was 66 kDa with a purity of 98% as observed by electrophoresis.

**Glucose syrup:** The material was a product of Cerestar, (Manchester, United Kingdom) with a dextrose equivalent of 42. The moisture content of sample was 18% (w/w), but the percentage of solid in this investigation refers to dry solids. The polydisperse nature of glucose syrup was confirmed by gel permeation chromatography.

Methods

**Sample preparation:** Samples were formulated using a fixed concentration of protein (15%, w/w) with concentration of glucose syrup ranging from 0-65% (w/w). Required concentration of glucose syrup was mixed with 10mM CaCl$_2$ using a magnetic stirrer until a clear solution was obtained. BSA was then added as batches to the glucose syrup solution and stirred for two hours until it was properly dispersed into the system. Samples were refrigerated overnight to ensure proper hydration.

**Rheology:** Samples were analysed for their viscoelastic characteristics as a function of temperature, time and deformation. Systems from low to intermediate level of solids (15-65% total solids) were measured on MCR 301 rheometer (Anton Paar, Virginia, USA.) using bob and cup geometry with dimensions 26.66 and 28.66 mm in diameter. Samples were loaded at 25 °C and heated to 85 °C at 1 °C/min, kept at 85 °C for 60 minutes followed by cooling to 5 °C at 1 °C/min. Solvent trap was placed throughout the experimentation to avoid moisture loss from the sample. A constant angular frequency of 1 rad/s and a strain of 0.1% were applied during the experiment. This helps to characterise the protein aggregates based on values of storage modulus ($G'$) and loss modulus ($G''$).

High solid samples (80%, w/w) were analysed using AR-G2 (TA instruments, Newcastle, DE) equipped with environmental test chamber. This expands the experimental temperature range to -90°C by purging liquid nitrogen at controlled rate. Thereby, apart from the denaturation and subsequent aggregation of protein molecules, it helps to investigate the relaxation of polymeric network at sub-zero temperature. Samples were analysed using
parallel plate geometry of 5mm diameter with a thin layer of silicone oil covering the edges so as to prevent the moisture loss during the thermal treatment. Thermal treatment of samples were carried out by heating to 85°C at 1°C/min followed by an isothermal at 85°C for 60 minutes. Once a mature protein gel was obtained, samples were cooled to sub-zero temperature at 1°C/min. This provides a spectrum which depicts different states of a protein matrix ranging from rubbery to glassy consistency. To visualise the sample at a broader time scale, frequency sweeps (0.1 – 100 rad/s) were performed at an interval of 4°C during heating from sub-zero temperatures to implement time temperature superposition.

**Differential Scanning Calorimetry:** Thermal events of protein molecules typically, unfolding of globular conformation at high temperatures was observed using differential scanning calorimetry which is considered as the first order phase transition of the material (endothermic event). The instrument was fitted with refrigerated cooling system (RCS90) with temperature range of -90 to 560°C. Samples from low to high solids were loaded in hermetic aluminium pans (~10mg). Systems were scanned between 25 and 95°C at a modulation rate of 0.53°C every 40s. For system with 80% total solids, experimental temperature range was extended to -90°C to observe the relaxation of material. This is shown as variation in heat flow signals arising from the changes in specific heat as the material passes from one state to the another (second order phase transition).
4.2 RESULTS AND DISCUSSION

4.2.1 Mechanical approach to characterise the protein aggregates

Presence of co-solutes alters the pattern of denaturation and polymeric aggregation of globular proteins upon thermal treatment. Chapter 3 of the thesis demonstrates the effect of glucose syrup on whey protein isolate where protein molecules achieve better stability and creates highly elastic network. β-lactoglobulin is the major ingredient of whey protein isolate which is predominantly composed of β-sheets. Previous work in literature demonstrates that β-sheet structures play a crucial role in aggregation and network formation of protein molecules (Wang & Damodaran, 1991; Clark, Judge, Richards, Stubbs, & Suggett, 1981). In the present investigation we analyse the thermo-mechanical properties of BSA in presence of glucose syrup. α-helix is the major content constituting approximately of 57 – 60% within the globular conformation of BSA (Wang & Damodaran, 1991).

Small deformation dynamic oscillation in shear was carried out as a function of temperature to observe the denaturation and subsequent aggregation of BSA molecules. Figure 4.1 shows the aggregation profile of 15% BSA in presence of glucose syrup from 0-50% (w/w). Dramatic increase in values of storage modulus at temperatures above 70 °C demonstrates aggregation of BSA molecules. Formation of three dimensional matrix is facilitated by unfolding of protein molecules which exposes non-polar hydrophobic core leading to protein aggregation (Bryant & McClements, 1998). The progel state of BSA transforms to a fully matured state after the isothermal experimental step for 60 minutes at 85°C.

It is evident from Figure 4.1 that increase in concentration of glucose syrup creates more elastic protein network. Sugar is considered to be a kosmotropic agent that stabilises protein molecules. Imaging using confocal laser scanning microscopy has earlier shown that aggregates of globular protein (whey protein) in presence of glucose syrup has reduced size and are uniformly arranged. Therefore it is believed that coherent protein network formation in presence of glucose syrup creates elastic network. Spectra shown in Figure 4.2 represent the strengthening of protein network during cooling from 85°C to 5°C at 1°C/min. This is due to the formation of non-covalent bonds such as van der Waals forces and hydrogen bonds which are temperature dependent (Baier, & McClements, 2001).

From the above observations it can be confirmed that presence of sugar creates a reinforced protein network upon thermal denaturation. Wang et al. argues that heating of globular protein system in presence of kosmotropic agent increases the amount of β-sheets in
globular conformation. This has been reported for both BSA and 11S soy protein. It can be therefore hypothesised that increased content of $\beta$-sheets during thermal treatment in presence of co-solute is the mechanism by which BSA forms highly elastic network as like whey protein isolate.

4.2.2 Thermal denaturation of bovine serum albumin as observed by differential scanning calorimetry

Thermal transitions associated with protein molecules were investigated using differential scanning calorimetry. Samples were heated from 25 to 95°C at 1°C/min. Figure 4.3 shows the thermal profile of 15% BSA with 0-50% sugar. A shift in the endothermic peak associated with protein denaturation can be observed as the concentration of sugar is sequentially increased in formulations. This observation proposes that sugar molecules acts as a stabiliser in protein system. According to McClement (2002) stabilising agent tends to oppose protein transitions by increasing the surface free energy. Figure 4.4 shows the mid-denaturation temperature of 15% BSA with different concentrations of sugar. As shown in figure, denaturation temperature for 15% BSA by itself is 58°C, and approximately a degree centigrade increase in mid-denaturation temperature can be seen for every 10% increase of sugar in formulations. In comparison with whey protein isolate/sugar in chapter 3, WPI had a dramatic increase in denaturation temperature by the addition of sugar. This suggests that $\beta$-lactoglobulin, the major component of whey protein isolate has been more stabilised than BSA by the presence of glucose syrup.

4.2.3 Behaviour of high solid material at sub-zero temperature

Over the last decade much interest has been shown to study the relaxation behaviour of materials at sub-zero temperatures. Amorphous systems with reduced moisture (<30%) or those which lack water molecules that crystalizes at low temperatures undergo vitrification where material from melt or rubbery state transforms into brittle or glassy consistency. Such a system has been formulated using 15% BSA and 65% glucose syrup making total solids of 80% (w/w). Provisions from differential scanning calorimetry have been used to record the heat flow signals as the sample passes through glass transition.

Figure 4.5 shows the thermogram associated with relaxation of protein/sugar system as represented by the change in heat flow signals during cooling and heating at sub-zero temperature. At low temperatures, molecular rotations and vibrations are restricted due to the
energy barrier that reduces the specific heat of the material. This reflects on the heat flow signal during the calorimetric observation. The mid-point of the transition is considered as an index to identify the glass transition temperature which is estimated to be \(-48^\circ\text{C}\). This is considered to be a perfectly thermo-reversible process as identical glass transition temperatures \((T_g)\) were recorded during cooling and heating process. In earlier chapter of thesis, identical trend in the relaxation of whey protein/sugar has been observed and this has been related to the vitrification of glucose syrup with no influence of protein molecules as 80% glucose syrup provides similar glass transition temperature (Kasapis, & Shrinivas, 2010).

To complement the above observation on calorimetric glass transition, mechanical studies were performed on the viscoelastic properties which provide information about the network relaxation as the material undergoes structural arrest at lower temperatures. Figure 4.6 show the mechanical spectra for 15% BSA with 65% glucose syrup when cooled from 85 \(^{\circ}\text{C}\) to \(-14\) \(^{\circ}\text{C}\) at 1 \(^{\circ}\text{C}\)/min at a constant frequency of 1 rad/s and a strain of 0.01%. Development in the values of storage and loss modulus as a function of temperature represents the transformation of sample as it passes through rubbery to glassy state through the phenomenon of glass transition. Initially at high temperatures (>\(40^{\circ}\text{C}\)), the spectra represents the sample in rubbery state, but on further reduction in temperature, there is a dramatic increase in moduli with values of storage modulus exceeding \(10^9\) Pa at temperatures below \(-10^{\circ}\text{C}\). The structural relaxation observed at the end of the cooling regime is due to the limited mobility of chemical bonds (bending and stretching) and restricted polymeric motions (Kasapis, & Salblani, 2005; Mitchell, 2000; Ward, & Hadley, 1993). The above investigation has been restricted on the grounds of time scale of observation as a constant frequency of 1 rad/s was used throughout the experimentation.

