Examination of a Novel Proteinaceous Extract from Winter Rye (*Secale cereale* L. cv Musketeer)

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ABSTRACT

A gel is a cross-linked polymer network that spans an entire liquid medium; its properties depend strongly on the interaction of the polymer and the liquid medium. There are various ways to induce gelation in different systems such as altering temperature or pH. In this study, phenol extracted protein fractions from non-acclimated (NA) and cold-acclimated (CA) winter rye (*Secale cereale* L. cv Musketeer) leaf tissue were subjected to freeze-thaw treatment. Gelation was induced in the NA and CA extracts after repeated freeze-thaw treatments, accompanied by a change in sample rheological properties. Further experimentation revealed that gel formation only occurred at high pH (pH 12.0) and that a minimum of 3 to 4 freeze-thaw cycles were required. The viscosity of the protein gel increased 5.7- to 9.5-fold in the NA and CA extracts respectively upon freeze-thaw. Experiments optimizing the extraction conditions and protein concentration were also performed. The gel was stable and only a specific combination of chaotropic agent, anionic surfactant and reducing agent such as urea, sodium docecyl sulfate (SDS) and β-mercaptoethanol (β-ME) with heating could disrupt the gel network. The gel was composed of several proteins in the extracts as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Based on SDS-PAGE analysis, ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) was identified as the major protein component in the gel. Various experiments were performed to assess the role of Rubisco in gel formation; however, the results were inconclusive. It is suggested that these extracts may contain antifreeze proteins (AFPs) that have been demonstrated to form amyloid gels upon freeze-thaw. Further studies examining the composition and mechanism of gel formation may result in a future role for this material in the food industry.

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LIST OF ABBREVIATIONS

AFP	Antifreeze protein
β-ME	β-mercaptoethanol
BSA	Bovine serum albumin
CA	Cold-acclimated
CD	Circular dichroism
cP	Centipoise
EDTA	Ethylenediaminetetraacetic acid
FDA	Food and Drug Administration
kD	Kilodalton
M_W	Molecular weight
NA	Non-acclimated
NMR	Nuclear magnetic resonance
PAR	Photosynthetically active radiation
PEI	Polyethyleneimine
PPFD	Photosynthetic photon flux density
PVPP	Polyvinylpolypyrrolidone
rpm	Revolutions per minute
Rubisco	Ribulose-1,5-bisphosphate carboxylase oxygenase
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
tBSA	Modified phenol extraction treated bovine serum albumin
TCA	Trichloroacetic acid
V1	Velocity 1
V2	Velocity 2
v’	Change in frequency
v_i	Raman shift
v_o	Initial frequency

1.0 INTRODUCTION

Cold-tolerant herbaceous plants, such as winter cereals, can grow at low temperatures and survive freezing events as a result of a process referred to as cold acclimation (Xin and Browse, 2000; Thomashow, 2001; Sung *et al.*, 2003). Cold acclimation is complex and involves numerous physiological and biochemical changes that result in improved freezing tolerance. The survival of frozen plant tissues depends on preventing damage caused by the growth of intracellular ice crystals. Winter rye (*Secale cereale* L.) is an over wintering annual plant that can survive temperatures below -30°C. This is due, in part, to ice formation only in the intercellular spaces which is mediated by endogenously produced AFPs (Pearce, 1988; Griffith and McIntyre, 1993; Brush, *et al*., 1994). Six AFPs have been found in the apoplast of winter rye leaves which function by inhibiting ice growth and recrystallization and are thought to be involved in maintaining the rheologic properties of the cellular matrix (Griffith *et al*., 1992; Griffith *et al.*, 1997; Yu and Griffith, 1999).

While extracting protein from cold acclimated winter rye for 2-D electrophoresis, researchers in the laboratory of Dr. Barbara Moffatt at the University of Waterloo made an interesting observation. After several freeze-thaw cycles, the protein extracts became more viscous and the formation of a gel was observed (Barbara Moffatt, personal communication). Interestingly, researchers examining winter flounder type I AFP also observed a translucent gel which formed upon freezing and thawing that was found to be amyloidotic in nature (Graether *et al.*, 2003; Graether and Sykes, 2009).

The food industry is continually searching for new food components to improve texture and in this aspect, food gels, especially those from natural (plant) sources, play an essential role (Roberfroid, 1999; Renard *et al.*, 2006). In addition, the quality of frozen foods decreases over time due to water sublimation and ice crystallization. Over wintering plants grow in similar sub-zero conditions as the storage of frozen food, by synthesizing compounds that limit the movement of water and growth of ice crystals.

By successfully identifying and characterizing the compounds responsible for gel formation in plant protein extracts, it may be possible to use this knowledge to improve the quality of frozen food products or use these materials as a thickener and gelling agent.

2.0 LITERATURE REVIEW

2.1 Gels

What is a gel? This is a question to which there is often no precise answer. This was noted by Dorothy Jordan-Lloyd decades ago (Lloyd, 1926), who stated "the colloidal condition, the gel, is one which is easier to recognize than to define, and even recognition is confused by the fact that the limits between gel and sol, on the one hand, and gel and what may be termed curd, on the other, are not precise, but consist of a gradual change". Almost sixty years ago, Bungenberg de Jong (1949) defined a gel as "a colloidal system of solid character, in which the colloidal particles somehow constitute a coherent structure, the latter being interpenetrated by liquid system". Whereas Tanaka (1987) defined a gel as "a cross-linked polymer network swollen in a liquid medium; its properties depend strongly on the interaction of the polymer and the liquid medium".

Currently, many different types of gels are being examined by a wide range of scientists from many disciplines. However, there is still a lack of consensus to reach a consistent definition of what constitutes a gel (Almdal *et al.*, 1993).

2.1.1 Types of Gels

Flory (1974) originally proposed a classification of gels in terms of the structural elements of the continuous gel network (Flory, 1974; reviewed by Horne, 1999). He proposed the following four classes indicated in Table 2.1.

Table 2.1 Classification of gels as proposed by Flory (1974).

Class	Definition	Description	Example
I	Well-ordered lamellar structures		Soap gels Inorganic gels from clay or minerals
II	Covalent polymeric networks; completely disordered	A continuity of structure is provided by a ramified three-dimensional network comprising structural units covalently linked to each other	Polyacrylamide gels Vulcanized rubbers
III	Polymer networks formed through physical aggregation; predominantly disordered, but with regions of local order	Polymer networks are formed when primary molecules, usually of a linear structure but finite size, come together to form junction zones at particular points along the protein chain	Gelatin gels
IV	Particulate, disordered structures	Particulate gels are clusters of aggregated particles which network to form continuous structures extending throughout an enclosing volume	Acidified milk gels

More recently, Burchard and Ross-Murphy (1990) reclassified gel networks into three main classes, which are covalently cross-linked materials, entanglement networks and physical gels (reviewed below). Despite the confusion and inconsistency in gel definition, in more modern terminology**,** a gel has been described as a viscoelastic solid, which depending on circumstances, it can flow like a viscous liquid and in others behave like an elastic solid (Horne, 1999).

2.1.1.1 Covalently Cross-Linked Materials

Covalent polymeric gels are associated with supermolecules, generally formed by covalently cross-linking simpler linear polymers. Their mechanical properties reflect this network structure (Ross-Murphy, 1995). The structure of a polymer gel consists of an "infinite" network of chains, from a macroscopic viewpoint. A container of gel consists of a macromolecule so large and branched that it actually fills the container. If one of the segments of a linear polymer in solution can cross-link with other segments on other chains a gel should

eventually be formed. After each intermolecular cross-linking, the average molecular weight (M_w) increases as the number of cross-linking sites has been increased. Over a period of time, as more and more cross-linking reactions occurs, the M_w increases even faster until it ultimately becomes "infinite" (Ross-Murphy, 1995). At this point, the largest individual molecule completely spans the entire volume in which it sits. This is known as the "gel point". Since M_w has become infinite, the longest relaxation time, which depends on M_w is also infinite. Thus, the gelled sample no longer flows like a polymer solution but instead has the properties of an equilibrium solid.

2.1.1.2 Entanglement Networks

Entanglement networks are formed by the simple topological interaction of polymer chains rather than by covalent cross-linking (Kavanagh and Ross-Murphy, 1998). Entanglement networks occur in the molten state or in solution when the product of concentration and relative molecular weight becomes greater than some critical entanglement molecular weight. Entanglement networks behave as pseudogels at frequencies higher (time scale shorter) than the lifetime of the topological entanglements. The rheological discrimination between entanglement networks and cross-linked gels can be made by the technique of dynamic mechanical analysis. Using small deformation, an oscillatory strain of frequency is applied to the material and shear storage modulus and shear loss modulus are measured. For the entanglement network, at very low frequencies, in the 'terminal zone' they flow as high viscosity liquids. However, the cross-linked gels response differently depends on the product concentration and relative molecular weight for the system before cross-linking was above or below the critical entanglement molecular weight. A further discrimination is that when excess solvent is added, the entanglement network system will dissolve to form a more dilute polymer solution whereas the covalently cross-linked gels will swell but not dissolve.

2.1.1.3 Physical Gels

Physical gels are polymer gels formed through physical aggregation or non-covalent cross-links (Burchard and Ross-Murphy, 1990). The presence of non-covalent cross-links

complicates any physical description of the network properties significantly, because unlike chemical bonds, their number and position will fluctuate with time and temperature (Clark and Ross-Murphy, 1987; Kavanagh and Ross-Murphy, 1998). The nature of the physical network is not known unambiguously. This is due to that disparate forces are often involved such as, Coulombic, dipole-dipole, van der Waals, charge transfer, hydrophobic and hydrogen bonding interactions as examples. In biopolymer gels non-covalent physical cross-links are formed by one or more of the disparate forces mentioned above, together with more specific and complex mechanisms involving junction zones of known, ordered secondary structure, for example, multiple helices or ion mediated "egg box" structures. Normally, there is a specific and often intricate, hierarchy of arrangements.

2.1.1.4 Hydrogels

Hydrogels are another classification of gels, which consist of three-dimensional, hydrophilic, polymeric networks capable of imbibing large amounts of water or biological fluids (Peppas *et al.*, 2000). Hydrogels were first introduced in the 1960's as novel materials having potential for a variety of biomedical applications, such as in soft contact lenses, drug delivery and tissue engineering (Wichterle and Lim, 1960; Hoffman, 2002). Since the introduction of hydrogels, they have gained popularity in a broad range of pharmaceutical and biomedical applications.

2.1.2 Factors Inducing Gelation

Gelation may be defined as that process (or series of process) which leads to the formation of a gel (Tan *et al.*, 2009). At the point in which the molecules in a solution react (cross-linking) to form larger molecules and eventually span the whole container in which it sits, is termed as the "gel point". In principle, any process which involves the cross-linking of chains can eventually lead to branching and finally gelation, provided that intermolecular bonds are favoured over intramolecular bonds. However, this depends on certain factors, such as sample concentration (Kavanagh and Ross-Murphy, 1998).

One of the most important functional properties of proteins is gelation (Avanza *et al.*, 2005). There are different ways to induce gelation in different systems. These include altering temperatures and pHs, adding cross-linkers, changing ionic strength, protein types and concentrations (Mulvihill and Kinsella, 1987). The most important factors in gelation are probably the protein concentration, heating temperature and pH. If either the temperature, protein concentration or pH are not favorable, gelation will not occur (Dunkerley and Hayes, 1980; Ross-Murphy, 1991; Sánchez and Burgos, 1996). Once temperature and protein concentration have surpassed their critical values, gel strength increases and gelation time decreases with increasing temperature and concentration (Dunkerley and Hayes, 1980; Ross-Murphy, 1991). For example, acidification and lowering temperature will induce gelation in milk (Raouche *et al.*, 2007). Caseins micelles are very stable under native milk conditions and are in equilibrium with soluble micelles and dissolved salts in the serum. However, changing pH and temperature induces aggregation or disintegration of casein micelles due to the solubilization of micellar calcium phosphate and β-casein release. The gelation of fish muscle protein is useful in food industry for making surimi, the main ingredient of artificial crab sticks. This involves thermally denaturing the protein and allowing the denatured protein to aggregate through the formation of a three dimensional structure (Stone and Stanley, 1992).

Gelation capacity and gel properties are directly related to their rheological properties (Avanza *et al.*, 2005). Protein gels are composed of a protein matrix within which the aqueous phase is occluded (Avanza *et al.*, 2005). The microstructure of the gel matrix determines the rheological properties, such as viscoelasticity and texture. Depending on the microstructure, the gels with a fine-stranded matrix, are harder and are able to retain more water than those with more open matrices, particulate gels for example (Foegeding *et al.*, 1995). The presence of covalent and non-covalent bonds within the gel matrix affects the nature of the gel differently in terms of gel structure and viscoelasticity.

2.2 Rheology

The term rheology is derived from the Greek words rheo, “to flow,” and logos, “science.” Rheology is therefore the study of the deformation and flow of materials under the influence of an applied stress. This definition was adopted when the American Society of

Rheology was founded in 1929 (Metzner, 1985; Barnes *et al.*, 1989; Amiji, 2003). Rheology is composed of three variables and their interaction with each other: applied force or shear stress; measured response or strain; and the time during which these events take place (Ross-Murphy, 1995).

Rheology can be applied to solid, liquid, and gaseous states of matter (Amiji, 2003). From a rheologic perspective, solids are classified as being completely resistant to deformation, liquids as being less resistant, and gases as being completely nonresistant. Rheology is used to describe the consistency of different products, normally by the two components viscosity and elasticity. Viscosity is defined as a measure of resistance to flow or thickness, and elasticity refers to the ability of a material to return to its original state once stress under which it deforms is removed. The higher the magnitude of viscosity is, the more resistant the material will be to flow. The friction of a fluid becomes evident when a layer of fluid is made to move in relation to another layer. The greater the friction the greater the amount of force required to cause this movement, which is called shear. Shearing occurs whenever the fluid is physically moved or distributed, as in pouring, spreading, or mixing and therefore, more force is required to move a more viscous than a less viscous material.

Isaac Newton proposed a model for the viscosity of a fluid in 1687 (Newton, 1687; Figure 2.1). Newton's model considered that two planes of fluid of equal area (A) are separated by a distance (dx) and are moving in the same direction at different velocities, V1 and V2 (Figure 2.1). Newton assumed that the force required to maintain this difference in speed was proportional to the difference in speed through the liquid, or the velocity gradient. Newton concluded that where 'h' is a constant for a given material and is called its velocity. The velocity gradient dv/dx is the measure of a material experiences a change in speed at which the intermediate layers move with respect to each other (Figure 2.1). This described the shearing the liquid experiences, termed shear rate (sec^{-1}). The force per unit area required to produce the shearing action are termed shear stress ($dynes/cm^2$). The viscosity can be defined as the shear stress divided by the shear rate. A material required a shear stress of one dyne per square centimeter to produce a shear rate of one reciprocal second has a viscosity of one poise or 100 centipoise (cP), poise is the basic unit of viscosity in the Absolute Metric system (Fox *et. al.*, 2009). For example, the viscosity of water at 20°C is 1 cP. Newton assumed that all fluids at a given temperature have a viscosity that is independent of the shear rate and time during which

the fluid is subjected to shear. In other words, twice the shear rate applied will move the fluid twice as fast. This type of flow behavior Newton described is called Newtonian fluids. Water and thin motor oil are the typical examples of Newtonian fluids.

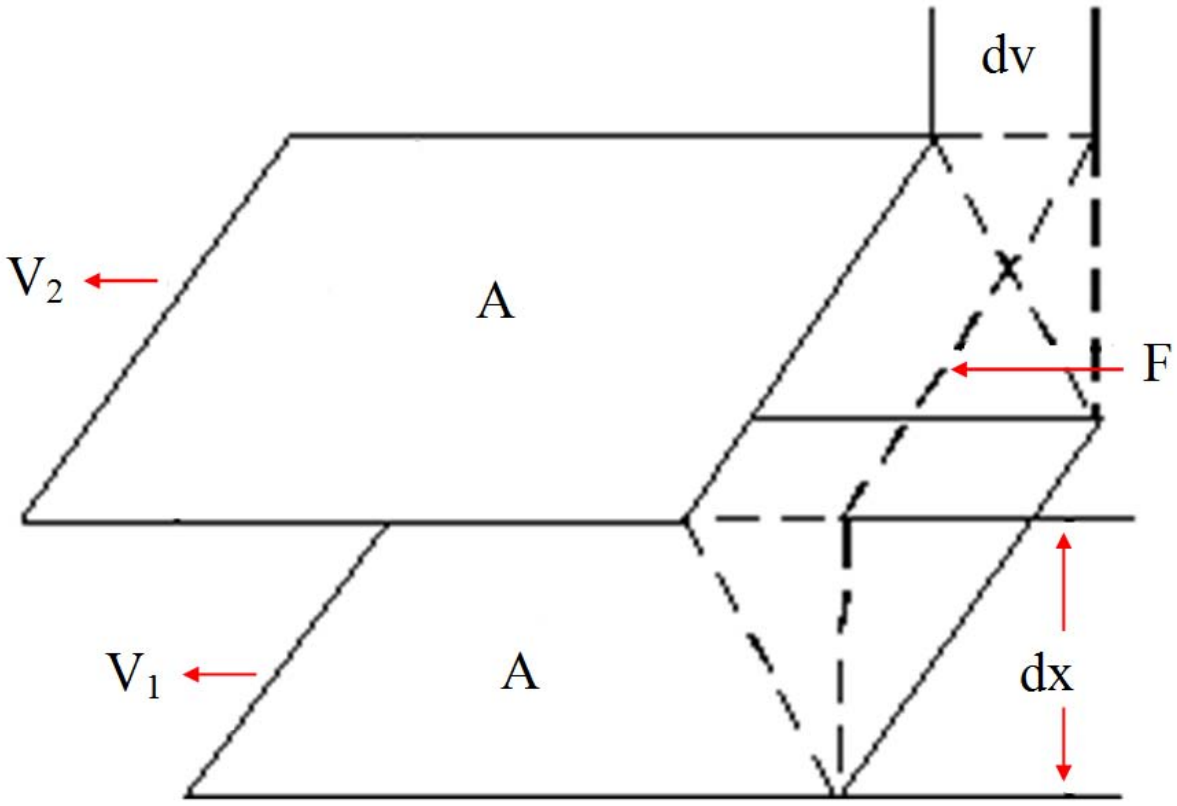

Figure 2.1 Model for the viscosity of a fluid. Proposed by Newton (1687). A, surface area; V1, velocity 1; V2, velocity 2; F, force; dv, difference in velocity; dx, difference in distance between two parallel planes.