4.2.4 Considering the concepts of polymeric free volume to understand network relaxation

To synchronise the effect of time and temperature on viscoelastic properties of the material, we implement the concept of time-temperature superposition (TTS). This provides detailed information on the role of polymeric free volume during the relaxation process and in determining the network \(T_g\) of the material. In addition, this approach helps to determine the physical properties of the material at broader time scale of observation ranging more than 10 decades of angular frequency. To design such a mathematical model, a series of frequency sweeps were performed within a temperature range of -14 and 22\(^{\circ}\text{C}\). Figure 4.7 a & b shows the values of storage and loss moduli within a range of 0.1-100 rad/s. The spectra obtained at
temperatures below 0 °C represent the sample in glassy state (upper half of spectra) and those below 0 °C represent sample as it undergoes glass transition process.

In an effort to obtain a mastercurve of viscoelasticity as a function of time, spectra obtained through series of frequency sweeps (Figure 4.7 a & b) were shifted horizontally along an arbitrarily chosen reference temperature (6 °C). Figure 8 represents the spectra with reduced variables as a function of extended frequency ranging 10 orders of magnitude (10^{-4} - 10^{6} \text{ rad/s}). This helps to derive a set of shift factors as we superimposed the frequency data in figure 4.7 to obtain a composite master curve of viscoelasticity (Figure 4.8). Plot in Figure 4.9 represents the set of shift factors aligned as a function of temperature. Theory of polymeric free volume suggests that vacant spaces arising from the irregular arrangement of the polymeric chains are temperature dependent. As the sample passes through the glass transition to the glassy state, there occurs a reduction in the free volume from 30 to 3% and vise versa during heating, which is related to the thermal expansion coefficient (Ferry, 1991). Williams Landel and Ferry coined the expression for the free volume which then incorporated with the dynamic mechanical variables gives the following expression (van der Put, 2010).

$$
\log \alpha_T = \log \left[ \frac{G'(T)}{G'(T_o)} \right] = \frac{(B/2.303f_o)(T - T_o)}{(f_o/\alpha_f) + (T - T_o)}
$$

where, \( f_o \) is the fractional increase in free volume at \( T_o \), \( \alpha_f \) is the thermal expansion coefficient, and the value of \( B \) is set to be about one. \( B/2.303f_o \) and \( f_o/\alpha_f \) are considered to be the WLF parameters and for 15% BSA with 65% glucose syrup those values were estimated to be 13 and 53 respectively. Shift factors derived from the WLF model gives perfect fit for data in the glass transition region at higher range of temperatures as shown in Figure 4.9 Further investigations into lower temperatures reveal a faulty fitting of mechanical data. From then on it is well explained by the theories of reaction rate theory or modified Arrhenius model (Gunning, Parker, & Ring, 2000).

$$
\log \alpha_T = \frac{E_a}{2.303R} \left( \frac{1}{T} - \frac{1}{T_o} \right)
$$

where \( R \) is the gas constant and \( E_a \) represents activation energy for the elementary flow of the material. The crucial temperature at which the complete seizure of polymeric free volume occurs within the protein/sugar system which then steps into the behaviour of elementary flow.
can be considered as the network glass transition temperature (3°C). During the cooling process, $T_g$ it can be considered as the point of completion for the glass transition phenomenon, further which the polymeric chains are totally constrained from physiochemical reactions and molecular mobility.

From the above observations it has been evident that addition of sugar to globular proteins provides better structural properties upon thermal denaturation. Apart from protein stabilisation and formation of highly elastic protein network at low levels of solids, it can exhibit early network vitrification which is considered to be a valuable observation for the novel product development and quality assurance.
Figure 4.1 Trend in the development of storage modulus for 15% bovine serum albumin with 0 (■), 10 (□), 20 (♦), 30 (◊), 40 (▲) and 50% (Δ) glucose syrup during heating to 85°C followed by an isothermal at 85°C for 60 minutes (scan rate: 1°C/min; frequency: 1 rad/s; strain: 1%).
Figure 4.2 Trend in the development of storage modulus for 15% bovine serum albumin with 0 (■), 10 (□), 20 (♦), 30 (◊), 40 (▲) and 50% (Δ) glucose syrup during cooling from 85°C to 5°C (scan rate: 1°C/min; frequency: 1 rad/s; strain: 1%).
Figure 4.3 DSC endotherms for 15% bovine serum albumin with 0, 10, 20, 30, 40, 50 and 60% glucose syrup arranged successively upwards.
Figure 4.4 Trend in the variation of mid-denaturation temperature for 15% bovine serum albumin by the addition of glucose syrup.
Figure 4.5 DSC thermograms for 15% bovine serum albumin with 65% glucose syrup at subzero temperatures (scan rate: 1°C/min).
Figure 4.6 Cooling profiles of storage (■) and loss (□) modulus for 15% bovine serum albumin with 65% glucose syrup scanned at 1°C/min (frequency: 1rad/s; strain: 0.01%).
Figure 4.7a

Figure 4.7b

Figure 4.7 Frequency variation of (a) $G'$ and (b) $G''$ for 15% bovine serum albumin with 65% glucose syrup; bottom curve is taken at 22°C (+), other curves successively upwards 18°C (○), 14°C (●), 10°C (x), 6°C (Δ), 2°C (▲), -2°C (▽) -6°C (♦), -10 (□) and -14°C (■).
Figure 4.8 Master curve of reduced shear moduli ($G'_p$ and $G''_p$) as a function of reduced frequency of oscillation ($\omega a_T$) based on the frequency sweeps of the preparations in 7 a & b. (reference temperature = 6°C).
**Figure 4.9** Temperature variation of the factor $a_T$ within the glass transition region (☐) and glassy state (■) for 15% bovine serum albumin with 65% glucose syrup, with the solid lines reflecting the WLF and modified Arrhenius fits of the shift factors throughout the vitrification regime (dashed line pinpoints the mechanical $T_g$ prediction).
4.3 CONCLUSIONS

Functionality of denatured bovine serum albumin in presence of glucose syrup were revealed by the above work. Presence of sugar elevated the denaturation temperature of BSA molecules as observed by calorimetry. Even though molecular conformation of BSA suggests an unfavourable environment for the network formation upon denaturation as it is being predominantly composed of $\alpha$-helix, but following the hypothesis from Wang et al. (1991) addition of Kosmotropic agent increases the content of $\beta$-sheets facilitating network formation and stabilisation of BSA molecules. Mechanical observations and concepts of free volume attained from the principle of time temperature superposition helped to understand the molecular processes involved in the relaxation of bio-polymeric material.
4.4 REFERENCES


CHAPTER 5
EFFECT OF THERMAL DENATURATION ON THE MECHANICAL GLASS TRANSITION OF GLOBULAR PROTEIN/SUGAR SYSTEMS.

ABSTRACT

This work attempts to explain the effect of thermal denaturation and subsequent aggregation on the structural relaxation of globular proteins in the presence of co-solute. The technique of small deformation dynamic oscillation has been widely used to explore the mechanical properties during vitrification process of condensed whey protein isolate and bovine serum albumin in presence of sugar (80% total solids, w/w) which inherits distinct level of network crosslinking achieved through manipulating the thermal regime of protein denaturation. In contrast to network relaxation of the polymeric constituents, molecular glass transition has been observed by differential scanning calorimetry. The principle of time-temperature superposition was adapted to understand the free volume and reaction rate theories involved during the vitrification process through Williams Landel and Ferry (WLF) and Arrhenius mathematical models. This can thereby provide better confidence in measuring and understanding the glassy properties of a material, which are close to reality as opposed to empirical indicators.

Keywords: Protein denaturation, molecular relaxation, network relaxation
5.1 INTRODUCTION

Glass transition can be described as the transformation of an amorphous material from a liquid to a glassy like behaviour which exhibits reduced molecular mobility (Badii, MacNaughtan, & Farhat, 2005). A glass can also be characterised as a liquid with high viscosity ($10^{12} - 10^{14}$ Pa.s) which flows with extremely low diffusion rate (Slade & Levine, 1991). The rubber to glass transition of materials can be considered as a kinetic phenomenon which is dependent on type of solute and external factors such as temperature, mechanical stress, hydrostatic pressure or time scale of measurement (Binder, Baschnagel, & Paul, 2003; Goff, 1994). Characteristic properties associated with the process of glass transition such as free energy, volume or stress/enthalpy relaxations enables to utilise techniques such as small deformation mechanical spectroscopy, differential scanning calorimetry, dilatometry, nuclear magnetic resonance etc. to understand the underlying molecular processes (Jiang, Kasapis, & Kontogiorgos, 2011).

Glass transition temperature of food components plays a vital role in their physicochemical properties that reflects on rates of deteriorative chemical reactions (Vrentas & Duda, 1978). As a result the architecture of amorphous matrix in frozen or dehydrated foods influences their stability as well as attributes during food processing and storage. This has led to more intensive investigation to provide a clear picture of vitrification for systems ranging from small organic molecules such as sugars, biopolymers including polysaccharides, proteins etc., and their mixtures.

Conventionally, technique of differential scanning calorimetry has been widely used to estimate the glass transition temperature by measuring the difference in energy inputs into a substance and its reference. Variations in thermal signals help to determine the range of glass transition temperature which includes onset, middle and completion of changes in heat flow trace. Previously, it has been described that there is no effect on calorimetric $T_g$ of solutes with molecular weight fractions above coil overlap and entanglement (Kasapis & Sablani, 2005).

Recently much interest has been developed in understanding the viscoelastic properties of materials as it undergoes transition from rubbery nature to glassy state. Recording the viscoelastic behaviour represented as storage modulus or elastic modulus ($G'$) and loss or viscous modulus ($G''$) as a function of temperature or time, reveals the mechanical behaviour of material as it passes through rubbery to glassy consistency via the phenomenon of glass transition. This mode of study will help to synchronise experimental temperature and timescale of observation through the concept of time-temperature superposition. This
approach has widely been used by material scientists to have a mechanistic understanding of the glass transition process through the concept of free volume. Irregular packing of polymeric segments creates vacant spaces or holes within the system known as free volume which facilitates molecular motions (Ferry, 1980). It is believed that free volume within polymer melts counts up to 30% of the total volume which then collapses to 3% of the total volume at a particular temperature known as glass transition temperature (Cangialosi, Schut, van Veen, & Picken, 2003).

Previously, Jiang et al. (2011) have demonstrated that molecular weight of solute is one among the governing factor that affects mechanical glass transition temperature, where they complied the viscoelastic data from four distinct molecular fractions of gelatin in mixture with cosolute. Fractions with higher molecular weight exhibited earlier vitrification as opposed those with lower molecular weight. In the present study, we exploit the phenomenon of globular protein aggregation upon thermal denaturation. We achieved distinct level of polymeric cross linking for 15% (w/w) whey protein isolate with 65% (w/w) glucose syrup, and 15% (w/w) bovine serum albumin with 65% (w/w) glucose syrup by manipulating the extent of thermal denaturation. This helps to understand the changing pattern of mechanical relaxation in relation to the dimension and density of the polymeric aggregates.
5.2 MATERIALS AND METHODS

Materials

Whey protein isolate: Whey protein isolate powder used for this investigation was purchased from MG Nutritionals, Murray Goulburn Co-operative Co. Ltd., Vic, Australia. The ingredient composition according to the supplier listed 91.3% protein, 0.7% fat, 3.5% moisture, 3.8% ash and 0.44% lactose.

Bovine serum albumin: The material used was a product from Sigma-Aldrich Pty. Ltd., NSW, Australia. According to the supplier, the lyophilised powder of albumin from bovine serum was of 98% purity with molecular weight of approximately 66 KDa., as observed by electrophoresis.

Glucose syrup: Glucose syrup was purchased from Cerestar (Manchester UK) with a dextrose equivalent (DE) of 42. As per the information from of the supplier, the level of solids (w/w) was 82% (percentage in formulations for this investigation refers to dry solids) and exhibited a polydisperse nature, as observed through gel permeation chromatography.

Methods

Sample Preparation: High solid system of whey protein/sugar was formulated using 15% whey protein with 65% glucose syrup. Glucose syrup was mixed with 10mM CaCl₂ solution using a magnetic stirrer until a clear solution was obtained. Further, required amount of whey protein was added in bathes at ambient temperature. Protein/sugar solution was further stirred for couple of hours to ensure proper mixing and refrigerated overnight before analysis. System with bovine serum albumin was prepared in a similar manner to that of whey protein system, where 15% bovine serum albumin was mixed with 65% glucose syrup in 10mM CaCl₂ solution followed by overnight refrigeration to ensure proper hydration.

Rheology: From chapter 3 of this thesis, we mechanically observed that whey protein system in presence of sugar completely denatures and aggregates by heating up to 85 °C at a controlled rate, followed by 30 minute isothermal at that temperature. Taking aboard this observation, we denatured the system completely by heating to 85°C at 1°C/min followed by isothermal for 30 minutes at 85°C, which was then cooled to sub-zero temperature at 1°C/min. To obtain a partially denatured system, protein/sugar sample was heated to 85°C (isothermal step at 85°C for 30 minutes was not implemented) and cooled at 1°C/min. Native
sample was devoid from any kind of heat treatment but cooled to -60°C from ambient temperature.

Similarly for albumin/sugar system, to achieve denaturation of different extents, varied thermal treatments were implemented. To obtain a completely denatured system, sample were heated to 85°C at 1°C/min followed by an isothermal for 60 minutes at that temperature (observation from chapter 4 of the thesis) which was followed by cooling to sub-zero temperature to observe structural relaxation. To obtain partially denatured system, protein/sugar sample was heated to 85°C/min. This was followed by cooling to sub-zero temperature. Native sample was directly cooled to sub-zero temperature.

The above procedure was implemented mechanically through the technique of small deformation dynamic oscillation in shear using a controlled stress rheometer AR-G2 (TA Instruments, New Castle, DE.) Viscoelastic measurements of the sample were made using parallel plate geometry of dimension 5 mm in diameter, with edges covered with silicone oil (50cS) to prevent moisture loss. A constant frequency of 1 rad/s and a strain of 0.01% were implemented during the initial heating (for denaturation) or cooling (to observe glass transition). During the subsequent heating from sub-zero temperatures, frequency sweeps were performed within a range of 0.1 – 100 rad/s at an interval of four degree centigrade.

For the simplicity of explanation within this manuscript, systems are abbreviated accordingly; completely denatured, partially denatured and non-denatured (native) whey protein system as W1, W2 and W3 respectively. Similarly, fully denatured, partially denatured and non-denatured (native) bovine serum albumin/sugar systems have been abbreviated as B1, B2 and B3 respectively.

Modulated differential scanning calorimetry: Calorimetric analysis of the sample were performed using Q 2000 (TA instruments, New Castle, DE) attached with a refrigerated cooling system RCS90. Samples were loaded in hermetic aluminium pans. Small amount of sample (~10mg) was cooled to -90 °C at 1 °C/min from ambient temperature, followed by heating to 90°C. Sample is then cooled from 90 °C to -90 °C at 1°C/min. Initial cooling helps to observe the calorimetric $T_g$ when protein is at native state whereas the second cooling helps to record the relaxation process when protein is in denatured state in the presence of sugar. A modulation rate of 0.53°C at every 40s is implemented throughout the experimentation process.
5.3 RESULTS AND DISCUSSION

5.3.1 Vitrification of globular protein/sugar system observed through a thermal window

The present investigation attempts to understand the vitrification phenomenon of globular protein/sugar system in low moisture environment (80% total solids, w/w), both thermally and mechanically. This section of the manuscript describes the calorimetric developments, as the system undergoes glass transition with conformation of protein either in native (globular) or denatured (aggregated) state in presence of glucose syrup, as co-solute.

Figure 5.1 shows the thermal profile of 15% whey protein with 65% glucose syrup as the sample undergoes vitrification process. System was initially cooled from ambient temperature to -90°C followed by heating to 95°C. Further, a second cooling step was introduced from 95°C to -90°C, followed by a subsequent heating to 90°C, at a constant ramping rate of 1°C/min throughout the experimentation. As shown in Figure 5.1, during the initial cooling and heating process (shown as dashed lines), a sigmoidal trace in the heat flow signal can be seen arising from the variation in specific heat of the matter as it undergoes glass transition. Protein fraction during this transition is considered to be in native state as the sample was devoid of any heat treatment. Later, Sample was heat treated to achieve protein aggregation through thermal denaturation which was then studied for vitrification process (shown as solid lines). Remarkably, the trace of heat flow for denatured and non-denatured sample are quite identical with mid-glass transition temperature recorded at -47°C during cooling and heating regime. Figure 5.2 shows the trend for 15% bovine serum albumin and 65% glucose syrup in native (dashed line) and denatured (solid line) state, with similar experimental protocol with mid glass transition temperature recorded at -48°C.

Previously in literature, the calorimetric glass transition temperature for 80% (w/w) glucose syrup has been recorded at (~-46°C) which is very similar to above observations (Kasapis & Srinivas, 2010). This emphasis the fact that presence of protein in either native or denatured state does not contribute to the calorimetric glass transition temperature, instead differential scanning calorimetry depicts the transition of small molecules, i.e. of co-solute (glucose syrup). Similar results have previously been reported, for high solid systems of gelling polysaccharides with sugar as co-solute, where DSC measured the $T_g$ of small molecules and was totally unaffected by the presence of network forming polysaccharides (Kasapis, 2008).
Glass transition, considered as the second order transition can be visualised as a very broad spectrum ranging ~40°C with transition being classified into onset, mid and completion. For convenience, temperature associated with the mid-point of this transition is considered to be glass transition temperature, but there are no relationships between local segmental motions which relate to the onset of glassy consistency of the material at this point during cooling. Thus mid glass transition temperature act as an empirical index rather being a fundamental indicator of molecular relaxation (Nagai & Roland, 2002; Kasapis, Al-Marhoobi, & Mitchell, 2003).

5.3.2 Temperature dependence on viscoelastic behaviour of matrix during rubber to glass transition

Small deformation dynamic oscillation in shear has been used to understand the mechanical behaviour of system as it undergoes glass transition. In doing so, we retrieve two sets of parameters, the in-phase and out of phase responses which are known as storage modulus ($G'$) and loss modulus ($G''$). In this work, we examine the effect of distinct levels of cross linking within globular system on structural relaxation as a function of temperature. Figure 5.3 shows pattern in the relaxation of whey protein (15%, w/w) in presence of sugar (65%, w/w) after denaturation to different extents. W1 and W2 samples were thermally treated to achieve aggregation, where W1 sample was completely denatured to achieve a mature network (heating to 85°C followed by isothermal at 85°C for 30 min), whereas W2 sample was partially denatured (heating to 85°C) so as to achieve relatively low intense network. W3 sample was preserved in its native state where the protein molecules retained its globular conformation.