However, many of the fluids do not fall into the category of a Newtonian fluid, therefore, those who do not satisfy Newton's criterion are known as non-Newtonian fluids. Non-Newtonian fluids exhibit a more complicated relationship between shear stress and velocity gradient. The viscosity of non-Newtonian fluids depends on the shear rate and/or the length of time which the material is subjected to stress. The shear stress of non-Newtonian fluids is not directly proportional to deformation rate (Fox *et. al.*, 2009). There are several types of non-

Newtonian fluids. They are classified according to the basis of the way in which their viscosity changes and are indicated in Table 2.2.

Table 2.2 Types of non-Newtonian fluids.

Type	Description	Example
Pseudoplastic (shear-thinning)	Viscosity decreases as the shear rate increases Most non-Newtonian fluids fall into this category	Polymer solutions Paint Shampoo
Dilatant (shear-thickening)	Viscosity increases as the shear rate decreases	A mixture of corn starch and water (mixed in correct proportions)
Bingham plastic	The transition from high viscosity (semisolid) to low viscosity takes place only after a shear stress exceeding a certain minimum value (yield value)	Ketchup

The viscosity of some non-Newtonian fluids is time-dependent (memory materials) (Fox *et. al.*, 2009). These fluids are referred to as rheopectic or thixotropic. The viscosity of rheopectic fluids increases over time with constant shear rate, for example, whipped cream, whereas the viscosity of thixotropic fluids decreases over time with constant shear rate, for example, honey.

The viscosity of a fluid can be measured by a viscometer. There are various laboratory viscometers available commercially such as a U-tube viscometer, a falling sphere viscometer, a falling piston viscometer, a vibrational viscometer and a rotational viscometer. A rotational viscometer was used in this study. A rotational viscometer measures the torque required to turn an object in a fluid and the torque is a function of the viscosity of the fluid. The torque is measured by rotating a disk (fixture) in a fluid at a known speed. There are different fixtures available in a rotational viscometer, such as parallel plate fixture and cone/plate fixture (Figure 2.2).

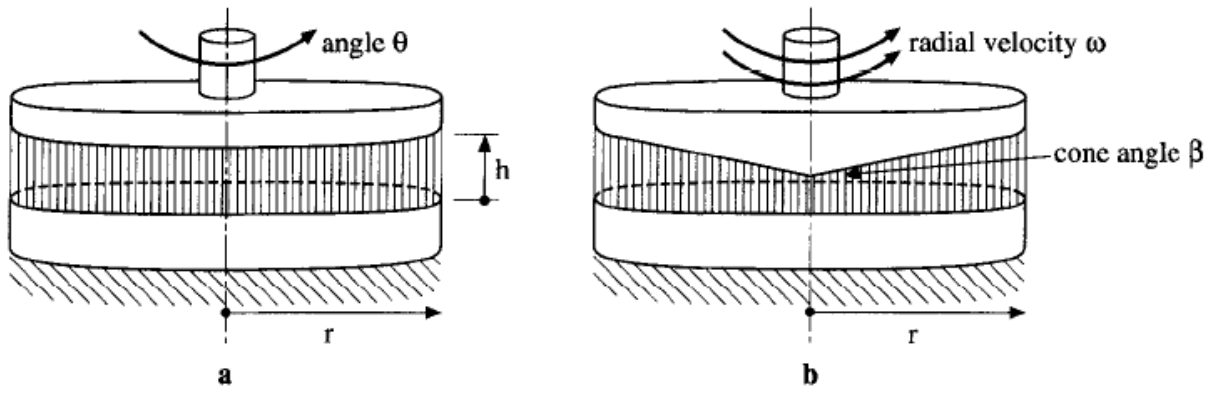

Figure 2.2 Plate fixtures used with rotational viscometers. (a) Parallel plate fixture. (b) Cone/plate fixture. Adapted from Krieger and Woods (1966).

A parallel plate fixture was used in this study. The advantages to the use of a parallel plate fixture include greater convenience in construction and alignment, and the ability to change the shear rate range by simply varying the gap width (Krieger and Woods, 1966). Parallel plane instruments are preferable to cone/plate fixture viscometers for the study of coarse mixtures and of suspensions which tend to destabilize when sheared in narrow gaps. They are also advantageous for measurements at high shear rates, because of superior heat transfer and lesser tendencies toward centrifugation and secondary flows.

2.3 Application of Gels

2.3.1 Biomedical Industry

In biomedical applications, *in situ* photopolymerization of hydrogels has been used for over a decade. Briefly, polyethylene glycol is used as a central block, which is flanked with oligo (α-hydroxy acids) and acrylate groups, coupled to the terminal hydroxyl groups (Sawhney *et al.*, 1993). The acrylate end-groups of polyethylene glycol show rapid polymerization upon irradiation with visible light in the presence of a suitable photoinitiator to yield a hydrogel structure. The incorporation of the oligo (α-hydroxy acids) ensures the degradability of the matrices under physiological conditions. It has been shown that a continuous release of bovine serum albumin (BSA) from the matrices for up to 2 months was achieved, making this system potentially suitable for protein delivery applications. These *in situ* polymerized gels have been

shown to be effective in the prevention of scar adhesion formation after pelvic surgery in animal models (Sawhney *et al.*, 1994). In addition, hydrogels have been shown to prevent thrombosis and reduced long-term intimal thickening when applied as a mechanical barrier on severely injured arteries (Hill-West *et al.*, 1994). Hydrogels such as polyethylene glycol- and polyvinyl alcohol-based polymers have the potential to serve as a replacement for damaged cartilage while new cartilage forms (Anseth *et al.*, 2002). Polymerizable hydrogels have a wide range of potential applications in tissue engineering (Nguyen and West, 2002).

2.3.2 Food Industry

In the food industry, gelation is regarded as one of the most important functional properties of polysaccharides and proteins. Functional properties of food proteins, such as solubility, surface activity, conformational stability, gel forming ability and emulsifying and foaming properties are affected by their interaction with polysaccharides (Makri *et al.*, 2006). In many food products, gelation of biopolymers is critical to the formation of desired texture that is preferable by consumers (Makri *et al.*, 2006). The texture depends on the mechanical properties, which in turn depends mainly on the structure of the gel network.

The gelling ability of fish muscle myofibrillar protein is utilized extensively in food, as the final texture conferred by gelled protein is a favoured sensory property (Rodger and Wilding, 1990). The gelling properties of fish myofibrillar protein led to the development of surimi, a modified fish muscle mince which is used for the production of a group of fish analog foods such as crabsticks and fish sausage (Lee, 1984). Sausages are an example of food product made from gelation of a mixture of poultry muscle tissue, solubilized proteins, fat, salt, and water (Foedgeding, 1988). During the manufacturing processes of sausages, heating the comminuted meat causes structural changes in the muscle proteins which favor intermolecular protein interactions. Protein aggregation progresses to gelation under favorable conditions. Recent studies showed that the addition of the cross-linking agent transglutaminase enhances the texture and gel strength of meat and meat proteins by forming a bond between glutamine and lysine, which improves the rigidity and gel elasticity of meat products (Ahhmed *et al.*, 2009). Yogurt gel is a fermented milk product. The formation of the texture of yogurt is achieved through gel formation in milk as a result of fermentation. Yogurt is prepared by

fermenting milk with bacterial cultures such as a mixture of *Streptococcus* subsp. *thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Lee and Lucey, 2010). There are two major types of yogurts which are the set and stirred type. Set yogurt (which includes fruit-on-the bottom) is formed in retail pots as lactic acid bacteria ferment lactose into lactic acid giving a continuous gel structure in the container. Whereas stirred yogurt, the acid gel formed during incubation in large fermentation tanks is disrupted by agitation, and the agitated product is usually filtered through a screen which gives the product a smooth and viscous texture. The lack of visual whey separation and perceived viscosity, are important properties for the quality and overall sensory acceptance of yogurts.

There is an increasing interest in the production of healthier foods with low fat content or with low levels of additives. However the additives in new functional foods, low-fat products or vegetarian foods do not always meet customers' satisfaction (Welman and Maddox, 2003; Piermaria *et al.*, 2008). As a result, the food industry is always searching for new compounds to improve texture and mouthfeel of food, especially those with a health-promoting capacity (Roberfroid, 1999).

Functional properties of food proteins, such as those from legumes and soybean, play an important role in food products (Chau and Cheung, 1998; Makri *et al.*, 2006; Zhu *et al.*, 2008). Legumes are of high nutritional value, contain a high amount of protein, have low lipid levels and are cholesterol free. Legumes have an important property for food texture improvement as they form gels with good water-holding capacity upon heating. In addition, legume protein can act as an emulsifier and stabilizer in the formulation of food emulsions and foams. Soybean protein is well known for its functional and nutritional properties (Singh *et al.*, 2008). Gelation of soybean protein is another important functional property and results in the incorporation of soybean protein in many food products.

Polysaccharides of microbial origin have been developed as food additives including xanthan gum from *Xanthomonas campestris* and gellan gum from *Pseudomonas elodea* which are used as gelling and emulsifying agents (Piermaria *et al.*, 2008). Xanthan gum is a heteropolysaccharide with a primary structure consisting of repeated pentasaccharide units formed by two glucose units, two mannose units, and one glucuronic acid unit (García-Ochoa *et al.*, 2000). Xanthan and gellan gums have been approved by the United States Food and Drug Administration (FDA) for food use (García-Ochoa *et al.*, 2000; Bajaj *et al.*, 2007). Xanthan

gum has been widely used in the food industry for a number of important reasons, including emulsion stabilization, temperature stability, compatibility with food ingredients and gelling agents (García-Ochoa *et al.*, 2000). Main food industry applications of xanthan gum include salad dressings (emulsion stabilizer; dispersant), syrups (thickener), dairy products (stabilizer; viscosity control of mix) and frozen foods (improves freeze-thaw stability). Gellan gum is a heteropolysaccharide composed of a tetracyclic repeating unit of one rhamnose, one glucuronic acid, and two glucose units substituted with an acyl group as O-glycosidically-linked esters (Bajaj *et al.*, 2007). Gellan gum is widely utilized in foods which require a highly gelled structure, and may also be suitable for uses in improving the mouth-feel of a food. Main applications of gellan gum in the food industry, include jams (thickener), dessert gels (gelling agent), and dairy products (bulking agent).

2.3.3 Biological Systems

Floyd and Ohlrogge (1970) observed that a naturally present mucilaginous gel formed on the surface of the nodal roots of corn. This mucilaginous gel was investigated and was found to be a mixture of uronic acids and polysaccharides. Recent studies have shown that mucilage gel can occur in high concentrations in different plant organs, but their physiological function in most cases is unclear (Clifford *et al.*, 2002). Mucilage found in rhizomes, roots, and seed endosperm may act as energy reserves. Foliar mucilage does not serve as storage carbohydrates and are usually assumed to be secondary metabolites. However, there are research groups that have suggested they may play a role in freeze and drought tolerance. The *Aloe vera* leaf also contains a mucilage gel which naturally occurs from the parenchyma cells of the plant (Eshun and He, 2004).

Plants that are freezing tolerant, such as winter rye (*Secale cereale* L.), can survive temperatures below -30°C by undergoing a process known as cold acclimation (Griffith and McIntyre, 1993) This involves growth and development at low, non-freezing temperature and results in numerous biochemical and molecular changes allowing the plant to acquire freezing tolerance. It has been shown that cold acclimated winter rye contains endogeneously produced AFPs which are involved in maintaining the rheologic properties of the cellular matrix during freezing (Griffith *et al.*, 1992). AFPs modify the normal growth pattern of ice crystals and

depress the freezing temperature of water non-colligatively by binding to the prism faces of ice, slowing growth and recrystallization (Griffith *et al*., 2005).

Type I AFP is found in fish (winter flounder) living in subzero seawater, and protects the organism from macromolecular ice growth by adsorption inhibition (Fletcher *et al*., 2001). Interestingly, while examining this AFP mechanism, Graether *et al*. (2003) found that the winter flounder AFP in solution formed a translucent gel upon freezing and thawing. Further, they proceeded to demonstrate that type I AFP was converted into amyloid fibrils upon freezing and thawing, conditions appropriate to its physiological function (Graether *et al*., 2003; Graether and Sykes, 2009). In addition, the authors speculate that this AFP could be used in applications such as cryopreservation, cryosurgery and as a food additive (Graether *et al*., 2003). Therefore, AFPs, at least in fish, appear to be natural protective amyloids (Iconomidou and Hamodrakas, 2008) and it is interesting to speculate if a similar phenomenon is occurring in winter rye.

2.4 Relevant Protein Analysis Methods

Substantial improvements in protein separation and identification techniques and the expansion of genomic knowledge have led to an increase in the application of proteomic methods to unravel biological questions. Although many biochemical and biophysical techniques exist, those particularly relevant to this study include protein extraction, SDS-PAGE and Raman spectroscopy. These are described in detail in the sections following.

2.4.1 Protein Extraction

For all proteomic analyses, sample quality is a critical factor, and so the protein extraction procedure is of prime importance. The ideal extraction method should reproducibly capture the most comprehensive repertoire of proteins possible, at the same time, minimizing degradation and contamination by non-proteinaceous compounds. Due to the diverse biochemical properties of cellular proteins, including their charge, size, hydrophobicity, susceptibility to proteolysis, ligand interactions and subcellular localization, no single protein extraction protocol can capture the full proteome (Isaacson *et al.*, 2006). Different protein

extraction protocols yield a subset of proteins, but each type of extract generated will include a subset of proteins that is not present in the other (Saravanan and Rose, 2004; Isaacson *et al.*, 2006). Comprehensive, uncontaminated and representative protein populations are known to be difficult to extract from plants. This is because plant cells produce a broad spectrum of secondary metabolites that contaminate protein extracts and severely interfere with downstream analysis (Rose *et al.*, 2004; Saravanan and Rose, 2004).

The trichloroacetic acid (TCA)-acetone protein extraction protocol is based on precipitating proteins from homogenized leaf tissues or cells with TCA and acetone (Dameval et al., 1986). Most proteomic studies of entire plant tissues use this simple method of TCA-acetone protein extraction to precipitate the proteins followed by resolubilization in a buffer containing chaotropes and detergents (Satoni *et al.*, 1994). Although the TCA-acetone extraction protocol has been found to be useful in young plants, it was found not to be necessarily the best method for more complex plant tissues that contain a high level of interfering compounds such as polyphenols and polysaccharides (Wang *et al.*, 2003; Saravanan and Rose, 2004; Zhou *et al.*, 2006).

The phenol protein extraction protocol is based on the solubilization of proteins in phenol, followed by their precipitation with ammonium acetate in methanol. This protein extraction method was developed and perfected in the 1980's and 1990's (Schuster and Davies, 1983; Hurkman and Tanaka, 1986; Meyer *et al.*, 1988; Usuda *et al.*, 1995; Isaacson *et al.*, 2006). This extraction method is effective with recalcitrant and resistant tissues such as banana, avocado, orange peel, olive leaves and wood, that contain high levels of interfering compounds such as polysaccharides and polyphenols (Mijnsbrugge *et al.*, 2000; Wang *et al.*, 2003; Saravanan and Rose, 2004). The phenol extraction method is more laborious and time consuming than the TCA-acetone extraction method but it has the advantages of generating a substantially higher purity and greater protein yield (Saravanan and Rose, 2004). The ability to generate a higher purity protein sample is due to the fact that water-soluble contaminants are partitioned into a discrete aqueous phase or are centrifuged into an insoluble pellet, well apart from the protein-enriched phenolic layer.

2.4.2 SDS-PAGE

The most popular technique for protein separation is currently gel electrophoresis, although alternative gel-free techniques which are based on fractionation by liquid chromatography are becoming increasingly popular (Wu *et al.*, 2006). Polyacrylamide gel electrophoresis in SDS (SDS-PAGE) was described for the first time in 1949 (Hoch, 1949; Bodzon-Kulakowska *et al.*, 2007). The SDS-PAGE is a useful and reliable method for molecular weight analysis of proteins; it is a powerful tool that can be used for protein identification or as an assay for protein purity (Shapiro *et al.*, 1967; Weber and Osborn, 1969; Bodzon-Kulakowska *et al.*, 2007).