Spectacular development in the master curve of viscoelasticity can be seen for all the systems as we lower the temperature at a controlled rate. Structured W1 and W2 systems upon cooling from 85°C shows an increase in values of moduli ($10^5$ Pa increase). Region of the master curve where mechanical response of the matter reaches $10^9$ Pa in storage modulus with loss modulus being separated represents the glassy state (Rahman, Al-Marhubi, & Al-Mahrouqi, 2007). These high values of moduli represent limited mobility of polymeric chains, stretching and bending of bonds and β transitions within the system (Ward, & Hadley, 1993; Mitchell, 2000; Kasapis, & Salblani, 2005).

It is evident from the observation in Figure 5.3 that, W1 sample with strong network had an accelerated vitrification (below 0°C) than fractions with partial (W2) and no polymeric structure (W3) respectively. W3 which contains protein in the globular conformation (native
state) shows delayed network relaxation with glassy consistency below -36°C. Similar trend can be seen in Figure 5.4 where bovine serum albumin (15%, w/w) at levels of denaturation (B1, B2 and B3) in presence of sugar (65%, w/w) shows vitrification at different range of temperatures. Albumin from bovine serum after complete denaturation (B1) by heating up to 85°C and by holding at that temperature for 60 minutes, displays trend for early vitrification with glassy consistency below 5°C during cooling. On the other hand B2 fraction shows later vitrification than B1 with partially developed network. B3 with native fraction of bovine serum albumin shows very late vitrification below -38°C which is very similar to W3.

Similar results have been reported for gelatin samples by Jiang et al. (2011) using gelatin fractions of varying molecular weight. It has been documented that systems with higher molecular weight of gelatin showed earlier vitrification as opposed to those with lower molecular weight. Spectrums in Figures 5.3 and 5.4 show a horizontal shift in the vitrification with diminishing intensity of protein aggregates. Doolittle et al. (1957) has documented that molecular dimensions within the system plays a significant role in the overall rheological behaviour in relation to free volume which is dealt extensively in the later part of this chapter.

5.3.3 Understanding network relaxation through the method of reduced variables

In the previous section we had an overview about the structural properties of samples as it passes through glass transition in relation to changing temperature and a constant frequency. To have a better understanding of the molecular processes involved during the network relaxation process, effects of temperature and frequency on the mechanical response from the material needs to be independently investigated (Ronan, Alshuth, Jerrams, & Murphy, 2007). This brings in the concept of molecular free volume and on how the properties of network influence the network relaxation. In doing so, mechanical spectra were obtained between 0.1 – 100 rad/s at an interval of 4°C for all the samples. Figure 5.5 a & b shows the storage and loss modulus data obtained between the temperatures of -52 and -24°C for W3 samples (non-denatured). Figure 5.5 c & d shows the spectra for partially denatured sample (W2) within the temperature range of -21 and 7°C. Results have been obtained for completely denatured sample of whey protein/sugar as shown in Chapter 3 of this thesis.

Similar results were obtained for bovine serum albumin/sugar samples as shown in Figure 5.6. Frequency sweeps were obtained within a range of temperature for native (B3) and partially denatured (B2) samples as shown in Figure 5.6 (a-b) & (c-d) respectively. Results for completely denatured bovine serum albumin/sugar sample are shown in Chapter 4 of the thesis. Within the spectrums in Figure 5.5 & 5.6, mechanical response shown in the top
half represents the glassy state of the sample and the bottom half represents sample passing through glass transition. This was followed by implementing the concept of time temperature superposition which helps to observe the system under extended timescale, thereby going beyond the technical limitations of instruments. This is carried out by shifting the spectra of frequency sweeps along the horizontal axis along a chosen reference temperature \((T_0)\) thus producing a master curve of viscoelasticity as shown in Figure 5.7 & 5.8. Table 1 shows the selected reference temperature \((T_0)\) for each sample along which horizontal shifting has been performed. Data of shear modulus in Figure 5.7 shows the master curve of viscoelasticity along the stretched timescale of observation (extended over ten decades of frequency) for W1, W2 and W3 samples of whey protein/sugar. The above protocol was repeated for BSA samples and the master curve with extended timescale was achieved as shown in Figure 5.8 for samples B1, B2 and B3 respectively.

Free volume has been widely used by the material scientists for polymeric characterisation. Amorphous polymers are composed of occupied volume and free volume which are dependent on temperature. According to Ferry (1991), it is this free volume arising from the packing irregularities of the polymeric segments that helps in string like movements within the system. It is estimated that the proportion of free volume in polymer melts is usually 30% of the total volume and as per the theory, below the glass transition temperature \((T_g)\) they collapses to 2-3%. This makes the concept of free volume an impeccable factor in the glass transition studies. Williams, Landel and Ferry designed a mathematical expression which incorporates fractional free volume and thermal expansion coefficient (van der Put, 2010). Integrating the rheological functions recasts the WLF equation as follows (Ferry, 1980).

\[
\log a_T = \frac{B}{2.303f_0}(T - T_0) \\
\frac{(f_0/\alpha_f)}{(T - T_0)}
\]

where, the fractional free volume, \(f_0\), is the ratio of free to total volume of the molecule, \(\alpha_f\) is the thermal expansion coefficient, and B is usually set to one. \(B/2.303f_0\) and \(f_0/\alpha_f\) are known as WLF parameters. Table 1 shows the WLF parameters for whey protein and bovine serum albumin based samples. Figure 5.9 and 5.10 represents the shift factors plotted against temperature for whey protein and bovine serum albumin samples respectively. It is quite evident that WLF equation provides identical trace to the derived shift factors in the glass
transition region. However, for temperatures below glass transition temperature WLF function becomes invalid. Instead they can be better explained by the mathematical expression of Andrade (Gunning, Parker, & Ring, 2000).

\[
\log a_T = \frac{E_a}{2.303R} \left( \frac{1}{T} - \frac{1}{T_0} \right) 
\]  

(2)

This estimates the activation energy (Ea) for the elementary flow of material in glassy state and R is the gas constant. The point of discontinuity where the sample passes from the theories of free volume to concepts of reaction rate can be considered as the network relaxation or glass transition temperature (T_g).

Figure 9 shows the variation in glass transition (point of discontinuity) for whey protein in the presence of sugar after being denatured to various extend. Fully denatured whey fractions displayed early vitrification than partially denatured and native samples. Similar observations can be derived from Figure 5.10 for bovine serum albumin where fully denatured system displayed glassy consistency below 3°C, whereas T_g for partially and non-denatured systems were estimated at -15 and -39 °C respectively.

The conformational freedom for the fully denatured protein systems are compromised as thermal denaturation induces the formation of thick polymeric aggregates. This can be the potential reason for early aggregation of proteins. As intensity of aggregates is reduced with partial or no denaturation, we thereby enhance the freedom of individual molecules, thus shifting the relaxation to lower temperatures. Therefore it appears that, similar to molecular weight effects on network relaxation, compromising the molecular mobility and conformation freedom by manipulating the denaturation of globular conformation changes the pattern in network relaxation.
<table>
<thead>
<tr>
<th>Sample</th>
<th>$C_1^0$</th>
<th>$C_2^0$ (deg)</th>
<th>$f_g$</th>
<th>$\alpha_f$ (deg$^{-1}$ x 10$^{-4}$)</th>
<th>$T_g$ (°C)</th>
<th>$T_0$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W1</td>
<td>12.9</td>
<td>51</td>
<td>0.033</td>
<td>6.4</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>W2</td>
<td>10.91</td>
<td>51</td>
<td>0.039</td>
<td>7.64</td>
<td>-10</td>
<td>-9</td>
</tr>
<tr>
<td>W3</td>
<td>14.93</td>
<td>52</td>
<td>0.028</td>
<td>5.38</td>
<td>-38</td>
<td>-36</td>
</tr>
<tr>
<td>B1</td>
<td>13.0</td>
<td>53</td>
<td>0.031</td>
<td>5.84</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>B2</td>
<td>12.27</td>
<td>52</td>
<td>0.034</td>
<td>6.5</td>
<td>-15</td>
<td>-13</td>
</tr>
<tr>
<td>B3</td>
<td>13.91</td>
<td>52</td>
<td>0.030</td>
<td>5.7</td>
<td>-39</td>
<td>-37</td>
</tr>
</tbody>
</table>

$W1$ – Fully denatured whey protein/sugar sample  
$W2$ – Partially denatured whey protein/sugar sample  
$W3$ - Non-denatured whey protein/sugar sample  
$B1$ - Fully denatured bovine serum albumin/sugar sample  
$B2$ – Partially denatured bovine serum albumin/sugar sample  
$B3$- Non-denatured bovine serum albumin/sugar sample

**Table 5.1:** Values of WLF parameters for protein/sugar samples
**Figure 5.1** Thermograms for denatured (solid lines) and native (dashed lines) whey protein isolate (15%, w/w) with glucose syrup (65%, w/w) at subzero temperatures.
Figure 5.2 Thermograms for denatured (solid lines) and native (dashed lines) bovine serum albumin (15%,w/w) with glucose syrup (65%,w/w) at subzero temperatures.
Figure 5.3 Cooling profiles of denatured (♦,◊), partially denatured (▲,Δ) and native (■,□) whey protein isolate (15%,w/w) with glucose syrup (65%,w/w) scanned at 1°C/min; storage modulus represented by closed symbol and loss modulus represented by open symbol (frequency: 1rad/s; strain: 0.01%).
Figure 5.4 Cooling profiles of denatured (♦,◊), partially denatured (▲,Δ) and native (●,○) bovine serum albumin (15%, w/w) with glucose syrup (65%, w/w) scanned at 1°C/min; storage modulus represented by closed symbol and loss modulus represented by open symbol (frequency: 1rad/s; strain: 0.01%).
Figure 5.5a

Figure 5.5b
Figure 5.5c

Figure 5.5d

Figure 5.5 Frequency variation of $G'$ and $G''$ for native and partially denatured 15% whey protein isolate with 65% glucose syrup. Bottom curve for native sample (a&b) is taken at $-24^\circ C$ (■), other curves successively upwards $-28^\circ C$ (□), $-32^\circ C$ (♦), $-36^\circ C$ (◇), $-40^\circ C$ (▲), $-44^\circ C$ (Δ), $-48^\circ C$ (●) and $-52^\circ C$ (○) and bottom curve for partially denatured sample (c&d) is taken at $7^\circ C$ (■), other curves successively upwards $3^\circ C$ (□), $-1^\circ C$ (♦), $-5^\circ C$ (◇), $-9^\circ C$ (▲), $-13^\circ C$ (Δ), $-17^\circ C$ (●) and $-21^\circ C$ (○).
Figure 5.6a

Figure 5.6b
Figure 5.6c Frequency variation of $G'$ and $G''$ for native and partially denatured 15% bovine serum albumin with 65% glucose syrup. Bottom curve for native sample (a&b) is taken at -21°C (■), other curves successively upwards -25°C (□), -29°C (♦), -33°C (◇), -37°C (▲), -41°C (Δ), -45°C (●) and -49°C (○) and bottom curve for partially denatured sample (c&d) is taken at -1°C (■), other curves successively upwards -5°C (□), -9°C (♦), -13°C (◇), -17°C (▲), -21°C (Δ), -25°C (●) and -29°C (○).
Figure 5.7 Master curve of reduced shear moduli as a function of reduced frequency of oscillation ($\omega_T$) based on the frequency sweeps for native (■, □), partially denatured (♦, ◊) and denatured (●, ○) whey protein isolate (15%, w/w) in presence of glucose syrup (65%, w/w); $G'_p$ represented with closed symbols and $G''_p$ represented with open symbols.
**Figure 5.8** Master curve of reduced shear moduli as a function of reduced frequency of oscillation ($\omega a_T$) based on the frequency sweeps for native (■, □), partially denatured (♦, ◊) and denatured (●, ○) bovine serum albumin (15%,w/w) in presence of glucose syrup (65%,w/w); $G'_p$ represented with closed symbols and $G''_p$ represented with open symbols.
**Figure 5.9** Temperature variation of the factor $a_T$ within the glass transition region (open symbol) and glassy state (closed symbol) for denatured W1 (●,○), partially denatured W2 (♦,◊) and native W3(■,□) 15% whey protein isolate with 65% glucose syrup, with the solid lines reflecting the WLF and modified Arrhenius fits of the shift factors throughout the vitrification regime (dashed line pinpoints the mechanical $T_g$ prediction).
Figure 5.10 Temperature variation of the factor $a_T$ within the glass transition region (open symbol) and glassy state (closed symbol) for denatured W1 (●,○), partially denatured W2 (♦,◊) and native W3(■,□) 15% bovine serum albumin with 65% glucose syrup, with the solid lines reflecting the WLF and modified Arrhenius fits of the shift factors throughout the vitrification regime (dashed line pinpoints the mechanical $T_g$ prediction).
5.4 CONCLUSIONS

Current work deals with the thermal effects on the network relaxation of globular proteins and about the existing discrepancies on concepts of glass transition. High solid systems of globular protein in mixture with sugar exhibit variation in network relaxation based on the conformation of protein molecules. Thermal spectrum obtained through differential scanning calorimetry provided identical traces of heat flow signals for systems with native and denatured protein fractions. Implementing the technique of dynamic oscillation in shear helped in understanding the variation in network glass transition as an effect of compromising the conformational freedom of the protein molecules through thermal denaturation. Samples with fully denatured protein samples exhibited early vitrification as opposed to those with globular conformation. This is witnessed by investigating systems of whey protein and bovine serum albumin in presence of sugar. Concept of free volume and time temperature superposition helped to logically understand the molecular processes involved during the vitrification process.
5.6 REFERENCES


EFFECT OF HIGH HYDROSTATIC PRESSURE ON THE STRUCTURAL PROPERTIES AND BIOACTIVITY OF IMMUNOGLOBULINS EXTRACTED FROM WHEY PROTEIN

ABSTRACT

High hydrostatic pressure was applied on immunoglobulin samples whose molecular, structural and glass transition properties were examined in comparison to thermal effects at atmospheric pressure. Immunoglobulins exhibit pressure stability throughout the experimental concentration range by conserving native conformation, which results in cohesive structure formation observed by small-deformation dynamic oscillation on shear, modulated differential scanning calorimetry and infrared spectroscopy. Application of the combined WLF/free volume theoretical framework demonstrates that pressurized immunoglobulin preparations are able to form glassy systems upon cooling at subzero temperatures. This has been attributed to a reduction in polymeric free volume under pressure and the development of an efficient friction coefficient amongst tightly packed particles that link to form a three-dimensional matrix. Pressure treated assemblies of condensed immunoglobulins demonstrate viscoelastic behaviour matching that of the thermally treated counterparts, but retain bioactivity, which is largely lost with thermal treatment.

Keywords: immunoglobulins, hydrostatic pressure, glass transition temperature, preservation of native conformation
6.1 INTRODUCTION

Whey proteins contain biologically active components such as immunoglobulins, which impart a protective shield against infectious diseases (Severin & Wenshui, 2005). Oral intake of bovine immunoglobulin concentrates (BIC) has been reported to protect the human body from several infections caused by microorganisms such as rotavirus, *Escherichia coli*, *Cryptosporidium*, *Streptococcus mutants*, *Candida albicans*, *Helicobacter pylori*, etc. (Mitra, Mahalanabis, Ashraf, Unicomb, Eeckels, & Tzipori, 1995; Tacket, Losonsky, Link, Hoang, Guesry, & Hilpert, 1988). Whey proteins can be used as a potential resource to extract immunoglobulins for the preparation of infant formulas as well as other hyperimmune foods that are effective against infectious diseases affecting children and immunocompromised individuals. Natural antimicrobials have advantages over synthetic and semi-synthetic antibiotics due to their low cost, efficient activity against microbes based on their polyclonal conformation and by imparting less stress to the microbial gut ecology. From a commercial point of view, their entry into the market is faster due to less stringent regulatory issues being natural ingredients (Jan, 2001).

The main molecular fractions are immunoglobulin A (IgA), immunoglobulin M (IgM) and immunoglobulin G (IgG), with the latter constituting 80% of the total milk immunoglobulins. This major immunoglobulin is a monomeric peptide, approximately 150 kDa, consisting of two heavy polypeptide chains and two light chains linked by disulphide bonds (Indyk, Williams, & Patel, 2008). Successful supplementation of immunoglobulins into food and nutraceutical formulations requires sound knowledge on their stability to various processing conditions that are employed industrially.

Conventional heat treatments are used in the food industry to attain acceptable microbial safety hence extending the shelf life of products. Commonly, this leads to loss of bioactivity in several ingredients including vitamins, antioxidants and proteins. Regarding the protein of interest in this work, investigations have been carried out to examine the thermal stability of immunoglobulins. This class of proteins is considered to be thermolabile, as they undergo thermal denaturation at temperatures above 75°C (Chen, Tu, & Chang, 2000; Domínguez, Perez, & Calvo, 1997; Li-Chan, Kummer, Losso, Kitts, & Nakai, 1995). Increasingly, high pressure processing (HPP) is considered as an alternative non-thermal processing technique to inactivate pathogenic and spoilage microorganisms without affecting the biological activity of food ingredients. For any protein, the expression (i.e. retention) of biological activity depends on the thermodynamic stability of its three-dimensional native conformation following processing (Price, 2000). Some studies have reported that immunoglobulins in low solid
systems are sensitive to HPP at a pressure greater than 276 MPa. Under these conditions, HPP results in a twenty five percentage loss in bioactivity of bovine IgG while heat treatment yields a significant reduction (close to 100%) in immunoactivity (Li et al., 2006).

This work deals with the determination of the effect of hydrostatic pressure on the structural properties of condensed preparations of immunoglobulins (> 60% solids) through thermomechanical analysis. A micromolecular investigation on their secondary state under both atmospheric and pressurised conditions using infrared spectroscopy is also carried out to reveal changes in the native conformation following processing. Detailed quantitative information is achieved by resolving the amounts of beta sheets and helical coils at each stage of the analysis for both types of materials. An index of physical significance (network $T_g$) for the rationalization of the mechanical properties of these high solid systems during vitrification is also developed and, as far as we are aware, this is the first report on rheological properties of immunoglobulins at atmospheric and high pressure conditions.
6.2 EXPERIMENTAL PROTOCOL

Materials

Whey protein isolate (WPI), for the extraction of immunoglobulins, was obtained from MG Nutritionals, Murray Goulburn Co-operative Co Ltd, Victoria, Australia. According to the supplier’s information, the composition of WPI was reported as 91.3% protein, 0.7% fat, 3.5% moisture, 3.8% ash and 0.44% lactose.

Extraction of bovine immunoglobulins from whey proteins

Immunoglobulins were extracted from whey protein isolate using a procedure of selective precipitation with ammonium sulphate. Saturated ammonium sulphate solution (SAS) was prepared by dissolving excess salt in distilled water, which was later refrigerated at 4°C to observe the recrystallization of ammonium sulphate thus confirming the degree of high saturation. SAS was diluted to 40% in distilled water and mixed with 20% WPI solution in equal proportion. The precipitate formed was separated through centrifugation at 1431g for 15 minutes at 4°C. The obtained pellet was dissolved in distilled water and dialysed for 24 hours at 4°C for the complete removal of salt. Solutions containing immunoglobulins were then freeze dried and stored at -20°C for further analysis (Nawar, 1999).