Briefly, the extracted protein sample is mixed with a sample buffer which usually contains Tris, glycerol, β-ME, SDS and a tracking dye (bromophenol blue; Laemmli, 1970). The protein mixture is heated with buffer for 2-5 minutes before loading into the polyacrylamide gel. The Tris acts as a buffer for the protein mixture while the glycerol helps to weigh down the sample into the wells while loading. β-ME is a reducing agent which reduces the intra- and inter-molecular disulfide bonds of the proteins. SDS is an anionic detergent that dissociates and denatures protein secondary and non-disulfide tertiary structures into individual polypeptides. The heating process denatures the protein and SDS binds around the polypeptide backbone to induce "reconstructive denaturation", where proteins adopt a conformational mixture of α-helix and random coil (Maizel, 1966; Laemmli, 1970; Rath *et al.*, 2009). In general, SDS is thought to bind with the polypeptides in a constant weight ratio of 1.4 g/g of polypeptides, which results in a net negative charge on each protein in proportion to its mass (Reynolds and Tanford, 1970). This treatment causes the polypeptides to become rod-like and possess the same net negative charge per unit length, thus the proteins will separate based on mass and not by charge during electrophoresis. A tracking dye is used to monitor the progress of protein migration, as it runs ahead of most sample proteins.

After the protein mixture is loaded into the wells of the polyacrylamide gel, an electric field is applied across the gel, causing the negatively charged proteins to migrate across the gel towards the anode. The migration speed of proteins depend on their size, smaller proteins will fit through the pores in the gel, while larger proteins will move slower as they encounter more resistance, and thereby a longer retention time in the gel. Thus, smaller proteins will travel

farther down the gel, whereas larger proteins will travel slower and remain closer to the origins. By varying the amount of acrylamide in the gel, generally in the range of 5 to 20% (w/v), will result in a different resolving power depending on the molecular weight of protein samples. Lower percentage acrylamide gels are generally used for proteins which have higher molecular weights, whereas higher percentage gels are good for resolving low molecular weight proteins.

The gel is usually stained with Coomassie brilliant blue so that the proteins can be visualized as distinctive bands, which have been separated according to their molecular weight. Coomassie brilliant blue was originally introduced to visualize protein bands in 1963 and is still the most widely used SDS-PAGE protein staining technique (Westermeier, 2006; Wang *et al.*, 2007). Coomassie brilliant blue gained its popularity due to its reproducibility, lower background, reasonable sensitivity and excellent compatibility with mass spectrometry (Candiano *et al.*, 2004).

2.4.3 Raman Spectroscopy

Raman spectroscopy is a technique used to study the vibrational and rotational transitions in a system (Raman and Krishnan, 1928; reviewed by Ferraro *et al.*, 2003). In principle, when a molecule is irradiated with light of a specific frequency (v_o), most of the photons are scattered from the molecule without a change in frequency ($v' = v_o$) or photon energy (Egawa and Yeh, 2005). This is called elastic or Rayleigh scattering (Figure 2.4a). A small fraction of the photons (approximately 1 in 10^7 photons) are scattered inelastically by losing or gaining a quantum of vibrational energy ($v' = v_o \pm v_i$), termed Stokes and anti-Stokes Raman scattering, respectively (Figure 2.3a). The Raman shift, (v_i), reflects the energy of an internal vibrational mode of the molecule. The scattering effect typically occurs in 10^{-14} seconds or less. In the spectrum of the scattered light, the frequencies of the Stokes and anti-Stokes are equally displaced with respect to the frequency of the incident light. The intensities of the Stokes lines are typically much stronger than the anti-Stokes lines because the anti-Stokes scattering originates from molecules in excited vibrational states (for example, v = 1 in Figure 2.3b) and depending on the Boltzmann distribution, the populations of the vibrational excited states are typically very small at room temperature. A change in the molecular polarizability or amount of deformation of the electron cloud with respect to the vibrational

coordinate is required for a molecule to exhibit a Raman effect. The amount of the polarizability change will determine the Raman scattering intensity. The pattern of shifted frequencies is determined by the rotational and vibrational states of the sample.

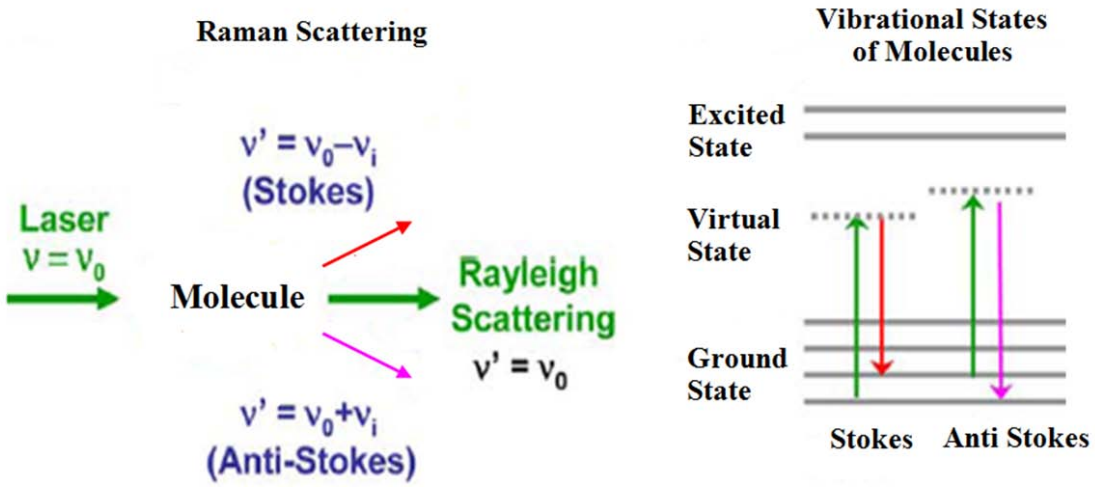

Figure 2.3 Raman scattering (a) and vibrational states (b) of molecules. Adapted from Egawa and Yeh (2005). (a) Red arrow, Stokes scattering; Green arrow, Rayleigh scattering; Pink arrow, anti-Stokes scattering. Rayleigh scattering has no Raman effect, the incident and emitted photons have the same energy. (b) In Stokes scattering, the atom or molecule absorbs energy, the emitted photon has less energy. In anti-Stokes scattering, the atom or molecule losses energy, the emitted photon has more energy.

Typical Raman spectroscopy uses a monochromatic source from a laser which is passed through a series of filters before it is focused on a sample cell, normally a cylindrical cuvette or Nuclear magnetic resonance (NMR) tube (Figure 2.4). The scattered light is typically at right angles to the excitation source and focused by an achromatic camera lens into the entrance slit of a polychromator. The incident laser light (the Rayleigh light) is filtered out through a notch filter placed between the camera lens and the polychromator. The dispersed light is collected by a liquid nitrogen cooled charge-coupled device attached to the exit port of the polychromator and analyzed by a computer (Figure 2.4). In a typical Raman spectrum, the intensity of scattered light is plotted as a function of $\Delta\nu$ cm $^{-1}$ (Raman shift), the relative energy of the scattered photon with respect to the incident photon.

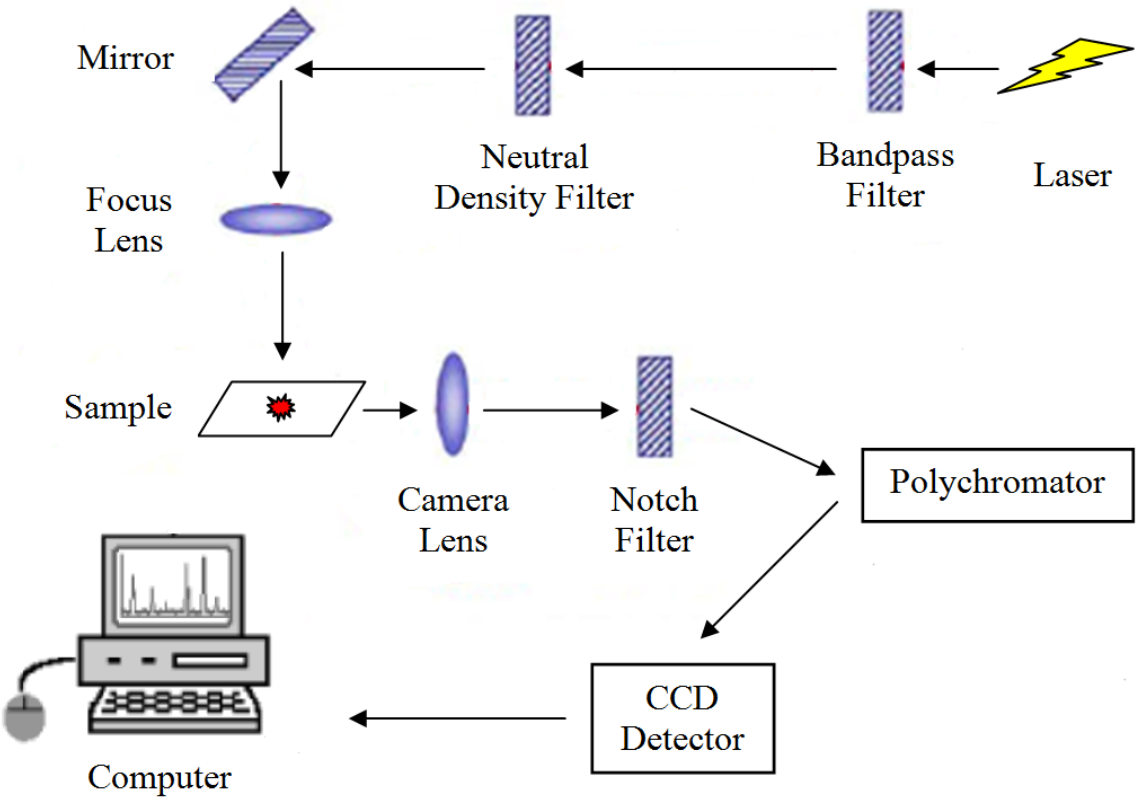

Figure 2.4 Schematic illustration of a typical Raman instrument. Adapted from Egawa and Yeh (2005).

Raman spectroscopy is capable of providing secondary structural information on proteins such as bond strength, angle and geometry of the molecule of interest similar to infrared absorption spectroscopy (Egawa and Yeh, 2005). However, there are several advantages of using Raman spectroscopy when compared to infrared spectroscopy including a smaller sample size, quantity of data obtained with a single recording and less interference from biological samples in aqueous solution (Ferraro *et al.*, 2003). The function, kinetics, and structure of proteins can be elucidated by Raman spectroscopy (Callender *et al.*, 1998; Zhao and Spiro, 1998). Analyzing protein amide band profiles provides information on the secondary structure of a protein and the feasibility of the method is not hindered either by size of the supramolecular assembly or by light-scattering artifacts that can severely restrict the use of other spectroscopic probes (Sane *et al.*, 1999). Raman spectroscopy also has several important advantages in characterizing the vibrational spectra and secondary structural

tendencies of natively unfolded proteins (Maiti *et al.*, 2004). The Raman spectra can be obtained in dilute aqueous solutions, thereby avoiding the tendency towards aggregation of these proteins at higher concentrations. Thus, Raman spectroscopy is a structure-rich probe that can be used to examine a sample in various states: aqueous solutions, insoluble aggregates, fibrils and crystals (Sane *et al.*, 1999; Dong *et al.*, 2003; Carey and Dong, 2004; Maiti *et al.*, 2004). The development of Fourier-transform Raman spectroscopy has been a significant advance in solving the fluorescence problem, due to phenolic compounds in plant substance (Meng *et al.*, 2003). Raman spectroscopy is more commonly used to determine the secondary structure or conformational change of homogenous protein samples. Meng *et al.* (2003) used Raman spectroscopy to study the conformation of red bean globulin protein in various buffers and on heat treatment, they suggested that extreme pH caused a changed in the protein conformation from an ordered structure to a random coil conformation. The mechanism for heme protein CO activation has also been investigated by Raman spectroscopy (Vogel *et al.*, 1999). The interaction of proteins with their substrates results in a shift in Raman band and/or change in the band intensity. Torreggianni and Fini (1998) investigated the conformational change and interaction between the binding of avidin with biotin derivatives. The study showed the importance of the side chains of biotin molecule in its interaction with avidin.

2.5 Thesis Objectives

In this thesis, I will examine protein extracts from non-acclimated and cold acclimated winter rye (*Secale cereale* L. cv Musketeer) to test the hypothesis that gel formation occurs preferentially in cold acclimated tissues and affects changes in sample rheological properties. This will be accomplished by characterizing the conditions required for gel formation as well as an analysis of the gelling and non-gelling components of the extracts to determine their biochemical and rheological properties. In this study, freezing and thawing are external stresses applied to the protein extracts in order to induce a change in sample rheology. It is hoped that this information will provide an important first step into the elucidation of protein gelation in winter rye and advance commercial applications of the resulting gel.

3.0 MATERIALS AND METHODS

3.1 Chemicals and Solutions

Distilled water was used for preparation of all buffers and solutions. Chemicals were obtained from suppliers as indicated in the text and were of the highest grade. Where commercially available kits were utilized, the suppliers are also indicated in the text.

3.2 Plant Materials and Growth Conditions

Winter rye (*Secale cereale* L. cv Musketeer) was germinated from seed (7-10 seeds/ pot) in four inch plastic pots containing coarse vermiculite in controlled environment growth chambers (Conviron E15; Controlled Environments Ltd., Winnipeg, MB, Canada). Non-acclimated plants (NA) were grown under 250 µmol photons m^{-2} s^{-1} photosynthetically active radiation (PAR), using fluorescent tubes (Cool White, 160 W, F72T12/CW/VHO, Sylvania), with a 16 h day length and day/night temperatures of 20/20°C for 21 days. The photosynthetic photon flux density was measured at pot height with a Li-Cor (Lincoln, NE, USA) Quantum/Radiometer/Photometer (model LI-189) equipped with a model LI-190SA quantum sensor (Li-Cor). For cold acclimated material (CA), plants grown under non-acclimating conditions for 7 days were shifted to a controlled environment chamber (Conviron E8) set for 5/4°C (day/night) temperatures with all other conditions constant for an additional 49 days. Plants were watered with a nutrient solution or water as required (Somerville and Ogren, 1982). All healthy leaves were harvested for analysis.

3.3 Protein Extraction and Quantitation

3.3.1 TCA-Acetone Extraction Protocol

Total leaf protein was extracted from NA and CA tissue as described by Isaacson *et al.* (2006). Leaf tissue (1 g) was frozen with liquid nitrogen and ground into a fine powder with a pre-chilled mortar and pestle. Five mL of ice cold TCA extraction buffer (10% [v/v] TCA in acetone, 2% [v/v] β-ME added immediately before use) was added and the mixture homogenized for 2 min. The slurry was transferred to a 15 mL plastic tube and stored at -20°C

overnight to allow protein precipitation. The mixture was centrifuged at 5,000 x g for 30 min at 4°C and the supernatant removed. The protein pellet was washed with 4 volumes (10 mL) of ice-cold acetone followed by centrifugation at 5,000 x g for 10 min at 4°C. This was repeated two more times and the resultant protein pellet was gently air dried in a fume hood at room temperature (22°C) for 30 min. The pellet was stored at -80°C for future analysis.

3.3.2 Phenol Extraction Protocol

Total leaf protein was extracted from NA and CA tissue using a modified protocol of Hurkman and Tanaka (1986). Leaf tissue (1 g) was frozen with liquid nitrogen and ground into a fine powder with a pre-chilled mortar and pestle using 2.5 mL of ice cold tissue extraction buffer (0.1 M Tris [pH 8.8], 10 mM EDTA, 0.9 M sucrose, 0.4% [v/v] β-ME added immediately before use) for 2 min. The homogenate was transferred to a 50 mL plastic tube and mixed in a 1:1 ratio (2.5 mL) of phenol saturated with Tris-EDTA, pH 8.0 (Omni Pur®, catalog no. 6710, Gibbstown, NJ, USA). The mixture was agitated for 30 min at 4°C followed by centrifugation at 6000 x g for 15 min. One mL of the phenol phase (top phase) was removed and placed in a new 15 mL plastic tube. This solution was mixed with 5 volumes of ice-cold 0.1 M ammonium acetate in methanol and incubated overnight at -20°C to allow for protein precipitation. The precipitate was collected by centrifuging at 6000 x g for 15 min at 4°C. Two volumes (based on the volume [2 mL] of the last collected phenolic phase) of ice-cold 0.1 M ammonium acetate in methanol were added to wash the pellet and remove phenol, lipids and pigments. The sample was centrifuged at 6000 x g for 10 min in ice-cold 80% (v/v) acetone to remove ammonium acetate and achieve faster, more effective drying. The protein pellet was gently air dried in a fume hood at room temperature (22°C) for 30 min and stored at -80°C for future analysis.

3.3.3 Modified Phenol Extraction Protocol

In order to optimize protein extraction, the phenol extraction method described above in section 3.3.2 was modified as discussed by Isaacson *et al.* (2006). Leaf tissue was ground using a ratio of 5 mL of extraction buffer per 1 g of leaf tissue and the amount of the phenolic phase

removed for precipitation was increased to 2 mL. All other steps were as described in section 3.3.2.