Preparation of immunoglobulin samples

Immunoglobulin dispersions of 30% solids were prepared by adding freeze-dried powder in distilled water at room temperature. Samples were stirred using magnetic stirrer for 2 hours to ensure proper mixing, and were then stored overnight at 4°C. Following this, materials were concentrated using rotary evaporator to achieve total solids of 60, 70 and 80% (w/w).

Immunoglobulin preparations were vacuum sealed and subjected to high hydrostatic pressure at 600 MPa at 22°C for 15 min within the cylindrical chamber of a high pressure vessel with 35 litre volume and 70 mm plunger diameter (Quintus Press – QFP 35L, Avure Technologies, Kent, WA, USA). Demineralised water served as the pressure transmission medium and the increment of pressure was at a rate of 100 MPa per 20 s.
Experimental Analysis

**Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis.** SDS-PAGE was performed according to the method of Laemmli (1970) in a Bio-Rad mini-Protein electrophoresis cell with a polyacrylamide gel concentration of 6% (w/w). A protein stock solution of 10 µg/ml was prepared in phosphate buffered saline which contains 137 mM NaCl, 2.7 mM KCl and 10 mM phosphate buffer of pH 7.4 at 25 °C. The solution was homogenised by 30 sec sonication cycles for 3 min. Part of the stock solution was thermally treated and three different samples were prepared at 30, 60 and 100% concentration of the original preparation. Thermal treatment was performed by boiling 10 mins in a water bath. In addition, an aliquot of 100% concentration of the non thermally treated stock solution was examined. All samples along with a 212 kDa protein ladder (New England Biolabs no. P7702, Hitchin, UK) were electrophoresed on 6% SDS PAGE gel at a constant voltage of 100 V and 400 mA for 2 hrs. The gel was stained with coomassie blue for 30 min and kept overnight at ambient temperature to enhance resolution of the various protein bands.

**Small Deformation Rheology:** Various samples being pressure treated or at atmospheric conditions were examined using the technique of small-deformation dynamic oscillation on shear. A controlled strain AR-G2 rheometer (TA Instruments, New Castle, DE, USA) with a 10 mm standard steel parallel plate geometry was employed to obtain measurements of the samples containing 60, 70 and 80% (w/w) total solids. Thermal gelation was carried out using Peltier element and the viscoelastic changes in immunoglobulin samples were monitored using small-amplitude oscillatory measurements at a constant strain of 0.1% and angular frequency of 1 rad/s. All measurements were carried out within the linear viscoelastic region (LVR) of the sample where stress is proportional to strain.

Samples of 60, 70 and 80% (w/w) solids were introduced to the peltier plate of the instrument and a thin layer of silicon oil was placed on the gap between the two measuring parallel plates to minimize moisture loss. Prior to analysis, samples were equilibrated at 35°C for 2 min. Then, they were heated from 35 to 85°C at a rate of 2°C/min and a frequency of 1 rad/s, kept at that temperature for 15 min under observation, and further monitored at 85°C with increasing frequency from 0.1 to 100 rad/s to examine the viscoelastic nature of formed systems. Thus, low-amplitude oscillatory measurements of the real (\(G';\) storage modulus) and imaginary (\(G'';\) loss modulus) parts of the complex shear modulus (\(G^* = G' + iG''\)), and tan δ (\(G''/G'\)) were recorded at atmospheric pressure.

For the glass transition studies, small-amplitude oscillatory measurements on 80% (w/w) atmospheric and pressurised immunoglobulin samples helped in obtaining master curve
of viscoelasticity as a function of temperature (Figure 7), at a constant strain of 0.001% and angular frequency of 1 rad/s using parallel plate geometry of 5mm diameter. In doing so, the atmospheric pressure samples were thermally denatured by heating from 35°C to 85°C at a rate of 1°C/min, kept at that temperature for 15 min and then cooled down at 2°C/min to subzero temperatures. Frequency sweeps were also carried out from 0.1 to 100 rad/s at constant temperature intervals of four degrees centigrade (between -41 and -1°C) to facilitate theoretical modelling of the molecular processes responsible for the observed viscoelastic behaviour in the glass transition region. Pressurised samples of immunoglobulin were cooled from 20°C to subzero temperatures at a controlled scan rate followed by frequency sweeps (between -40 and 0°C) to match the analysis implemented for the atmospheric pressure counterparts.

**Differential Scanning Calorimetry:** These were performed using Setaram Micro-DSC VII (Setu-rau, Caluire, France). All samples were placed in 850 mg cell and equilibrated against 850 mg reference cell that was filled with an appropriate blank solution (water). Cells were stabilized inside the microcalorimeter for 1 hour at 35°C before heating up to 95°C at a rate of 2°C/min. The denaturation temperature $T_{\text{mid}}$, corresponding to the maximum of the transition peak and the change in enthalpy ($\Delta H$), associated with this first order thermodynamic transition, were determined using Calisto, Setaram proprietary software.

**Fourier Transform Infrared Spectroscopy:** This type of work for both pressurized and atmospheric samples of immunoglobulins was carried out using a Perkin Elmer Spectrum 100 FTIR spectrometer. Sample spectra were obtained in the convenient absorbance mode. For each spectrum, an average of four scans was recorded at 8 cm$^{-1}$ resolution in the range of 800 – 1800 cm$^{-1}$ after atmospheric and background subtraction. Spectra within the range of 1600 - 1700 cm$^{-1}$ (Amide I) were deconvoluted, using the proprietary software Spectrum Version 6.0.2, to obtain the characteristic peaks that represent the secondary conformation of immunoglobulins.
6.3 RESULTS AND DISCUSSION

6.3.1 Structural Functionality of Pressurised and Atmospheric Immunoglobulins

Immunoglobulins are the major bioactive fraction of whey proteins along with lactoferrins and other glycoproteins, which are highly thermolabile materials. In an attempt to overcome the shortcomings of thermal processing, we obtained immunoglobulins from WPI through selective salt precipitation and carried out first a typical electrophoresis run in an SDS-PAGE gel to assess the quality of our protein extracts. Figure 6.1 reproduces the picture of SDS-PAGE for native and thermally treated immunoglobulin samples. These glycoproteins are composed of one or more units, each containing four polypeptide chains; two identical heavy chains and two identical light chains, which are held together by a combination of non-covalent interactions and covalent interchain disulfide bonds, forming a bilaterally symmetric structure. Electrophoretic patterns in this investigation show typical heavy chains (bands between 50 and 70 kDa) and light chains (bands between 17 and 30 kDa) of IgG, IgA and IgM molecules obtained from whey proteins (Felipe, Capellas, & Law, 1997).

Once the authenticity of our materials is established, we turn our attention to their structural properties under various “environmental” conditions using first small-deformation dynamic oscillation. Figure 6.2 represents mechanical data of immunoglobulins during controlled heating to 85°C at 2°C/min and holding at 85°C for 15 min. Globular proteins aggregate as they lose native conformation, which results in the formation of a polymeric network, and from the rheological perspective, the values of elastic modulus increase once the sample overcame the energetic barrier for denaturation.

During the initial heating regime (35 - 60°C), the dense solid like behaviour of condensed preparations of atmospheric immunoglobulin samples were compromised, due to formation of increasingly unstable hydrogen bonds, a process that results in reduced values of elastic modulus (Dissanayake, Kasapis, Chaudhary, Adhikari, Palmer, & Meurer, 2012). Once the experimental temperature exceeds 70°C, immunoglobulin molecules denature by losing their globular conformation and converting to thermally stable polymeric associations. Values of elastic modulus at the end of the experimental routine approach $10^4$ Pa, and the final network strength is dependent on the protein concentration for each preparation. As depicted in Figure 2, pressurised samples show higher strength attributed to the compression cycle of 600 MPa for 15 min that eliminates free volume to create a dense system of proteinaceous material. Similar to observations for the atmospheric counterparts, initial softening of the system is observed for pressurised materials due to destabilisation of hydrogen bonding,
followed by structure recovery in the denatured protein with increasing temperature (Dissanayake et al., 2012).

With the increasing interest in hydrostatic pressure protocols in the dairy industry, several studies have been reported showing substantial differences, following thermal or pressure treatments, in protein denaturation pathways with respect to their molecular mechanism and ingredient functionality (Considine, Patel, Anema, Singh, & Creamer 2007; Hinrichs & Rademacher, 2005; Patel, Singh, Havea, Considine, & Creamer, 2005). The distinct effects of heat and pressure on protein structure are controlled by enthalpy and volume changes, respectively, which are associated with the transition to the unfolded state. Pressure treatment is known to primarily destabilise non-covalent bonds whereas heat treatment disrupts both covalent and non-covalent associations (Balny & Masson, 1993). Regarding bovine IgG, the precise mechanism by which the native protein unfolds remains unknown, although the variable effects of heat and high pressure on the secondary structure have been discussed in relation to the apparent loss of immunoactivity (Li, Bomer, & Zhang, 2005; Li, et al., 2006).

Taking on board the above knowledge on the subject, we continued the rheological investigation on immunoglobulin gelation with or without pressurisation by performing frequency sweeps to extend the range of viscoelastic observations. Figure 6.3 reproduces the spectra of storage modulus for all samples monitored as a function of oscillatory frequency within the range of 0.1 – 100 rad/s at 85°C. All preparations exhibited frequency dependence in the development of storage modulus within the plateau region of the viscoelastic master curve hence highlighting the “rubbery nature” of these materials. In accordance to Figure 6.2, \( G' \) values of pressurised samples are higher than those for the atmospheric systems. Data from Figures 6.2 and 6.3 indicate that the native conformation of immunoglobulin at high levels of solids (> 60%) was retained after our pressurisation protocol leading to denaturation upon subsequent heating. Resistance to high pressure could be due to the absence of free SH groups (Felipe, Capellas, & Law, 1997; Howlett, Ismail, Armstrong, & Wong, 1992), and the relatively high molecular weight of the immunoglobulin fractions.