3.3.4 Protein Quantitation

Protein concentrations were determined using either the Bradford assay (Bradford, 1976; Bio-Rad Laboratories, Hercules, CA, USA) or the Pierce® BCA protein assay (Smith *et al.*, 1985) (Thermo Scientific, Rockford, IL, USA) following the manufacturer's instructions with BSA as the standard. Absorbance measurements were read using a SmartSpec Plus spectrophotometer (Bio-Rad, Mississauga, ON, Canada).

3.4. Induction of Gel Formation

3.4.1 Freeze-Thaw Cycles

Protein pellets from NA and CA winter rye were suspended in 400 to 800 µL of an appropriate buffer (depending on experiment) by sonication (VirSonic 600 Ultrasonic Cell Disrupter, VirTis Company, Gardiner, NY, USA) using a microtip probe for two times at 10 s each (intensity 0.5) and once or twice for 10 s (intensity 1.0) until the protein pellet was fully suspended in the buffer. Gel formation was induced by subjecting the protein extracts to multiple freeze-thaw cycles in a freezer (Revco Technologies, model ULT2586, Asheville, NC, USA) at -80°C. Freezing occurred for a minimum of 3 h or usually overnight followed by thawing on the bench at room temperature (22°C). The freeze-thaw cycles were repeated 6 to 9 times until protein precipitation or gel formation was visible.

3.4.2 Effect of pH on Gel Formation

To determine the effect of pH on gel formation, the NA and CA winter rye protein pellets were suspended in 800 µL of 50 mM Tris (pH 2.0, 3.0, 7.0, 10.0 or 12.0) or 50 mM borate buffer (pH 12.0) followed by multiple freeze-thaw cycles as described in section 3.4.1. The freeze-thaw cycles were repeated 6 times to assess the gel formation of the sample. The experiment on effect of pH on gel formation was repeated 4 times.

3.4.3 Effect of Protein Concentration on Gel Formation

To determine the effect of protein concentration on gel formation, the NA and CA winter rye protein pellets were suspended in 50 mM borate buffer (pH 12.0) as described in section 3.4.1. The protein concentration was determined by the BCA protein assay (section 3.3.4). A series of samples with different protein concentrations (2.0 to 13.0 mg/mL) were prepared and subjected to 6 freeze-thaw cycles as described in section 3.4.1 to assess the gel formation of the sample. The experiment on effect of protein concentration on gel formation was repeated 4 times.

3.5 Viscosity Measurements

To determine viscosity, the NA and CA winter rye protein pellets were suspended in 50 mM borate buffer (pH 12.0) to a concentration of 6 mg/mL and subjected to freeze-thaw cycles to induce gel formation as described in section 3.4.1. In total, 4 freeze-thaw cycles were performed until gel formation was visible. The viscosity of the entire sample (gelling and non-gelling components) after each freeze-thaw cycle was determined with Digital Viscometer (model: DV-1+; Brookfield, Middleboro, MA, USA) using a parallel plate fixture. The sample (600 µL) was loaded into the sample holder using a syringe and viscosity was measured at 25°C with a shear rate (rotational speed) of 3 or 6 rpm. The sample holder was washed with acetone between sample analyses. The instrument was calibrated with a mineral oil viscosity standard (95.5 cP at 25°C; Brookfield, Middleboro, MA, USA). When the sample reading had stabilized the data was collected manually.

3.6 SDS-PAGE Analysis of Gelling and Non-Gelling Components

3.6.1 Separation of Gelling and Non-Gelling Components

The NA and CA winter rye protein pellets were suspended in 50 mM borate buffer (pH 12.0) and subjected to 6 freeze-thaw cycles as described in section 3.4.1. Following gel induction, the gelling and non-gelling components of the extract were mechanically separated by passing of the sample mixture through a 250 µm thread diameter nylon mesh. The gel was

washed a minimum of two times with 50 mM borate buffer (pH 12.0) before storage at -80^{o}C. The non-gelling component was also stored at -80°C prior to subsequent SDS-PAGE analysis (see section 3.6.3).

3.6.2 Solubilization of Gel

The solubility of the gelling component from NA and CA protein pellets was assessed in the presence of a variety of reducing agents and detergents, this included anionic (SDS) and nonionic surfactants (Triton X-100), a chaotropic agent (urea), SDS-PAGE loading buffer (Laemmli, 1970), reducing agent (β-ME), and base (0.1-1 M NaOH). These agents were examined with and without heat incubation for periods up to 6 min at 95°C in a waterbath.

3.6.3 SDS-PAGE Analysis

Following solubilization, samples were combined with 2X sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-ME, 0.001% (v/v) bromophenol blue) (Laemmli, 1970). Protein separation was resolved by SDS-PAGE using a 4% (w/v) stacking gel and a 12% (w/v) separating gel for approximately 70 min at 30 mW using a Mini-protean II (Bio-rad) vertical electrophoresis system in a running buffer consisting of 25 mM Tris, 192 mM glycine and 0.05% (w/v) SDS (Laemmli, 1970). Samples were loaded on either an equal volume or equal protein basis and boiled in a waterbath at 100°C for 3 min prior to loading. Pre-stained molecular mass standards (PageRuler® Prestained Protein Ladder; Fermentas, Burlington, ON, CA) were utilized to monitor migration and assess molecular mass. Once separation was completed, gels were stained with 0.1% (w/v) Coomassie brilliant blue R-250 (Sigma, St. Louis, MO, USA) in 50% (v/v) methanol and 7% (v/v) glacial acetic acid for a minimum of 1.5 h followed by overnight destaining in a solution containing 20% (v/v) methanol and 7% (v/v) glacial acetic acid.

3.7 Role of Rubisco in Gel Formation

3.7.1 Removal of Rubisco

Protein extracts devoid of Rubisco were obtained as described by Holler *et al.* (2007) using polyethyleneimine (PEI) precipitation. Leaf protein was extracted from NA and CA tissue using an extraction buffer (50 mM sodium phosphate [pH 7], 0.1% [v/v] β-ME and 1 mM EDTA) at a ratio of 5 mL extraction buffer to 1 g of leaf tissue. The sample was homogenized in the extraction buffer with a cold mortar and pestle for approximately 2 min until it was free of large particulates. Immediately after homogenization, 2% (w/v) pre-hydrated insoluble polyvinylpolypyrrolidone (PVPP) was added to the sample. The extract was then vortexed vigorously for 15 s and allowed to set at room temperature for 15 min. The mixture was centrifuged at 17,000 x g for 20 min at 4°C and the supernatant (containing total leaf protein) was collected and filtered through a 0.22 µm syringe filter (Millipore, Billerica, MA, USA).

PEI precipitation was used to remove Rubisco from the protein extract (Holler *et al.*, 2007). A 25% (v/v) PEI stock solution in distilled water was prepared. PEI was added to the leaf extracts to final concentrations of 0.5%, 1% and 2% (v/v) and the samples were vortexed vigorously and allowed to set at 4°C for 30 min. Following centrifugation at 8,000 x g for 5 min, the supernatant was collected for SDS-PAGE analysis as describe in section 3.6.3. The sample, with Rubisco successfully removed and confirmed by SDS-PAGE was subjected to 6 freeze-thaw cycles to assess its gel forming ability. The experiment was repeated adjusting the pH of the final extract to pH 12.0 using NaOH.

3.7.2 Rubisco Partial Purification

Rubisco was partially purified according to Kwanyuen *et al.* (2002). Leaves from NA winter rye were homogenized in a blender with ice-cold 2% (v/v) sodium meta-bisulfite solution at a tissue to buffer ratio of 1:10. The homogenate was filtered through cheesecloth and a crude leaf extract was collected. The pH of the crude leaf extract was adjusted to pH 5.6 with 1 M NaOH. The crude leaf extract was centrifuged at 30,000 x g for 30 min and the supernatant was collected, followed by 48 h incubation at 4°C. The solution was then

centrifuged at 3,000 x g for 10 min and the pellet (partially purified Rubisco) and supernatant were collected for SDS-PAGE analysis as described in section 3.6.3.

The partially purified Rubisco pellet, confirmed by SDS-PAGE, was suspended in 50 mM borate buffer (pH 12.0) or 50 mM Tris buffer (pH 8.0) at a concentration of either 5 or 10 mg/mL and subjected to 6 freeze-thaw cycles to determine its ability to form a gel. To ensure that sodium meta-bisulfite was removed, samples as described above were desalted against their respective buffers overnight using a Slide-A-Lyzer dialysis cassette (3.5 kD molecular-weight cutoff; Thermo Scientific, Rockford, IL, USA) according to manufacturer's instructions. These samples were also subjected to 6 freeze-thaw cycles to determine their ability to form a gel.

3.7.3 Catalytic Effect of Rubisco with BSA

The gel forming ability of commercially available BSA Fraction V (EMD Chemicals, Gibbstown, NJ, USA) was assessed using samples (2 to 20 mg/mL) prepared in 50 mM borate buffer (pH 12.0). The BSA samples were subjected to freeze-thaw cycles as described in section 3.4.1 up to 6 times to assess the gel formation of the samples.

To determine if Rubisco could initiate catalytic effects on gel formation, different concentrations (5, 8 and 15 mg/mL) of BSA were mixed with 8 mg/mL of purified Rubisco from NA tissue (obtained as described in section 3.7.2) and subjected to 6 freeze-thaw cycles. The catalytic effect was assessed by the ability of the mixture to form a gel.

3.8 Spectroscopic Analysis

3.8.1 Absorbance Measurements

Absorbance measurements were performed in an Applied Photosystems PiStar-180 circular dichroism (CD) spectrometer using 1 cm path length cuvettes. NA and CA protein samples without freeze-thaw treatment and NA and CA protein samples with 2 freeze-thaw cycles treatment were measured in 50 mM borate buffer (pH 12.0). Absorbance measurements were taken from the far UV region (180-260 nm) at 1 nm steps, and a scan rate of 20 nm/min.

The entrance and exit slits were set at 2 nm. The background baseline used was an empty chamber.

3.8.2 Raman Spectroscopy

Raman measurements were performed using a Renishaw inVia ® Raman microscope (Gloucestershire, UK) which consisted of a semiconductor diode near-infrared laser that operates at 785 nm. Raman spectra were collected on the gel extracts (obtained as described in section 3.6.1) and protein extracts without freeze-thaw treatment obtained from NA and CA samples. The samples were placed on an Au-plated Si wafer. For these measurements a 20 or 50X long working distance objective was used. Raman measurements were taken from the spot that generates a clear spectrum. Raman measurements (entire range of 0 to 3400 cm^{-1}) were taken with a 10 s integration time and 4 to 6 accumulations for each measurement.

Two grams of commercially available BSA Fraction V (EMD Chemicals Inc., Gibbstown, NJ, USA) was used as a protein source and subjected to the modified phenol extraction protocol as described in section 3.3.3. The BSA pellet obtained from the treatment was labeled as "tBSA"; this was used as a control to determine the potential effects that the phenol extraction may have had on the Raman spectra. Raman measurements of the tBSA pellet and commercially available BSA were obtained with a 50X long working distance objective. The samples were placed on an Au-plated Si wafer. Raman measurements were taken from the spot that generates a clear spectrum. The extended Raman measurements (entire range of 0 to 3400 cm^{-1}) were taken with a 10 s integration time and 4 to 5 accumulations for each measurement. Raman measurements were also collected for 50 mM borate buffer (pH 12.0) to assess background.

4.0 RESULTS

4.1 Conditions for Gel Formation

Protein was extracted from leaf material of NA and CA winter rye using three different protein extraction methods. The ability of the extracted protein to form a gel in response to repeated freeze-thaw cycles was used as an indicator of the protocol which would be used for all further experiments (Figure 4.1). Gel formation from each extraction method was assessed in 50 mM Tris adjusted to various pH values. The effects of various freezing durations ranging from 3 h to 4 days at -80°C were also assessed. The number of freeze-thaw cycles was also varied from 6 to 9 in total. No gel formation was observed when protein was extracted using either the TCA-acetone (Isaacson *et al.*, 2006) or phenol extraction protocols (Hurkman and Tanaka 1986) (Table 4.1). In both cases, protein precipitation was observed after thawing, indicating that the protein was not in solution. These results were consistent irrespective of pH (Table 4.1), freezing temperature, freezing duration or number of freeze-thaw cycles employed.

When a modified phenol extraction protocol was utilized (Isaacson *et al.*, 2006), similar results were obtained for extracts at adjusted pH values of 2.0, 3.0, 7.0 and 10.0 (Table 4.1). In contrast, extracts adjusted to pH 12.0 demonstrated gel formations between three and four freeze-thaw cycles (Table 4.1). Generally, a greater amount of gel was observed in the CA samples, although this was not always the case. However, the protein concentrations were not standardized in these experiments when comparing NA and CA samples. This experiment was repeated using a borate buffer which possesses a greater buffering capacity at pH 12.0 than Tris and identical results were obtained (Table 4.1). Based on these results the modified phenol protein extraction protocol and the use of borate buffer (pH 12.0) for gel formation was utilized for all further experiments unless otherwise indicated.

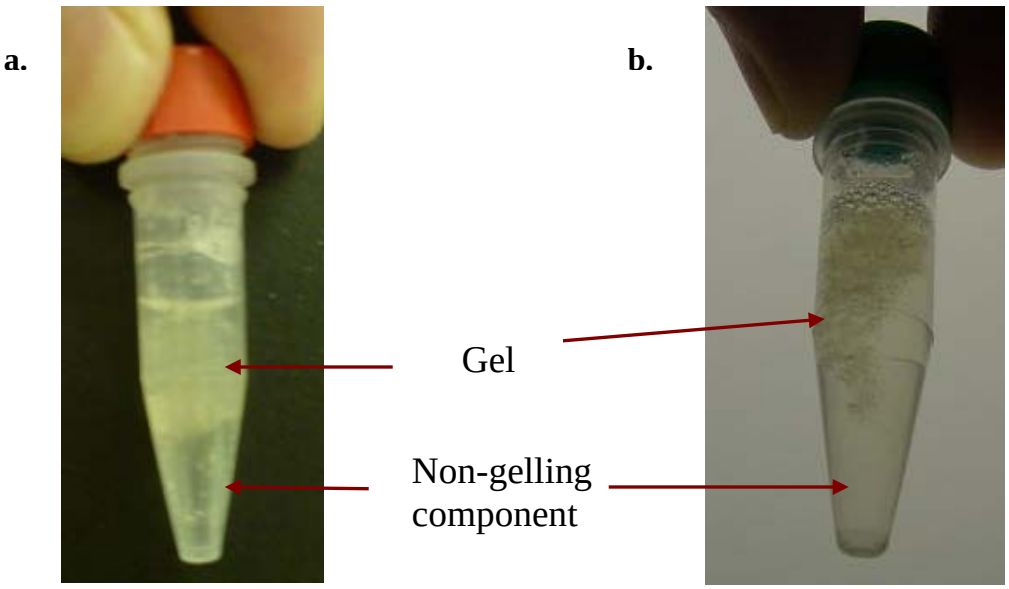

Figure 4.1 Gel formation in NA (a) and CA (b) protein extracts. Extracts were prepared from leaf tissue of winter rye and suspended in borate buffer (pH 12.0).

The physical appearance of the gel was white or yellowish in color and it was opaque (Figure 4.1). It was soft in texture like gelatin, but more elastic, and not as brittle. There was no clear distinction in physical appearance between the gels formed from NA or CA protein extracts (Figure 4.1). After the protein extracts were subjected to freeze-thaw cycles in pH 12.0 borate, the protein extract formed into gelling and non-gelling components. However, only a portion of the extract formed a gel not the entire extract. The protein gel had a lower density separating itself from the non-gelling component of the extract (Figure 4.1).

Table 4.1 Summary of protein extraction protocols and subsequent gel formation. NA, non-acclimated; CA, cold acclimated; nd, not detected; +, qualitative assessment of gel formation. + + + denotes a gel with a volume of 1/3 of the protein extract; + + + + denotes a gel with a volume of 1/2 of the protein extract. This experiment was repeated 12 times.

Extraction protocol	Buffer	pH	Gel formation	
			NA	CA
TCA/acetone	Tris	2.0	nd	nd
		7.0	nd	nd
		12.0	nd	nd
Phenol	Tris	2.0	nd	nd
		3.0	nd	nd
		7.0	nd	nd
		10.0	nd	nd
		12.0	nd	nd
Modified phenol	Tris	2.0	nd	nd
		3.0	nd	nd
		7.0	nd	nd
		10.0	nd	nd
		12.0	+ + +	+ + + +
Modified phenol	Borate	12.0	+ + +	+ + + +

Although the gel was obtained from both NA and CA extracts by using modified phenol extraction and borate buffer (pH 12.0), there were still extracts incapable of forming a gel under these conditions. These extracts occurred more often in NA than in CA samples. Based on visual observation, the protein extracts were generally more transparent which could mean they contained a lower protein concentration. This effect was examined by assessing gel formation in extracts from NA and CA winter rye which were adjusted to various protein concentrations. Gel formation was observed in extracts from both NA and CA winter rye at all concentrations examined, however gel formation increased with increasing protein concentration up to 13.0 mg/mL (Table 4.2). These results suggested that the gel formation was directly correlated with protein concentration.

Table 4.2 Effect of protein concentration on gel formation. NA, non-acclimated; CA, cold acclimated; +, quantitative assessment of gel formation. + denotes a gel with a volume of 1/5 of the protein extract; + + denotes a gel with a volume of 1/4 of the protein extract; + + + denotes a gel with a volume of 1/3 of the protein extract; + + + + denotes a gel with a volume of 1/2 of the protein extract. This experiment was repeated 4 times.

Sample	Protein concentration (mg/mL)	Gel formation
NA	2.0	+
	4.0	+ +
	8.0	+ + +
	10.0	+ + +
CA	2.0	+
	4.0	+ +
	6.0	+ +
	13.0	+ + +

These experiments demonstrate that specific conditions were required for gel formation. Specifically, the protein extraction method, protein concentration, and the pH of the suspension buffer were crucial factors. While freezing temperature and freezing duration appear to have no effect, a minimum of 3 freeze-thaw cycles are required for gel formation. In addition, the gel can be obtained from both NA and CA extracts.

4.2 Viscosity Measurements

Both NA and CA protein extracts were subjected to a total of 4 freeze-thaw cycles and their viscosity determined after each cycle (Table 4.3). The viscosity was measured at 2 different shear rates and the viscosities were taken manually when the value had stabilized. Gel formation occurred in the third freeze-thaw cycle with the viscosity increasing approximately 1.6- and 2.8-fold in NA and CA samples respectively, in comparison to values obtained after the first freeze-thaw cycle (Table 4.3). There was a dramatic increase in viscosity of NA and CA samples in the fourth freeze-thaw cycle in which the viscosities increased 3.5- and 3.4-fold respectively, in comparison to values obtained after the third freeze-thaw cycle.

From Table 4.3, there were no significant changes in viscosities with respect to a change in shear rate applied to the samples; this suggested that the samples could be a Newtonian fluid. However, during the measurement of viscosity, the samples exhibited properties of a

thixotropic liquid, as the sample fluids displayed a decrease in viscosities over time at a constant shear rate (Fox *et. al.*, 2009). The final stabilized viscosities are presented in Table 4.3. The decrease in viscosity over time could be resulting from the shearing and disruption of the gel network in the sample.

Table 4.3 Viscosity changes as a function of freeze-thaw cycles. Values reflect means ± standard deviation of 2-4 independent experiments. NA, non-acclimated; CA, cold acclimated; nd, not determined. * This experiment was only performed once as a shear rate of 3 rpm is not optimal for viscosity measurements using this viscometer.

Shear rate (rpm)	Freeze-thaw cycles	Viscosity (cP)	
		NA	CA
6	1	10.10 ± 1.48	8.73 ± 0.98
	2	13.55 ± 6.21	10.13 ± 1.94
	3	16.10 ± 5.61	24.53 ± 4.52
	4	57.10 ± 33.83	82.85 ± 19.30
3*	1	10.8	15.1
	2	16.9	18.9
	3	12.2	68.2
	4	nd	nd

4.3 SDS-PAGE Analysis of Gelling and Non-Gelling Components

In order to examine the protein composition of the samples through SDS-PAGE, adequate solubilization of the gelling and non-gelling fractions in Laemmli sample buffer (Laemmli, 1970) was required prior to protein separation. However, the gel exhibited heat stability and was insoluble over a range of conditions including chaotropic agent (urea), surfactants (Triton-X and SDS), reducing agent (β-ME), base (NaOH) and Laemmli sample buffer (Laemmli, 1970; Table 4.4).

Table 4.4 Summary of solubilization treatments prior to SDS-PAGE. Heat incubation at 95^{o}C was performed for up to 6 minutes. "✓" denotes complete solubilization; "×" denotes incomplete solubilization.

Treatment	Solubilization of gel	
	Without heating	Heating
Distilled water	×	×
0.1% Triton-X	×	×
Up to 8 M urea	×	×
Up to 10% β-ME	×	×
Up to 4% SDS	×	×
3 M urea + 1% β-ME	×	×
3 M urea + 4% SDS	×	×
2X Laemmli sample buffer	×	×
6 M urea + 0.0125% SDS + 0.5% β-ME	×	×
3 M urea + 2% SDS + 5% β-ME	×	✓
4 M urea + 4% SDS + 1% β-ME	×	✓
0.1 M NaOH	×	✓
1 M NaOH	×	✓

In all the chemical treatments applied to the gel, with or without heating, only the base (0.1 M and 1 M NaOH) and a combination of chaotropic agent, anionic surfactant and reducing agent (urea, SDS and β-ME) with heating for a minimum of 2 min could achieve complete solubilization for downstream SDS-PAGE analysis (Table 4.4). In contrast, the non-gelling component was readily soluble in Laemmli sample buffer (Laemmli, 1970)

To determine which of the solubilization methods were feasible for downstream analysis, gel samples solubilized by 0.1 M NaOH with heating and the combination of 4 M urea, 4% SDS and 1% β-ME with heating were chosen for SDS-PAGE analysis (Figures. 4.2 and 4.3). These two treatments were chosen to perform the SDS analysis because they were the least harsh of those which achieved complete solubilization.

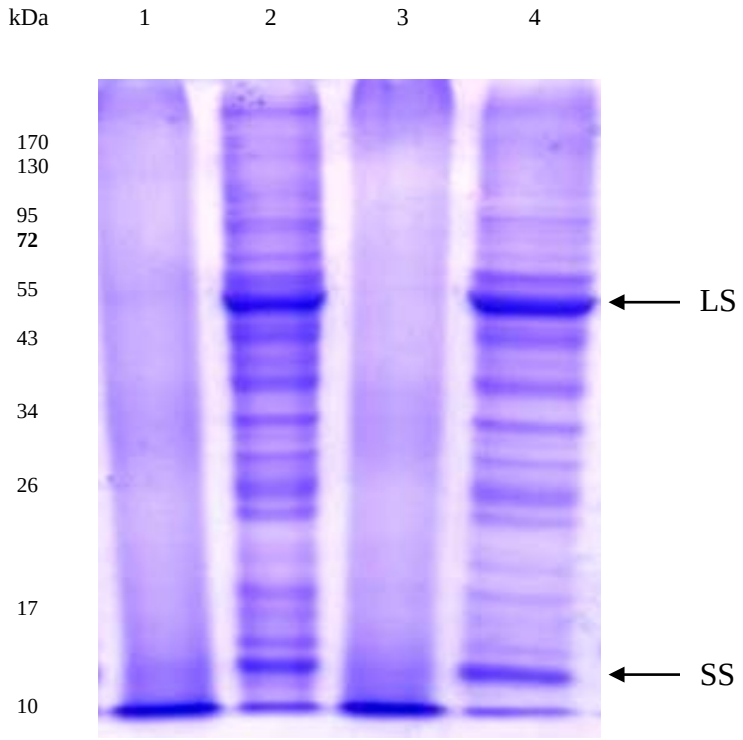

Figure 4.2 SDS-PAGE showing solubilization of gelling components with 0.1 M NaOH. Lanes 1 and 2 represent gelling and non-gelling components from NA tissue, respectively. Lanes 3 and 4 represent gelling and non-gelling components from CA tissue, respectively. Seven μg of protein was loaded into each lane and were resolved in a 12% polyacrylamide gel stained with Coomassie blue. A MW standard (protein ladder) was also run in a separate lane. Gelling components were solubilized in 0.1 M NaOH with heating. LS, large subunit of Rubisco; SS, small subunit of Rubisco.

From the SDS-PAGE analysis, the protein gel was completely degraded by the 0.1 M NaOH solubilization treatment (Figure 4.2). This occurred in gel obtained from NA or CA protein. The non-gelling component solubilized well in sample buffer and was readily separated with good protein resolution by SDS-PAGE (Figure 4.2). Thus, the NaOH was not a suitable treatment to solubilize the gel for downstream analysis.

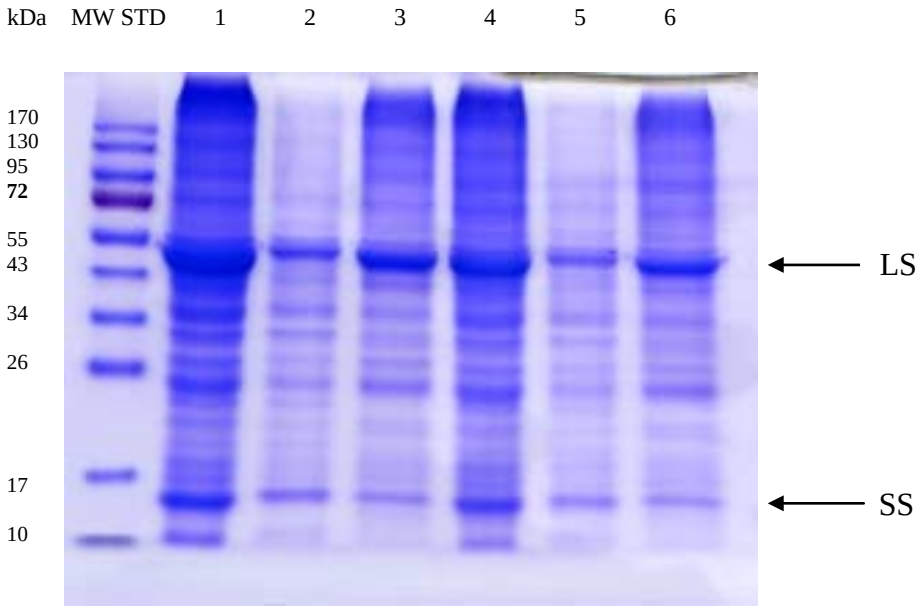

Figure 4.3 SDS-PAGE showing solubilization of gelling components with 4 M urea, 4% SDS, and 1% β-ME. Lanes 1 and 4 represent total protein extracts from NA and CA tissues**.** Lanes 2 and 5 represent gelling components from NA and CA extracts respectively. Lanes 3 and 6 represent non-gelling components from NA and CA extracts respectively. Samples were loaded on an equal volume basis and were resolved in a 12% polyacrylamide gel stained with Coomassie blue. Gelling components were solubilized in 4 M urea, 4% SDS and 1% β-ME with heating for 2 min. LS, large subunit of Rubisco; SS, small subunit of Rubisco.

The feasibility of using urea, SDS and β-ME as solubilization agents for the gel was also determined by SDS-PAGE (Figure 4.3; Table 4.4). In contrast to the NaOH solubilization treatment, this combination of chaotropic agent, anionic surfactant and reducing agent was able to solubilize the gelling component and allowed for excellent protein separation and resolution (Figure 4.3; lanes 2 and 5). The non-gelling component solubilized well in sample buffer (Figure 4.3; lanes 3 and 6), as did the total protein extract (Figure 4.3; lanes 1 and 4). The protein profile for the total protein extract, gelling and non-gelling components appeared to be similar (Figure 4.3), suggesting that the gel formed was likely due to interactions of multiple proteins in the total leaf protein extract under specific conditions. In addition, the protein composition appeared to be the same from both NA and CA samples (Figure 4.3) which supports the theory that gel formation was not the result of induced or degraded proteins as a result of cold acclimation.

4.4 Role of Rubisco in Gel Formation

Rubisco catalyzes the first step of carbon fixation and is the most abundant protein in leaf tissues. Not surprisingly, Rubisco was also shown to be a major protein present in our SDS-PAGE analyses, as indicated by the presence of the small and large subunits (Figures 4.2 and 4.3). Due to the preponderance of this protein, attention was focused towards determining the role of Rubisco, if any, in gel formation from leaf protein extracts of NA and CA winter rye.

4.4.1 Removal of Rubisco

In order to access the role of Rubisco in gel formation an attempt was made to remove Rubisco from the total protein extracts using PEI precipitation. This PEI precipitation protocol was attempted on protein pellets (obtained as described in section 3.3.3) from NA winter rye and proved to be unsuccessful. Further attempts utilized the leaf protein extraction protocol of Holler *et al.* (2007) described in section 3.7.1.

As shown in Figure 4.4, PEI precipitation was unsuccessful in selectively removing Rubisco from the phenol extracted protein samples. After addition of PEI, almost all proteins were precipitated (Figure 4.4). This may due to non-specific precipitation or the surface structure of the sample proteins was modified by the phenol extraction method causing a reduced solubility.

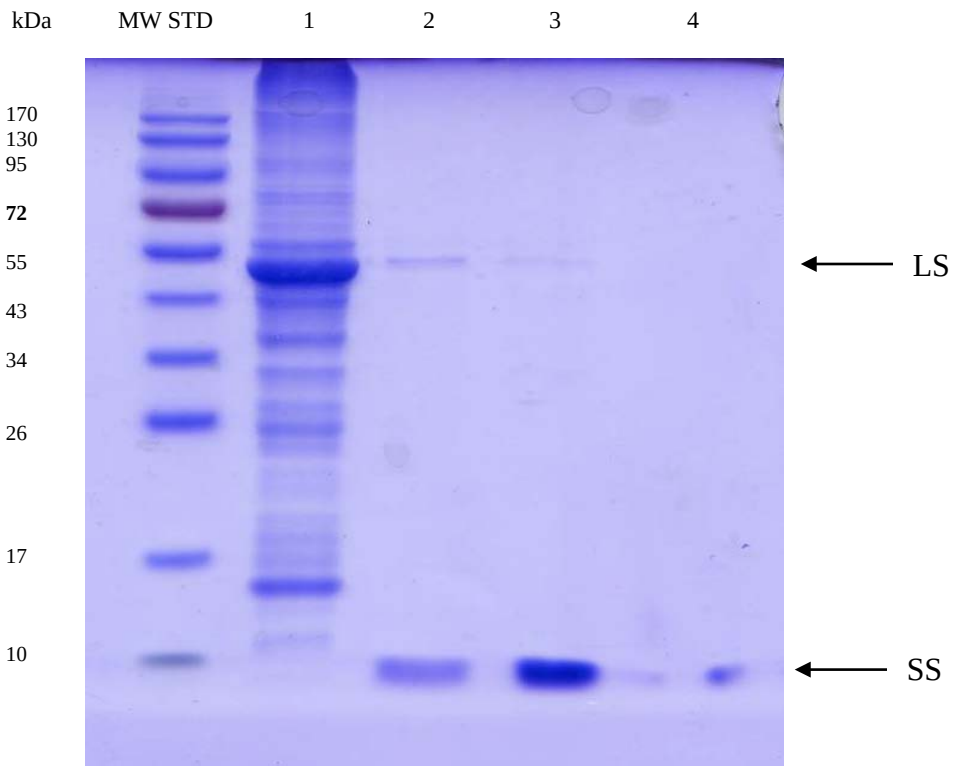

Figure 4.4 SDS-PAGE showing Rubisco removal from phenol extracted proteins. Phenol extracted protein samples were precipitated with PEI. Lanes 1-4 represent protein samples with 0, 0.5, 1.0 and 2.0% (v/v) PEI concentrations respectively. Samples were loaded on an equal volume basis and were resolved in a 12% polyacrylamide gel stained with Coomassie blue. LS, large subunit of Rubisco; SS, small subunit of Rubisco. The PEI precipitation in phenol extracted protein samples was unspecific, almost all proteins were precipitated from the samples.

In order to complete these experiments the extraction protocol of Holler *et al.* (2007) was employed, realizing that this may have an effect on subsequent gel formation.

SDS-PAGE analysis revealed that Rubisco was successfully removed from the protein extracts using PEI precipitation with as low as 0.5% (v/v) PEI (Figure 4.5). However, higher concentrations of PEI (2% [(v/v]) were not as successful (Figure 4.5).

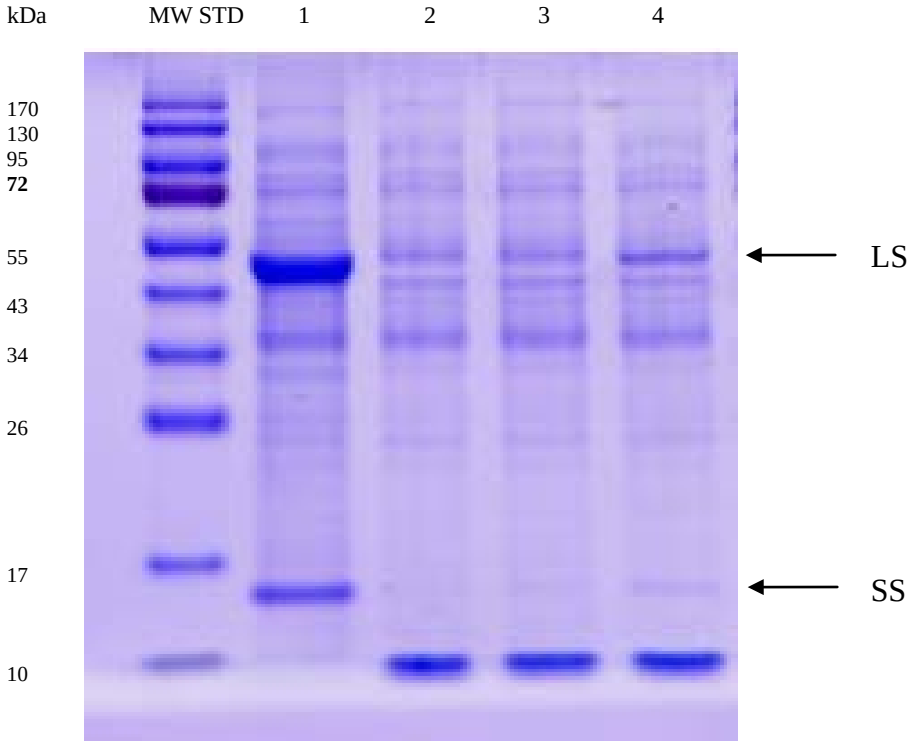

Figure 4.5 SDS-PAGE showing Rubisco removal from proteins extracted with sodium phosphate. Protein samples were extracted according to Holler *et al.* (2007) and precipitated with PEI. Lanes 1-4 are protein samples with 0, 0.5, 1.0 and 2.0% (v/v) PEI concentrations respectively. Samples were loaded on an equal volume basis and were resolved in a 12% polyacrylamide gel stained with Coomassie blue. LS, large subunit of Rubisco; SS, small subunit of Rubisco. The removal of Rubisco was successful as the PEI precipitation was specific, only Rubisco was precipitated from the samples.