### 6.3.2 Thermal Analysis of Immunoglobulins

To broaden our understanding on the denaturation patterns of immunoglobulin based materials, and to confirm the rheological observations of the preceding section, thermal events were recorded using heat flow signals of micro differential scanning calorimetry (mDSC). The technique offers insights into vital parameters of material structure alteration, which can
be related to the temperature band and energy required to achieve protein denaturation. The effect of high pressure processing on immunoglobulin is examined presently by obtaining thermograms for atmospheric systems and after pressurizing at 600 MPa for 15 min. Figure 6.4 reproduces the corresponding mDSC traces during heating from 35 to 95°C at a controlled scan rate that matched the kinetics of the rheological routine.

As illustrated in Figure 4, atmospheric samples display typical endothermic peaks upon heating. These thermal events demarcate the denaturation boundaries of immunoglobulin whereby loss of secondary conformation occurs. Changes in enthalpy ($\Delta H$), associated with this first-order thermodynamic transition, are around 4.1 J/g, shown in Table 6.1 The figure also depicts the corresponding thermograms for pressurised systems at the same level of solids (60 to 80%, w/w). Gratifyingly, peaks appear to be similar with those for the atmospheric samples in terms of temperature range (mid-denaturation temperature, $T_{mid}$, is about 81°C in Table 6.1) and energy associated with the DSC transition. This outcome complements the mechanical observations reported previously in Figure 6.2, where the secondary structure of immunoglobulins withstands the pressure cycle of 600 MPa for 15 minutes.

6.3.3 Conformational Analysis of Immunoglobulins Using Infrared Spectroscopy

Fourier transform infrared spectroscopy is a useful technique for identifying the physicochemical bonds associated with protein structures of beta sheets, beta turns, alpha helix and random coils, and it has been used presently to delve further into conformational changes occurring on condensed immunoglobulin preparations as a function of treatment. In general, the amino acid or polypeptide repeating units give rise to nine characteristic bands, which include amide I – VII and amide A & B “fingerprints” (Krimm & Bandekar, 1986; Surewicz & Mantsch, 1988). From these, the most sensitive spectral region that represents the secondary conformation is the amide I band (1600 - 1700 cm$^{-1}$) formed by the C=O stretching vibrations of the peptide linkages. Figure 5 represents the FTIR spectra of 60 and 80% (w/w) immunoglobulins under atmospheric and pressurised conditions.

Overlap in the absorbance of atmospheric and pressurised samples at the amide I region supports our earlier position from thermomechanical observations that immunoglobulins have retained their secondary conformation after pressure treatment. Quantitative estimation of the secondary structure is based on the assumption that protein conformation is the linear sum of certain fundamental secondary elements. Thus, deconvolution of a specific band (e.g., amide I) generates a series of secondary peaks that represent different components within the
secondary structure of the protein. The approach helps considerably with determination of the proportion of secondary elements in the molecule as a result of processing or physicochemical environment. Figures 6.6a and 6.6b display the changes in secondary structure obtained at ambient temperature for native preparations or samples that have been previously pressurized at 600 MPa for 15 min by assigning band frequencies to the secondary conformation of proteins (Dong, Huang, & Caughey, 1992; Dong, Caughey, Caughey, Bhat, & Coe, 1992).

The secondary conformation of 60% immunoglobulin samples where beta sheets are confined to about 35% of the total conformation and alpha helix increases its percentage to about 40% of the molecule following pressurisation is shown in Figure 6.6a. Work was repeated for preparations at 80% solids producing similar patterns of changing conformation with the non-thermal treatment (Figure 6.6b). Therefore, it appears that pressurisation increases the amount of alpha helix but decreases the beta sheet concentration in immunoglobulin preparations throughout the 60 to 80% solids range of this investigation.

In dissolved native proteins, it is more favourable for the peptide units at the aqueous periphery to form hydrogen bonds with water molecules than among each other. During pressurisation, part of the hydrophobic interior of the immunoglobulin may be exposed to the solution, which causes the formation of aggregates (Dissanayake, Kasapis, Chaudhary, Adhikari, Palmer, & Meurer, 2012). At the interfaces between the building blocks of these aggregates, hydrogen bonds between peptide units in the polypeptide chain can be formed inducing the formation of alpha helices (Vermeer & Norde, 2000). Recorded trends in Figure 6.6 argue that immunoglobulins retain largely the secondary conformation of alpha helix or beta sheet following application of high pressure. Such adaptation to non-thermal environmental stress indicates retention of bioactivity and structural functionality in condensed systems of immunoglobulin, with the latter property being further pursued in the ensuing section.

6.3.4 Vitrification Phenomena in Condensed Immunoglobulin Systems

Preliminary work for this part of the manuscript produced smooth and continuous patterns of shear modulus development as a function of temperature for condensed immunoglobulin samples indicating vitrification phenomena, as for the physical state and morphology of amorphous synthetic polymers (Andreozzi, Castelvetro, Faetti, Giordano, & Zulli, 2006; Haque, Kawai, & Suzuki, 2006). Figure 6.7 reproduces small-deformation oscillatory profiles of the system at 80% solids spanning a wide range of temperature (between -50 and 90°C) at a controlled rate of 2°C/min, frequency of 1 rad/s and strain
amplitude of 0.001%. Heating the atmospheric samples to 85°C results in protein denaturation and network formation, which is further reinforced with continuous cooling to subzero temperatures. Pressurised samples were directly cooled from 20°C to also show formation of a coherent structure and a monotonic development in storage modulus to the end of the experimental routine.

Working within the dynamic range of the mechanical oscillatory measurements, the next stop of this type of analysis requires that frequency sweeps are taken at regular temperature intervals (every four degrees centigrade presently). Figures 6.8a and 6.8b reproduce the outcome of this protocol covering the isochronal temperature range in Figure 6.7 at subzero temperatures, but extending the effective time scale or frequency of observation from 0.1 to 100 rad/s. Results are illustrated for pressurised immunoglobulin samples but similar trends were observed for the atmospheric counterparts (not shown here).

Mechanical spectra in Figure 6.8 were processed by choosing arbitrarily a point as the reference temperature ($T_o = -21^\circ C$ and $-24^\circ C$ for atmospheric and pressurized samples, respectively) and shifting the remaining spectra horizontally along the log frequency axis until a uniform curve was obtained. In doing so, temperature essentially plays the role of changing the time or frequency scale of observation and assumes that a change in temperature shifts the time or frequency scale of all molecular mechanisms by the same amount (concept of thermorheological simplicity). Reduced composite curves put together are known as master curves of viscoelasticity and are reproduced in Figure 6.9 for both types of material. They cover an extensive frequency of oscillation of about 11 orders of magnitude from $10^{-5}$ to $10^6$ rad/s.

The method of reduced variables employed in the construction of composite curves in Figure 6.9 yields a set of shift factors ($a_T$) that can be treated with the concept of free volume in the form of the Williams, Landel and Ferry (WLF) equation (Ferry, 1991):

$$
\log a_T = - \frac{(B/2.303f_o)(T - T_o)}{(f_o \alpha_f) + T - T_o}
$$

(1)

where, $f_o$ is the fractional increase in free volume at $T_o$ (or $f_g$ at $T_g$), $\alpha_f$ is the thermal expansion coefficient, and the value of $B$ is set to be about one.

An alternative approach to the theory of free volume is the predictions of the reaction rate theory that can be formulated in terms of the modified Arrhenius equation (Gunning, Parker, & Ring, 2000):
\[
\log a_T = \frac{E_a}{2.303R} \left( \frac{1}{T} - \frac{1}{T_0} \right) \tag{2}
\]

where, \( R \) is the gas constant. This mathematical expression follows the progress in shift factors by integrating two sets of temperature data. It returns a constant activation energy \( (E_a) \), which argues that relaxation processes in the temperature range of interest are heavily controlled by the specific chemical features of the material (Kasapis, Al-Marhoobi, Deszczynski, Mitchell, & Abeysekera, 2003).

Figure 6.10 reproduces the outcome of the application of this school of thought (equations 1 and 2) to the viscoelastic functions of the immunoglobulin samples tested at atmospheric and high hydrostatic pressure. Two distinct patterns of structural relaxation feature prominently, as documented in the temperature dependence of factor \( a_T \) for the horizontal superposition of mechanical spectra in Figure 8 and the atmospheric counterparts. It appears that free volume is the molecular mechanism dictating diffusional mobility in the upper range of temperatures that correspond to the mechanical glass transition region. Use of the WLF equation within this region yields \( f_g = 0.034, \alpha_t = 6.5 \times 10^{-4} \) deg and \( T_g = -23.0^\circC \) for the atmospheric material, and \( f_g = 0.028, \alpha_t = 5.3 \times 10^{-4} \) deg and \( T_g = -26.0^\circC \) for the pressurized ones.