This procedure was utilized for NA and CA tissue and the subsequent protein extracts, devoid of Rubisco, were assessed for their ability to form a gel upon 6 freeze-thaw cycles. Gel formation was not observed in extracts from either NA or CA tissue in extraction buffer at pH 7.0. The experiment was repeated using extraction buffer at pH 12.0 and identical results were obtained. However, protein precipitation was observed in the pH 12.0 extract (Figure 4.6).

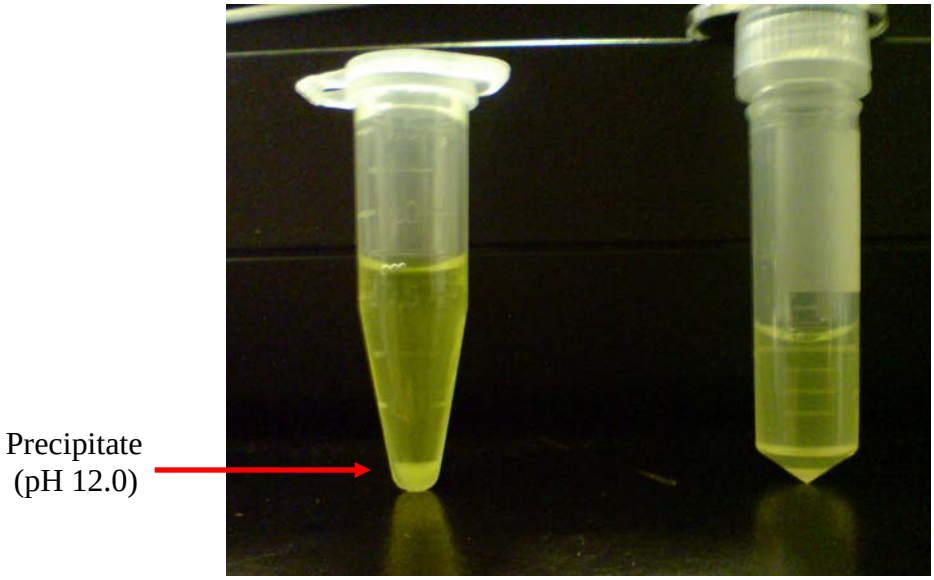

Figure 4.6 Precipitate formation in protein extracts devoid of Rubisco. Extracts are from NA tissue suspended in extraction buffer adjusted to pH 12.0 (left) or pH 7.0 (right). Precipitate is observed in the pH 12.0 sample.

4.4.2 Partial Purification of Rubisco

To determine whether Rubisco itself can form a gel in response to freeze-thaw cycles, a Rubisco isolation and purification from NA leaf tissues was performed. These experiments used the protein extraction protocol of Kwanyuen *et al.*, 2002 which we adopted, once again realizing this may have an effect on subsequent gel formation. SDS-PAGE analysis revealed that Rubisco was successfully partially purified (Figure 4.7). In this protocol, Rubisco is crystallized out of soluble protein solution during a 48 h incubation at 4°C in an acidic extraction buffer (pH 5.6). The Rubisco crystals are collected by centrifugation and suspended

in an appropriate buffer. Figure 4.7 (lane 2) demonstrates almost complete removal of the large and small subunits of Rubisco from the soluble fraction (lane 3) and a significant enrichment in the collected product (lane 4) as a result of the crystallization process.

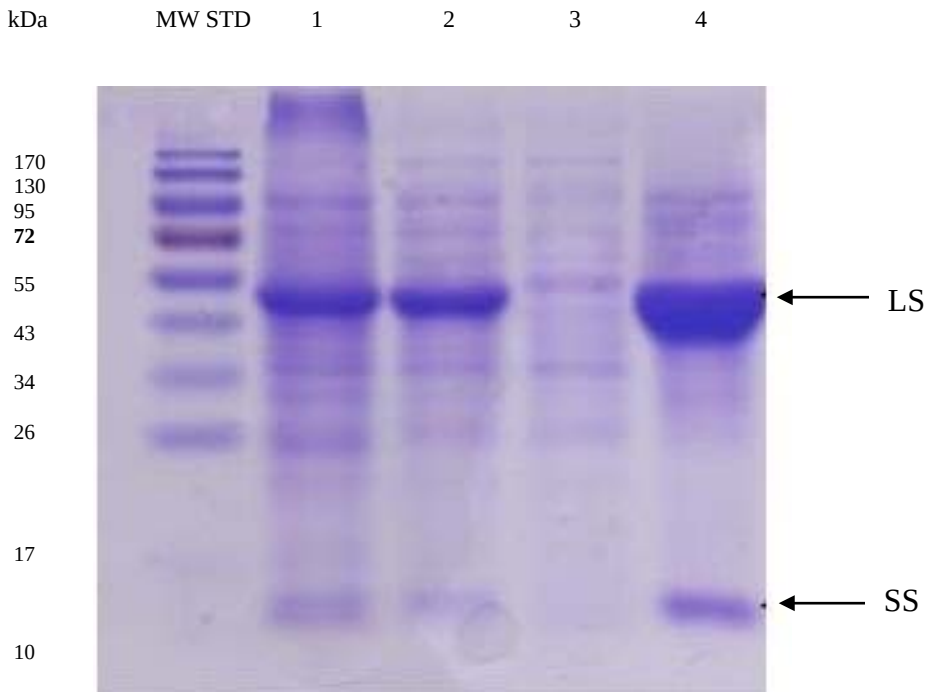

Figure 4.7 SDS-PAGE demonstrating partial purification of Rubisco. Lane 1, crude leaf extract; lane 2, soluble proteins (including Rubisco); lane 3, soluble proteins (after crystallization); lane 4, Rubisco (crystallized). Rubisco was loaded on an equal volume basis and were resolved in a 12% polyacrylamide gel stained with Coomassie blue. LS, large subunit of Rubisco; SS, small subunit of Rubisco. In lane 4, the Rubisco was partially purified as determined by the SDS-PAGE, both large and small subunit of Rubisco were greatly enhanced in the sample.

The partially purified Rubisco extracts from NA tissue were assessed for their ability to form a gel upon freeze-thaw cycles. Crystallized Rubisco (5 and 10 mg/mL) was suspended in Tris buffer (pH 8.0) or borate buffer (pH 12.0) and subjected to 6 freeze-thaw cycles. Prior to freeze-thaw cycles, dialysis was performed on the enriched Rubisco samples to remove salts from the process of protein purification. Samples were also subjected to freeze-thaw cycles

without dialysis. This was to assess whether the salts present from the crystallization steps had an effect on protein gelling. Unfortunately, no protein gel was observed from dialyzed or non-dialyzed samples in either buffer. This experiment was performed at partially purified Rubisco concentrations of 5 and 10 mg/mL which still did not result in the formation of a gel.

4.4.3 Catalytic Effect of Rubisco

To determine if Rubisco was acting as a catalyst for gel formation, the partially purified samples were added to BSA, a commercially available protein. Prior to this experiment, BSA was assessed for its gel forming ability. BSA was suspended in 50 mM borate buffer (pH 12.0) at protein concentrations from 2 to 20 mg/mL and subjected to 6 freeze-thaw cycles. No gel formation was observed in any of the samples. Thus, BSA itself was incapable of gel formation in response to freeze-thaw under these experimental conditions.

To determine the possible catalytic role of Rubisco in gel formation, partially purified Rubisco (with or without dialysis) at a standardized concentration (8 mg/mL) in borate buffer (pH 12.0) was mixed with commercially available BSA at 5, 8 or15 mg/mL. The mixtures were subjected to a minimum of 6 freeze-thaw cycles. No gel formation was observed under these experimental conditions. These results indicated that Rubisco did not possess the ability to catalyze gel formation when examined with BSA.

4.5 Absorbance Measurements and Raman Spectroscopy

Initially, experiments were attempted using CD spectroscopy, however, these proved unsuccessful. According to the absorbance measurements prior to CD spectroscopy, the samples were found to be highly absorbing (absorbance > 1.5) in the UV region (180-260 nm), thus CD measurement was not feasible. Raman spectroscopy was then utilized which yielded improved results shown in Figures 4.8 – 4.14.

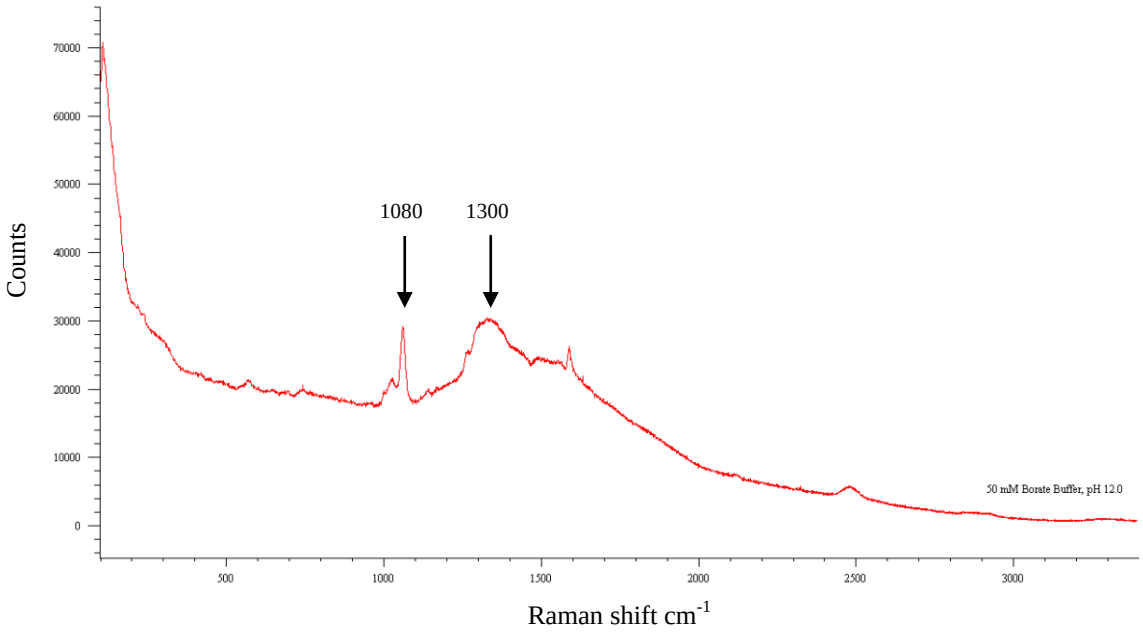

Figure 4.8 Raman spectrum of 50 mM borate buffer (pH 12.0). The intensity (Counts) is arbitrary. Major peaks at 1080 cm^{-1} and 1300 cm^{-1} were observed.

Raman spectra were collected for the borate buffer utilized in this study from 0 to 3400 cm^{-1} and very little background signal was observed (Figure 4.8). A sharp peak was observed at around 1080 cm^{-1}, and a wider peak was observed at 1300 cm^{-1}. A sharp peak was observed at about 100 cm^{-1}, this was due to the lattice vibration of crystallized borate buffer. This peak at about 100 cm^{-1} was observed in all the samples (Figures 4.8 – 4.14).

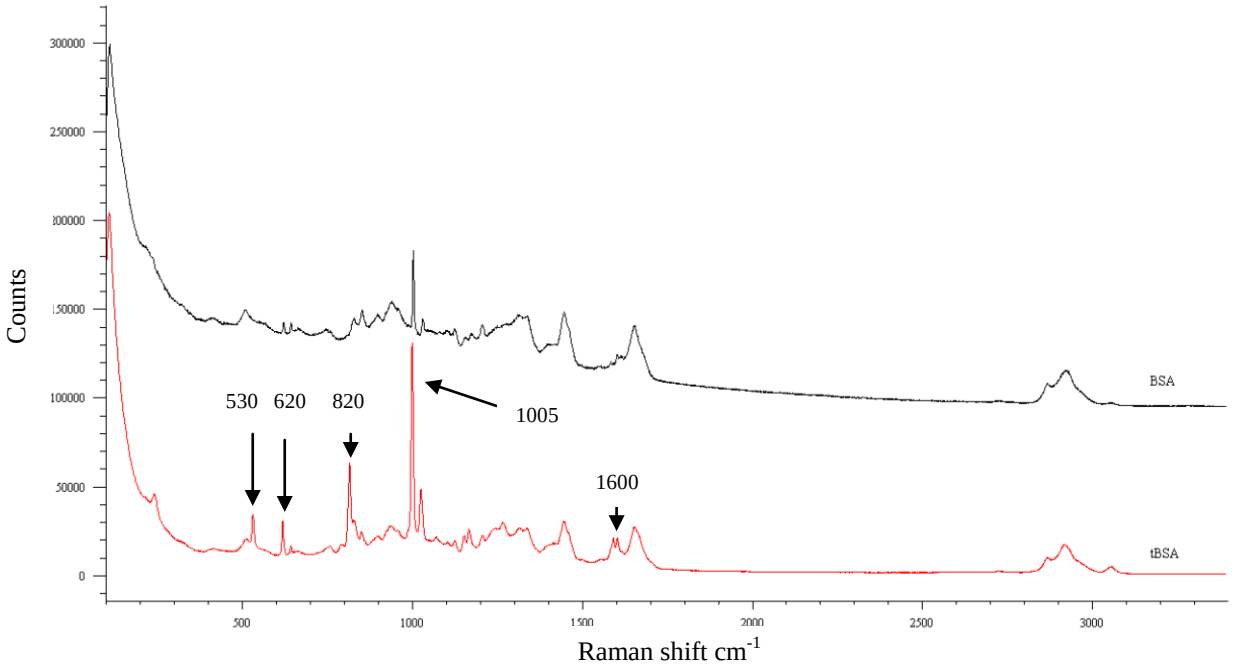

Figure 4.9. Raman spectra of BSA and tBSA. BSA is a commercially available purified sample; tBSA is commercially available BSA that has been subjected to the phenol extraction protocol. The intensity (Counts) is arbitrary. Major differences between spectra were observed at 530, 620, 820, 1005 and 1600 cm^{-1}.

Raman spectra were also obtained for commercially available BSA and BSA which had been subjected to the phenol extraction protocol (tBSA; Figure 4.9). By comparing the protein fingerprint region (500 – 2000 cm^{-1}) between the two BSA samples, sharper peaks were observed at around 530 (disulfide bond), 620 (Phe), 820, 1005 (Phe) and 1600 (Amide I) cm^{-1} in the phenol extracted BSA sample (Table 4.5).

The phenol extracted BSA sample showed that Raman spectroscopy analysis was acceptable and no sample preparation was required as it did not result in spectroscopic interferences in the range studied.

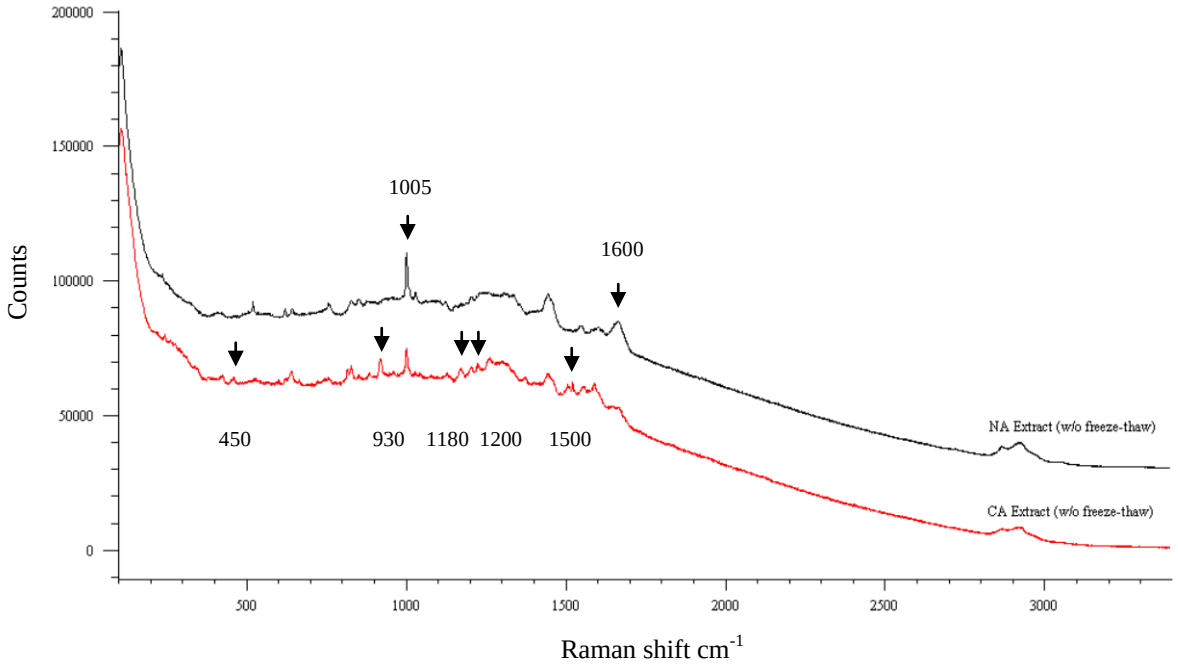

Figure 4.10 Raman spectra of protein extracts from NA and CA tissues. Samples have not been subjected to freeze-thaw treatment. The intensity (Counts) is arbitrary. Major differences between spectra were observed at 450, 930, 1005, 1180, 1200, 1500 and 1600 cm^{-1}.

Raman spectra of protein extracts without freeze-thaw treatment from both NA and CA tissues were compared (Figure 4.10). Generally both NA and CA extracts have similar Raman profiles; however the CA extract has additional peaks at around 450, 930 (stretching C-C α-helix), 1180, 1200 (Phe) and 1500 cm^{-1} (Table 4.5). These additional peaks may be due to the presence of cold acclimated induced protein expression which resulted in differences in the sample's structural conformation. However, the NA extract has sharper peaks at around 1005 cm^{-1} (Phe) and 1600 cm^{-1} (Amide I region) (Table 4.5).

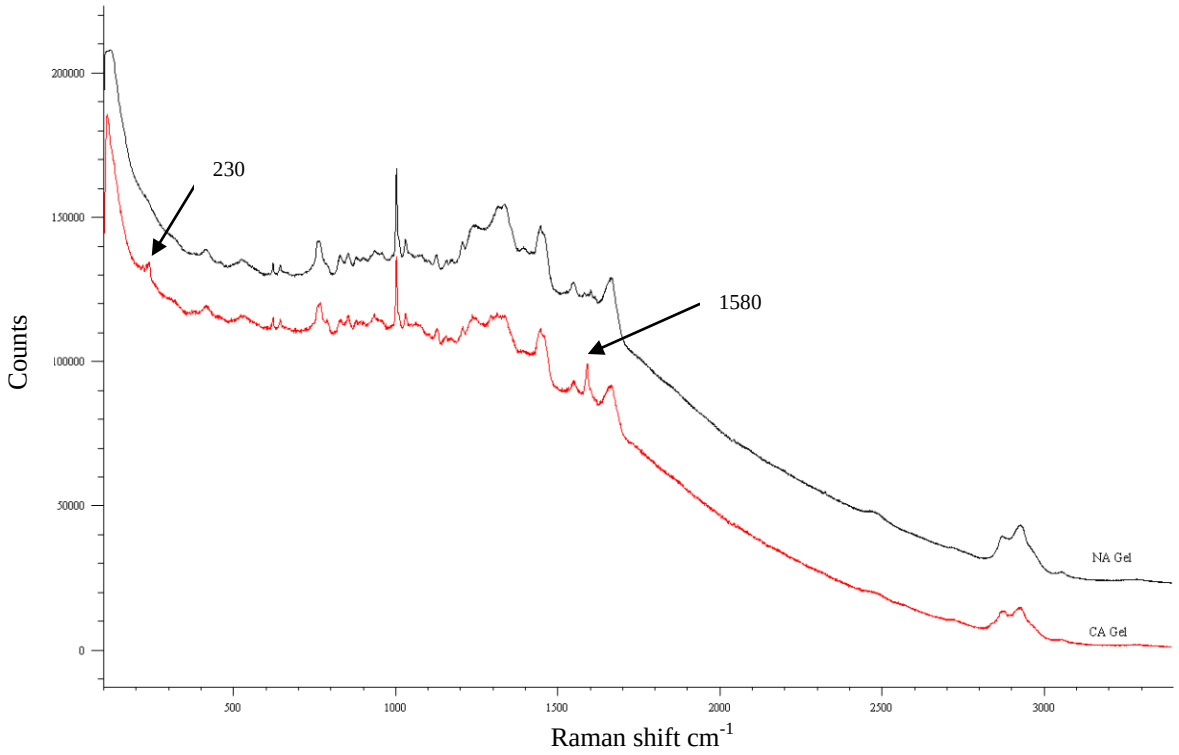

Figure 4.11 Raman spectra of gelling components from NA and CA protein extracts. The intensity (Counts) is arbitrary. Unexpected higher baseline fluorescence was observed from the gelling components. Major differences between spectra were observed at 230 and 1580 cm^{-1}.

The Raman spectra of the gel from both NA and CA tissues were obtained (Figure 4.11). The CA gel has a minor peak at 230 cm^{-1} and a sharper peak at around 1580 cm^{-1} (Trp; Table 4.5) compared to NA gel. Besides that, both NA and CA gels showed almost identical Raman profile in the protein fingerprint region (500 – 2000 cm^{-1}). This demonstrates that the two samples have similar chemical bonding and conformation and suggests that the gel formed by the NA and CA samples occurs through similar protein-protein interactions.

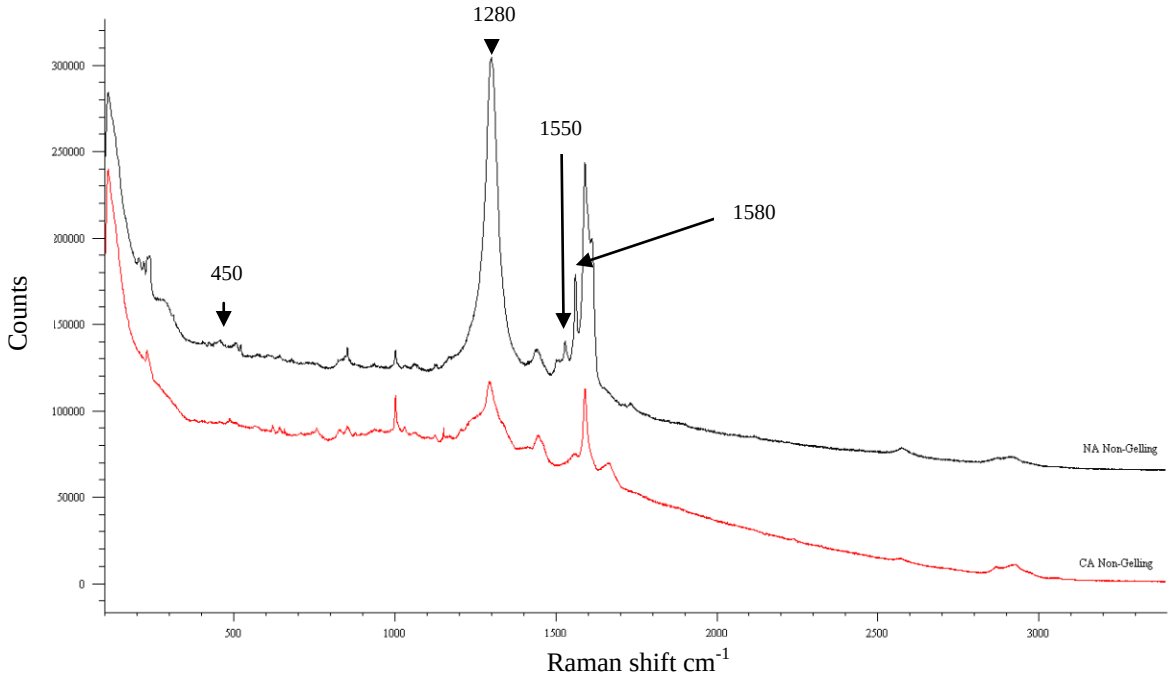

Figure 4.12 Raman spectra of non-gelling components from NA and CA protein extracts. The intensity (Counts) is arbitrary. Major differences between spectra were observed at 450, 1280, 1550 and 1580 cm^{-1}.

Comparison of Raman spectra of the non-gelling components of NA and CA protein extracts (Figure 4.12), revealed that there was an additional minor peak at around 450 cm^{-1}, a sharper peak at 1280 cm^{-1} (Amide III region) and peaks at around 1550 (Trp) and 1580 cm^{-1} (Trp) (Table 4.5) in the NA non-gelling protein extract. The NA non-gelling sample has different secondary structure conformation in the Amide I region showed by the increase in intensity and broader peaks at around 1600 cm^{-1} (Table 4.5).

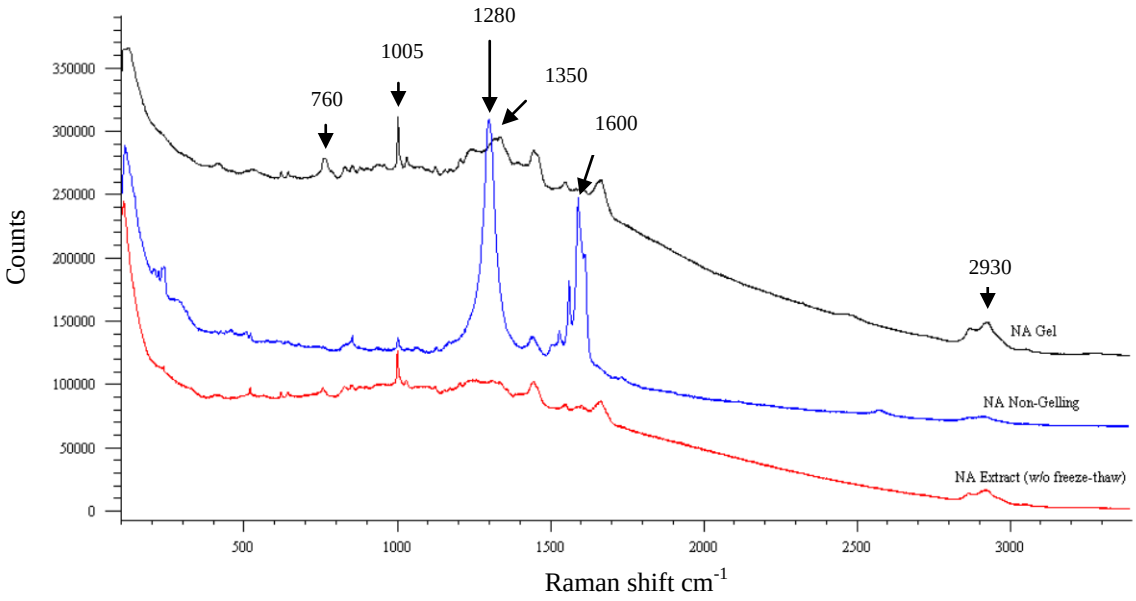

Figure 4.13 Raman spectra of protein extract, non-gelling component and gelling component from NA tissue. The protein extract has not been subjected to freeze-thaw treatment. The intensity (Counts) is arbitrary. Major differences between spectra were observed at 760, 1005, 1280, 1350, 1600 and 2930 cm^{-1}.

Figure 4.13 shows the offset Raman spectra of the protein extract (without freeze-thaw treatment), non-gelling component and gelling component from NA tissue. The protein extract and gelling component demonstrated similar Raman profile, however, the gelling component showed an increase in intensity at around 760, 1005 and 1350 cm^{-1} which represents Trp and Phe (Table 4.5). The gelling component also showed an increase in intensity in the C-H stretching region (2930 cm^{-1}; Table 4.5). The non-gelling component showed a change in the secondary structure conformation in the Amide I (1600 cm^{-1}) and Amide III (1280 cm^{-1}) region (Table 4.5).

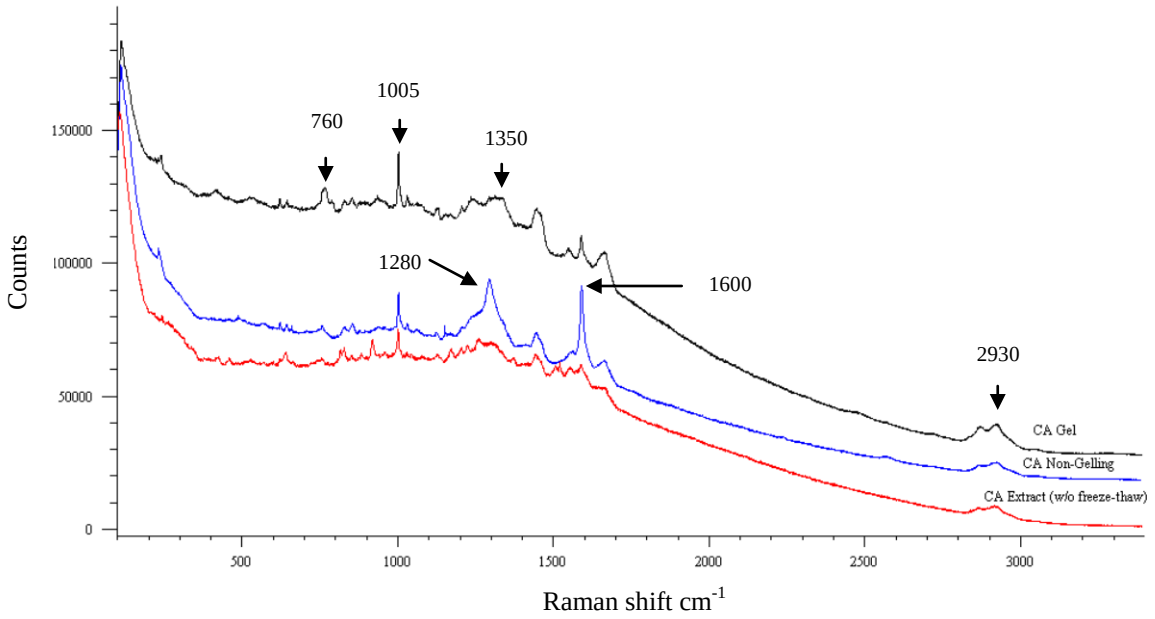

Figure 4.14 Raman spectra of protein extract, non-gelling component and gelling component from CA tissue. The protein extract has not been subjected to freeze-thaw treatment. The intensity (Counts) is arbitrary. Major differences between spectra were observed at 760, 1005, 1280, 1350, 1600 and 2930 cm^{-1}.

From Figure 4.14, the Raman profile of the gel and protein extract showed high similarity, it again showed increased in intensity at around 760, 1005 and 1350 cm^{-1} which represents Trp and Phe (Table 4.5). The intensity in the C-H stretching region at 2930 cm^{-1} (Table 4.5) was increased in the gel sample. Whereas the non-gelling component showed a change in the secondary structure conformation near the Amide I (1600 cm^{-1}) and Amide III (1280 cm^{-1}) (Table 4.5) region.

Despite the fact SDS-PAGE showed similar protein compositions in protein extract, non-gelling component and gel (Figure 4.3), Raman spectroscopy revealed that there were differences in the secondary structure conformation between the protein extract, non-gelling component and gel. These conformational differences were similar between NA and CA samples. This suggests that the gels from NA and CA tissues have very similar protein conformation and formed through similar protein-protein interactions.

Table 4.5 Summary of Raman band assignment.

Raman Band (cm^{-1})[1,2]	**Assignment**	**Major Raman Bands Observed (cm^{-1})**
1203(w), 1032 (w), 1004(s), 624(w)	Phe	1005, 620
1623(w), 1582, 1555(s), 1436(s), 1016(s), 882(w), 762 (s)	Trp	1580, 1550, 1350, 760
540 – 510	Disulfide (S-S) bond	530
2982 (s), 2942, 2880	Aliphatic C-H stretching region	2930
936	Stretching C-C α-helix	930
1645 – 1600	Amide I (α-helix)	1600
1300 – 1260	Amide III (α-helix)	1280

[1] Li-Chan *et al.*, 1996.
[2] Ferraro *et al.*, 2003.

5.0 DISCUSSION

5.1. Conditions for Gel Formation

Protein gelation occurs under conditions that allow ordered interchain, intermolecular or interparticle interactions. A classic two-step mechanism of gelation was proposed by Ferry (1948) which involves an initiation step of unfolding or dissociation of the protein molecules and an aggregation step involving network association resulting in gel formation under appropriate conditions. In this research, the thawing process during the freeze-thaw cycles initiates the unfolding or denaturation of the protein molecules and the freezing process results in network aggregation of the dissociated protein molecules. Gelation only took place in pH 12.0 buffer, regardless of plant tissue (NA or CA) samples. Sánchez and Burgos (1996) have studied the effect of pH and protein concentration on thermal gelation of trypsin hydrolysates of sunflower proteins. The authors found that the sunflower protein gelation is only possible in the pH range of 7 – 11. The gel reaches its maximum gel strength at pH 8 and gel formed at pH 7 or above pH 9 are very weak. They also found that gelation time increases with pH and decreases with increase protein concentration. In addition, Knipe and Frye (1990) showed that the protein gel properties of bovine plasma gel were strongly affected by pH. These previous studies have shown that gelation was highly pH dependent (Knipe and Frye, 1990; Sánchez and Burgos 1996). Our results are consistent with the previous study performed by Sánchez and Burgos (1996) which gelation from plant proteins was occurred at basic pHs. Thus, it is possible that protein gelation is limited in a range of pH; due to the fact that pH can alter the charge distribution on amino acid side chains and affect interchain attractive and repulsive forces. The gel formation observed at pH 12.0 suggests that very high pH was required to increase protein-protein interactions by increasing attractive forces between protein molecules.

Both TCA-acetone and phenol extraction methods employed in this study are popular protein extraction method for proteomic analysis (Saravanan and Rose, 2004; Carpentier *et al.*, 2005). However, protein gelation only occurred in the protein samples generated by the modified phenol extraction (Table 4.1). It has been documented that both of the extraction methods yield a substantial subset of proteins with relatively low amounts of contaminating compounds, but each extract type will only include a subset of proteins that is not present in the other (Saravanan and Rose, 2004). This suggests that a different subset of proteins were

extracted using the modified phenol extraction method and that this particular subset of proteins associated to form the gel prior to freeze-thaw cycles in pH 12.0 borate buffer.