There is a clear change in the pattern of shift-factor development towards a linear behaviour that cannot be followed by the WLF equation at the lower temperature range in Figure 6.10. In here, the contribution of the mechanism of free volume is minimal and instead the modified Arrhenius equation offers a viable alternative to describe molecular dynamics. Based on the assumption that the free volume decreases linearly with temperature, the glass transition temperature \( (T_g) \) is defined as the point where the thermal expansion coefficient undergoes a discontinuity and below which thermal motions become extremely slow. Thus, the \( T_g \) should be located at the end of the glass transition region where the free volume declines to about three percent of the total volume of the material (Ferry, 1980; Kasapis, 2012). This is reproduced in the present work with vitrification parameters exhibiting an \( f_g \) value of \( 0.031 \pm 0.003 \). It seems that pressure-induced vitrification patterns appear to be largely irreversible due to the stabilisation of densely packed particles in a cohesive network yielding glass transition temperature predictions \( (-24.5 \pm 1.5^\circC) \) similar to those of the thermally treated matrices in Figure 6.7.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Change in enthalpy (ΔH, J/g)</th>
<th>Mid-denaturation temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>60% Ig – A</td>
<td>4.186</td>
<td>79.75</td>
</tr>
<tr>
<td>70% Ig – A</td>
<td>4.101</td>
<td>79.79</td>
</tr>
<tr>
<td>80% Ig – A</td>
<td>4.104</td>
<td>80.15</td>
</tr>
<tr>
<td>60% Ig – P</td>
<td>4.088</td>
<td>80.65</td>
</tr>
<tr>
<td>70% Ig – P</td>
<td>4.112</td>
<td>81.15</td>
</tr>
<tr>
<td>80% Ig – P</td>
<td>4.031</td>
<td>83.04</td>
</tr>
</tbody>
</table>

*A – Atmospheric sample  P – Pressurised sample at 600 MPa for 15 min*

**Table 6.1** Thermal properties of 60 – 80% (w/w) immunoglobulin systems detected by differential scanning calorimetry.
Figure 6.1 SDS-PAGE of immunoglobulins with lanes: (A) marker protein, (B) 30% heat treated immunoglobulin, (C) 60% heat treated immunoglobulin, (D) 100% heat treated immunoglobulin and (E) 100% native immunoglobulin.
Figure 6.2 Changes in storage modulus ($G'$) of 60 (□, ■), 70 (◇, ◆) and 80% (△, ▲) (w/w) immunoglobulin samples during heating from 35 to 85°C at a rate of 2°C/min and holding at 85°C for 15 min at a frequency of 1 rad/s and strain of 0.1%; samples at atmospheric pressure (open symbols), samples after pressurizing at 600 MPa for 15 min (closed symbols).
Figure 6.3 Changes in storage modulus ($G'$) of 60 (□, ■), 70 (◇, ◆) and 80% (△, ▲) (w/w) immunoglobulin samples during a frequency sweep from 0.1 to 100 rad/s at 85°C and 0.1% strain; samples at atmospheric pressure (open symbols), samples after pressurizing at 600 MPa for 15 min (closed symbols).
Figure 6.4 MicroDSC thermograms of 60 (□, ■), 70 (◇, ◆) and 80% (△, ▲) (w/w) immunoglobulin samples during heating from 35 to 95°C at a heating rate of 2°C/min; samples at atmospheric pressure (open symbols), samples after pressurizing at 600 MPa for 15 min (closed symbols).
Figure 6.5 FTIR spectra of 60 and 80% (w/w) immunoglobulin samples either at atmospheric pressure or after pressurizing at 600 MPa for 15 min.
Figure 6.6a

Figure 6.6b

Figure 6.6 Secondary conformation of immunoglobulin at (a) 60 and (b) 80% total solids as observed by infrared spectroscopy.
Figure 6.7 Variation of storage modulus ($G'$) for 80% immunoglobulin samples at frequency of 1 rad/s and strain of 0.001%; samples at atmospheric pressure (shown in open symbols) were heated from 35 to 85°C, held at 85°C for 15 min and cooled down to -48°C, and samples after pressurizing at 600 MPa for 15 min (shown in closed symbols) were cooled from 20°C to -44°C (scan rate of 2°C/min).
Figure 6.8a

Figure 6.8b

**Figure 6.8** Frequency variation of (a) $G'$ and (b) $G''$ for 80% immunoglobulin samples after high pressure processing; bottom curve was taken at 0°C (◇) and other curves successively upwards -4 (+), -8 (◆), -12 (●), -16 (▲), -20 (■), -24 (●), -28 (★), -32 (○), -36 (□) and -40°C (△).
Figure 6.9 Master curves of reduced shear modulus \([G'_p (\bigcirc, \bullet); G''_p (\triangle, \blacksquare)]\) for 80% immunoglobulin samples at atmospheric pressure (shown in open symbols) and pressurized at 600 MPa for 15 min (shown in closed symbols), as a function of reduced frequency of oscillation \((\omega\sigma_T)\) using data from Figures 8, and corresponding data from atmospheric materials, with reference temperatures for the horizontal superposition of mechanical data being -21 and -24°C for atmospheric and pressurised samples, respectively.
Figure 6.10 Temperature variation of factor $a_T$ within the glass transition region (□, ●) and the glassy state (△, ●) for 80% immunoglobulin samples at atmospheric pressure (shown in open symbols) and pressurized at 600 MPa for 15 min (in closed symbols); solid lines reflect the WLF and modified Arrhenius fits of the shift factors during the vitrification process, with dashed lines indicating the corresponding $T_g$ predictions.
6.4 CONCLUSIONS

Protein systems at high levels of solids remain underresearched and, consequently, molecular mechanisms responsible for their structural properties are poorly understood. The present work is part of an effort to provide a comprehensive picture of the structural behaviour of such systems by examining the behaviour of immunoglobulins at atmospheric conditions and following application of a high-pressure protocol. Mechanical properties of both types of immunoglobulin preparations reflect coherent gels, which upon cooling transform into glassy matrices. Experimental observations are further supported by theoretical modeling, which produces comparable predictions for the glass transition temperature and free volume of atmospheric and pressurised systems. Estimates of denaturation enthalpy and infrared spectroscopy data argue that immunoglobulin samples subjected to high hydrostatic pressure retain a stable secondary conformation comprising mainly alpha helix and beta sheets, an outcome that argues for retention of bioactivity in the condensed pastes. The works suggests opportunities for the utilisation of high-pressure treated immunoglobulins in starch or dairy based formulations of functional foods in an effort to initiate replacement of thermally treated dairy powders with limited biofunctionality.
6.5 REFERENCES


7.1 CONCLUSIONS AND FUTURE WORK

High protein diet is recommended in an era where life style diseases such as cardiovascular problems, diabetes etc. pose a serious threat to human health and wellbeing. Dairy proteins have been identified as a highly nutritive and functional ingredient in food and pharmaceutical sector benefiting both consumers and industries. Whey from bovine milk which includes β-lactoglobulin, α-lactalbumin, bovine serum albumin and immunoglobulins has been widely used as food ingredients, nutraceuticals, encapsulant and so forth. These functional and biological properties of proteins are influenced by environmental stress such as temperature, pressure, pH, processing conditions, and also by the presence of salts, sugars, alcohol etc. Therefore this investigation focuses on the effect of co-solutes and processing conditions on the globular conformation and structural behaviour of dairy proteins from low to high solid systems.

Stability of globular conformation against thermal treatment has been investigated on whey protein isolate and bovine serum albumin in the presence of glucose syrup. Calorimetric studies on the denaturation of protein molecules in the presence of co-solute witnessed an increase in the unfolding temperature of globular conformation. This suggests that presence of co-solute increases the surface free energy of globular conformation making it more stable against high temperature. This trend extends from low to high solid formulations which represent products from low viscous fluids to highly condensed pastes. Mechanical and microscopic observation of these protein/sugar systems during thermal treatment and subsequent aggregation suggests that sugar reduces the size of the protein aggregates, thus making a coherent network with high elasticity. This observation can have wide application in food industry, especially for the manufacture of dairy desserts where globular protein in sugar can act as textural modifiers or fat replacers. High solid aggregated systems of protein/sugar exhibits early vitrification (network relaxation) at around 0°C, i.e. transformation from rubbery to glassy consistency.

Further investigations into high solid systems suggest that, phenomenon of network relaxation temperature is dependent on the intensity of protein aggregates. In order to observe the sample at broader time scale, as well as to understand the molecular processes involved during the relaxation process, time temperature superposition (TTS) was implemented along with concepts of polymeric free volume from the expression of Williams Landel & Ferry (WLF). The mechanical glass transition was estimated at the temperature where sample shifts from the kinetics of free volume theory to the predictions of reaction rate theory. This mode of investigation clearly indicates that protein in native state exhibit late glass transition as
opposed to fully denatured system where an early vitrification was witnessed. This highlights the fact that glass transition temperature of a material is dependent on its physical and chemical properties and cannot be generalised or modelled based on molecular weight or moisture content within a system.

High pressure processing is an emerging non-thermal technique which leaves less carbon foot print on the environment. Immunoglobulins from bovine source were investigated for their conformational stability after high pressure processing. A comprehensive picture was obtained on the structural behaviour of immunoglobulins at atmospheric conditions and following application of a high-pressure protocol. Experimental observations are further supported by theoretical modeling, which produces comparable predictions for the glass transition temperature and free volume of atmospheric and pressurised systems. Estimates of denaturation enthalpy and infrared spectroscopy data argue that immunoglobulin samples subjected to high hydrostatic pressure retain a stable secondary conformation comprising mainly alpha helix and beta sheets, an outcome that argues for retention of bioactivity in the condensed pastes. The works suggests opportunities for the utilisation of high-pressure treated immunoglobulins in starch or dairy based formulations of functional foods in an effort to initiate replacement of thermally treated dairy powders with limited bio-functionality.