Ferry (1948) proposed that when the aggregation rate between protein molecules is slow, the resulting gel will form a finer network of protein chains, will be less opaque, and will exhibit less syneresis than one with a faster aggregation rate. On the other hand, a coarser protein network will form an opaque gel with large interstices capable of holding solvent which is easily expressed from the matrix. Recently, Lakemond *et al.* (2003) illustrated similar findings that in fine stranded networks the proteins are attached to each other like a string of beads. This type of gel is usually transparent, indicating that the protein aggregates within the gel are smaller than about 50 nm. Coarse networks are usually non-transparent and are thought to be formed by random aggregation of proteins into clusters, which aggregate to form thick strands. The protein aggregation in this study was facilitated by freezing the extract at -80°C which would greatly increase the rate of protein aggregation. This may explain the turbidity of the gel formed from the freeze-thaw cycles (Figure 4.1) as the protein molecules have formed a coarse gel network due to the higher rate of aggregation induced by the extreme low temperature.

Protein concentration is one of the most important factors in protein gelation. Protein concentration will influence the gelation time and the rheological behavior of gels (Oakenfull, 1984; Ross-Murphy, 1991). Ross-Murphy (1991) showed that gelation will occur only if the protein concentration of a sample is above its critical value. The data in this thesis also shows that the gelation process in our samples is highly dependent on sample concentrations as the amount of gel formed was dependent on the protein concentration (Table 4.2). The absence of gelation in some of the samples may be due to the lower protein concentration in these samples.

5.2. Gel Properties and Composition

Viscosity is an important property that is useful in many applications of Food Science, such as the design of food process and processing equipment, quality evaluation and control of food products, and understanding the structure of food materials (Barbosa-Cánovas *et al.*, 1996; Krokida *et al.*, 2001). After the protein samples were subjected to freeze-thaw cycles, their viscosities were determined and it was shown that when the protein gel had started forming,

after the third freeze-thaw cycle, the viscosity increased in both NA and CA samples (Table 4.3). Upon gel formation after the fourth freeze-thaw cycle, the viscosities of NA and CA samples were greatly increased once again (Table 4.3). This shows that upon gelation that the viscosity of the sample increased, and that gelation had a positive correlation with sample viscosity. Viscosity is a measure of resistance to flow or internal friction of a fluid. The observed increase in viscosity upon gelation was due to the presence of the gel in the sample causing the additional resistance to flow. Although there were no significant changes in viscosities with respect to a change in shear rate applied to the samples (Table 4.3), sample viscosities decreased over time during these experiments. The decrease in viscosity over time could have resulted from the disruption of the gel into smaller fragments, thus reducing the internal friction of the sample.

To achieve adequate solubility of the gel for downstream electrophoresis analysis, several chemical treatments were performed on the gel (Table 4.4) which included base (NaOH) or a combination of chaotropic agent, anionic surfactant and reducing agent, with or without heating in a waterbath. Once the gel was formed after the freeze-thaw cycles, the gelation process was found to be thermally irreversible. The gel was able to maintain its structural stability regardless of storage under freezing temperature or heating the gel in buffer (borate, pH 12.0), water or select chemicals. Adequate solubility of the gel was only be obtained from solubilizing the gel in NaOH or a solution containing urea, SDS and β-ME with heating (Table 4.4). However, solubilizing the gel in NaOH was not feasible for protein analysis by SDS-PAGE as strong base denatured the proteins, obviating their resolution (Figure 4.2). The addition of base increased protein solubility through hydrolysis; the solubility increased as the denaturation proceeds, as indicated by a decrease in size of the protein molecule (Wu and Yen, 1924).

Chaotropes can exert profound effects on all interactions by altering solvent parameters (dielectric constant, hydrogen bond formation, polarizability) and all resulting interaction energies will change (Rabilloud, 1996). Urea is a chaotrope, which is less efficient in breaking hydrophobic interactions, but more efficient in breaking intra- and/or intermolecular hydrogen bonding of proteins, so as to improve protein solubilization (Herskovits *et al.*, 1970). However, denaturation by urea induces the exposure of the proteins' hydrophobic residues to the solvent (Rabilloud, 1996), which increases the potential for hydrophobic interactions. SDS is an

anionic detergent which can disrupt protein secondary structure and non-disulfide-linked tertiary structure by interfering with non-covalent interactions, especially hydrophobic interactions. β-ME is a reducing agent, used to disrupt the disulfide interactions between protein molecules. By breaking disulfide interactions, both tertiary and quaternary structure of proteins can be disassociated. In terms of gel formation, disulfide bonds usually play an important role in increasing gel matrix hardness whereas hydrogen and hydrophobic interactions are responsible for keeping the network structure intact (Zheng *et al.*, 1993; Puppo and Añón, 1998). The gel produced in this study could only be solubilized in a solution containing all three: urea, SDS and β-ME, but not individual denaturant with heating. This shows that heating unfolds the proteins in the gel network, facilitating the binding of urea, SDS and β-ME to the proteins to speed up the disruption of the gel's structure. The use of all three denaturants in solubilizing the gel suggests that the gel network is mediated through disulfide interactions and non-covalent interactions especially hydrogen bonding and hydrophobic interactions. Solubilization of the gel in a solution with a combination of 4 M urea, 4% SDS and 1% β-ME was accomplished as the protein components of the solubilized gel were resolved by SDS-PAGE (Figure 4.3). However, difficulty was encountered in protein quantitation for samples solubilized in this chemical cocktail. The high concentration of urea, SDS and β-ME contained in the solution were incompatible with most of the commercially available protein assays. Thus, the protein extracts were not resolved by SDS-PAGE on an equal concentration basis. By comparing the total protein extract, gelling component and non-gelling component from both NA and CA plant materials; there was no observable difference in protein composition (Figure 4.3). It can be concluded that the gel was formed from the proteins present in the winter rye leaf tissue extracts through multiple protein-protein interactions.

The absorbance measurements required prior to CD revealed that the protein samples were highly absorbing (absorbance > 1.5), making it difficult to determine any conformation changes in the gel. As indicated by Matsuura and Manning (1994), there are certain restrictions in using CD for sample analysis. The authors showed that CD spectroscopy requires clear samples and this limits its application to dilute protein solutions or transparent gels. In addition, the interference due to absorbance by various salts and buffers in the far-UV region also limits the use of CD spectroscopy for analyzing the effects of chaotropic salts and protein perturbants on protein conformation (Stanley and Yada, 1992; Ma *et al.*, 2000). CD was shown to be

incompatible with the samples in this study due to these limitations. Thus, in order to investigate possible protein conformational changes in gelation by freeze-thaw treatment a more direct technique, Raman spectroscopy was utilized.

In general, α-helix exhibits lower amide I and higher amide III frequencies than β-sheet and random coil (Ferraro *et al.*, 2003). Amide III is more structure-sensitive than amide I. Amide I and Amide III regions at different Raman band could represent either an α-helix, β-sheet or random coil. To determine whether sample preparation was required for the phenol extracted gel samples and to understand the effect of phenol extraction on structural changes in protein samples with Raman spectroscopy was carried out on commercially available BSA and phenol extraction treated BSA (tBSA). As shown in Figure 4.9, Raman spectra of BSA and phenol extraction treated BSA were obtained with acceptable background noises. This showed that phenol extracted samples can be analyzed by Raman spectroscopy and no sample preparation was required prior to Raman spectroscopy. By comparing Raman spectra between BSA and tBSA, it revealed that the phenol extraction had altered the chemical bonding and conformation of the tBSA sample as sharper peaks were observed at around 530, 620, 820, 1005 and 1600 cm^{-1}. The changes in intensity and sharpness of these peaks suggest conformation changes in the aromatic residues (Trp and Phe), disulfide bonding and Amide I (α-helix) (Table 4.5). This may be one of the reasons the protein gel was only able to form in the phenol extracted protein samples, as the phenol extraction had altered the chemical bonding of the protein to a conformation that allowed gelling to occur.

As shown in Figure 4.10, both NA and CA extracts without freeze-thaw treatment have similar Raman profiles. However, the NA extract showed sharper peak at 1005 (Phe) and 1600 cm^{-1} (Amide I), whereas, the CA extract has additional peaks at around 450, 930 (stretching C-C α-helix), 1180, 1200 (Phe) and 1500 cm^{-1} (Table 4.5). These additional peaks in the CA extract may be due to the presence of cold acclimated induced protein expression such as AFPs which resulted in differences in the sample's structural conformation.

The Raman spectra of NA and CA gel (Figure 4.11) showed near identical Raman peaks and intensity, suggesting very similar secondary structural conformations were observed between the samples. As shown in Figures 4.13 and 4.14, the NA and CA gel samples have a higher intensity in the C-H stretching band (2930 cm^{-1}) than the non-gelling component and protein extract. Larsson and Rand (1973) showed that intensity of the 2930 cm^{-1} band

increased with increasing polarity of the environment around hydrocarbon chains. Recent studies suggested that the increase in intensity of the C-H stretching band of vibration was an indication of protein denaturation and increasing exposure of aliphatic side chains to the aqueous environment (Li-Chan, 1996; Ma et al., 2000). The increasing exposure of aliphatic amino acid residues could suggest the involvement of hydrophobic interaction in stabilizing the gel network.

SDS-PAGE (Figure 4.3) showed that similar protein compositions were present in protein extract, non-gelling component and gelling component. Although there were similar protein compositions in the different components of the sample, Raman spectroscopy revealed that there were differences in the secondary structure conformation between the protein extract without freeze-thaw, non-gelling component and gelling component (Figures 4.13 and 4.14). However, the Raman spectra of the same components between NA and CA samples showed similar structural conformation. These results could suggest that the similar structural conformation of gels from NA and CA samples were stabilized through similar protein-protein interactions. It is also possible that the changes observed in the spectra are a result of the formation of covalent bonds. In conclusion, these results showed that freeze-thaw treatment had altered the secondary structure conformation of the proteins and chemical bonding leading to the formation of a gel network.

5.3. A Role for Rubisco

Rubisco is the most abundant protein in leaf tissues and has been estimated it can comprise up to 50% of the total protein in the plant leaf (Mummenhoff and Hurka, 1986; Andersson and Backlund, 2008). Photosynthetic CO_2 reduction, involving the combination of CO_2 with ribulose-1,5-bisphosphate to form two molecules of 3-phosphoglycerate is catalyzed by Rubisco. The Rubisco holoenzyme complex has a molecular weight close to 550 kDa and is composed of eight identical catalytic large subunits (50 – 55 kDa) and eight identical small subunits (12 – 18 kDa) (Andersson and Backlund, 2008). Plant Rubisco is composed of large (L) and small (S) subunits in a haxadecameric structure (L8S8). The isoelectric point of native Rubisco is close to 6 as determined by electrofocusing while the isoelectric points of the

subunits determined after dissociation by urea are 7.5 and 6.6 – 6.9 for the small and large subunits respectively (Libouga *et al.*, 1996).

Rubisco was shown to be the major protein present in the gel as indicated by the presence of the small and large subunits (Figure 4.3). To confirm whether Rubisco plays a key role in freeze-thaw cycle induced gel formation, experiment were conducted to remove Rubisco from the total winter rye leaf extract by PEI precipitation (Holler *et al.*, 2007). The successful removal of Rubisco from total leaf protein extracts was confirmed by SDS-PAGE (Figure 4.5). The protein extracts devoid of Rubisco were not able to form a gel after freeze-thaw treatment. In addition, the protein samples suspended in pH 12.0 borate buffer precipitated out of solution during the freeze-thaw treatment, whereas samples in pH 7.0 Tris buffer remained soluble (Figure 4.6). The precipitation of protein in pH 12.0 borate buffer may have been due to the increase of both electrostatic and hydrophobic interactions between protein molecules. It is well known that pH will affect the surface charge of proteins and cause protein denaturation or unfolding which leads to increased electrostatic and hydrophobic interactions between protein molecules, resulting in increased protein-protein interactions and the formation of a precipitate (Rabilloud, 1996; Kristinsson and Hultin, 2003). These experiments suggest that Rubisco plays an important role in the gel formation.

To determine if Rubisco itself can form a gel, it was partially purified from winter rye leaf tissues and used in subsequent experiments. Dialysis was performed on the purified Rubisco sample prior to freeze-thaw treatment to remove the salts used in the purification process. Both dialyzed and non-dialyzed Rubisco samples were prepared in pH 7.0 (near physiological pH) and pH 12.0 buffer. After the Rubisco samples were subjected to freeze-thaw cycles, no gel formation was observed. The absence of gel formation in the purified Rubisco samples may be due to lack of hydrogen bonding and hydrophobic interactions between Rubisco subunits and other proteins in the original protein extract which were required to produce a gel network structure (Puppo and Añón, 1998).

While Rubisco itself was not able to form a gel by the freeze-thaw treatment it is possible that it may have a catalytic effect on gel formation. The aggregation of β-lactalbumin is dependent upon the presence of BSA and/or β-lactoglobulin (Calvo *et al.*, 1993). Therefore, BSA was used to investigate the possible catalytic effect of Rubisco on gel formation. The gelation ability of BSA was first assessed and it was not able to form a gel upon freeze-thaw,

regardless of protein concentration. Different concentrations of BSA were mixed with both dialyzed (desalted) and non-dialyzed purified Rubisco and assessed for gel formation. Regardless of the different Rubisco to BSA protein ratios, no gel formation was observed after freeze-thaw treatment, thus indicating Rubisco may not be acting as a catalyst in gel formation.

These Rubisco experiments must be interpreted with caution. While care was taken to maintain similar buffer conditions when performing the freeze-thaw treatments to induce gelation, conditions were not identical. In order to successfully perform these experiments, different protein extraction protocols needed to be utilized. In light of the fact that it has already been demonstrated that extraction protocol is an important parameter in gel formation, this could have negatively affected the ability of the extract to form a gel.

5.4. Antifreeze Proteins and Amyloid Formation

The plant material used in this study was winter rye (*Secale cereal* L.), a freezing tolerant, over wintering annual plant that can survive temperatures below -30°C. This is accomplished, in part, by allowing ice formation only in the intercellular spaces (apoplast) which has been shown to be the result of ice nucleating proteins (Pearce 1988; Griffith and McIntyre, 1993; Brush, *et al.*, 1994). However, the survival of plant tissue also depends on preventing damage caused by the growth of intracellular ice crystals. Cold acclimated winter rye contains endogenously produced AFPs which are involved in maintaining the rheologic properties of the cellular matrix (Griffith *et al.*, 1992). These AFPs are immunologically related to pathogenesis proteins, some of which also contain antifreeze activity. AFPs function by depressing the freezing temperature of water noncolligatively, binding to prism faces of ice and modifying the normal growth pattern of the ice crystals. AFPs will form oligomeric complexes which are thought to inhibit ice growth and recrystallization more effectively than the individual polypeptides (Yu and Griffith, 1999). Six AFPs have been identified in apoplastic extracts from cold acclimated winter rye leaves, ranging from 16-35 kDa (Grifith *et al.*, 1997). The accumulation of AFPs in winter rye are can be induced at warm temperatures by other factors such as drought or treatment with pathogenic fungi, salicylic acid, or abscisic acid (Yu and Grifith, 2001; Stressmann *et al.*, 2004).

A type I AFP is found at high concentrations in the circulatory system and skin of fish (winter flounder) living in subzero seawater, and protects the organism from macromolecular ice growth by adsorption inhibition (Fletcher et al., 2001). While studying AFP mechanism and structure, it was found that high concentrations of winter flounder type I AFP in solution formed a translucent gel upon repetitive freeze and thaw cycles (Graether et al., 2001). Gel formation of a protein at or above the critical concentration of polymerization (Harper and Lansbury, 1997) may indicate the formation of amyloid fibrils. It was further demonstrated that freezing and thawing caused the AFP to be converted from a soluble, single α-helical protein into amyloid fibrils in the form of an amyloidotic gel (Graether et al., 2003).

5.5. Conclusions and Future Studies

Protein-polysaccharide interactions play a significant role in the structure of many processed foods. A gel is a cross-linked polymer network that spans the entire liquid medium; its properties depend strongly on the interaction of the polymer and the liquid medium. Gelation is one of the most important functional properties of polysaccharides and food proteins in food products. In many food products, gelation of these biopolymers is critical to the formation of desired texture that is preferable by consumers.

Protein extracts from winter rye leaf tissues have demonstrated the ability to form gels. This novel proteinaceous extract possesses a gelling ability upon freeze-thaw cycles and may have a potential commercial use in the food industry as a thickener and gelling agent in food products.

Most studies on winter rye have focused on the freezing tolerance of the plant and few have examined rheological properties of cellular extracts in response to freezing. Based on previous studies, it is possible that the protein extracts utilized in this study are enriched with AFPs that are forming, or contributing to, amyloid fibril formation. Examination of the current gel by electron microscopy, fluorescence staining with the amyloid-specific dye Thioflavin T, and/or solid-state 13C-NMR spectroscopy would verify this suggestion. This could also be confirmed by examining apoplastic extracts containing AFPs and assessing their gel forming abilities. The quality of frozen foods decreases over time due to water sublimation and ice crystallization. If gelation in these plant extracts is indeed due to AFPs, it may be possible to

use this knowledge to improve the quality of frozen food products subjected to dehydrating conditions.

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