EXAMINATION OF THE GELLING PROPERTIES OF CANOLA AND SOY PROTEIN ISOLATES

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ABSTRACT

Canola protein isolate (CPI) has tremendous potential as a protein alternative to soy within the global protein ingredient market. The overall goal of this thesis was to compare and contrast the gelling mechanism of CPI with a commercial soy protein isolate (SPI) ingredient. Specifically, the gelation properties of CPI and SPI were evaluated as a function of protein concentration (5.0–9.0%), destabilizing agent [0.1 – 5.0 M urea; 0.1 and 1.0% 2-mercaptoethanol], ionic strength (0.1, 0.5 M NaCl) and pH (3.0, 5.0, 7.0, 9.0). The fractal properties of CPI were evaluated as a function of protein concentration (5.0 – 9.0%) and pH (3.0, 5.0, 7.0, 9.0).

In the first study, the gelling properties of CPI and SPI as a function of concentration were evaluated, along with the nature of the interactions within their respective gel networks. Overall, the magnitude of the storage modulus (G′) of the gel was found to increase with increasing concentration at pH 7.0, whereas the gelling temperature (T_{gel}) remained constant at ~88ºC. As the NaCl level was increased from 0.1 to 0.5 M, the zeta potential was found to be reduced from ~-20 to -4 mV, but with little effect on T_{gel} or network strength. In the presence of 2-mercaptoethanol, networks became weaker, indicating the importance of disulfide bridging within the CPI network. Disulfide bridging, electrostatics and hydrogen bonding are all thought to have a role in CPI gelation. In the case of SPI, the magnitude of the storage modulus (G′) and T_{gel} were found to increase and decrease (~80ºC to 73ºC), respectively, with increasing urea concentration at pH 7.0. Increases in NaCl from 0.1 to 0.5 M reduced the zeta potential from ~-44 to -13 mV and caused a shift in T_{gel} from ~84ºC to 67ºC, and increased G′. No gels were formed in the presence of 2-mercaptoethanol.

In the second study, the effect of pH on the gelling properties of CPI and SPI was evaluated. Surface charge (i.e., zeta potential) measurements as a function of pH found CPI to be positively (+18.6 mV), neutral and negatively (-32 mV) charged at pH 3.0, ~5.6 and 9.0, respectively. On the other hand, SPI was observed to be positively (+35.4 mV), neutral and negatively (-51 mV) charged at pH 3.0, 5.0 and 9.0, respectively. An increases in NaCl concentration from 0 M to 0.1 M resulted in a reduction in surface charge at all pHs for both CPI and SPI. Differential scanning calorimetry was performed to determine the thermal properties of CPI. The gelation temperature was found to be above the onset temperature for denaturation. For

CPI, the onset of denaturation was found to occur at ~68ºC and then increased to ~78-79ºC at pH 7.0-9.0. With respect to rheological properties, SPI did not gel at pH 9.0, and G′ declined as pH increased from 3.0 to 7.0. CPI did not gel at pH 3.0, however the network formed at pH 5.0 became stronger (higher G′) as pH increased. The SPI gelling temperature at pH 3.0, 5.0 and 7.0 was observed to be ~85.6, ~46 and ~81ºC, respectively. SPI gels formed at pH 5.0 earlier due to increased protein aggregation near its isoelectric point (pI). The gelation temperature for CPI at pH 5.0 and 7.0 were similar (~88ºC), then declined at pH 9.0 (~82ºC). Network structure of CPI as a function of pH also was investigated using confocal scanning light microscopy (CSLM). As the pH became more alkaline from pH 7.0 to pH 9.0, there was a decrease in lacunarity (~0.41→~0.25). However, the fractal dimension was found to increase (from ~1.54 to ~1.82) showing that increasing the pH resulted in a more compacted CPI network.

In summary, protein-protein aggregation induced either by increasing concentration or changing the pH resulted in network formation for both CPI and SPI, where both networks were thought to be stabilized by disulfide bridging and hydrogen bonding. SPI underwent protein aggregation earlier than CPI near its pI value, whereas CPI gels formed the strongest networks away from its pI under alkaline conditions. In all cases, CPI grew in diffusion-limited cluster-cluster aggregation to from the gel network.

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LIST OF ABBREVIATIONS AND SYMBOLS

ANOVA	Analysis of variance
CLSM	Confocal laser scanning microscopy
CPI	Canola protein isolate
d	Euclidean dimension (= 3)
d.b.	Dry basis
D_f	Fractal dimension
DLCA	Diffusion-limited cluster-cluster aggregation
DSC	Differential scanning calorimetry
g	Gravitational acceleration
G′	Storage modulus
G″	Loss modulus
GP	Genipin
Hz	Hertz
J	Joule
kDa	Kilodalton
M	Molar
ME	2-Mercaptoethanol
mol	Mole
mV	Milliovolt
N	Normality
p	Probability
Pa	Pascal
PAGE	Polyacrylamide gel electrophoresis
pI	Isoelectric point
PMM	Protein micellar mass
q	Heat flow
R	Thermoelectric disk resistance
RITC	Rhodamine B Isothyocyanate
RLCA	Reaction-limited cluster-cluster aggregation

rpm	Rotational per minute
S	Svedberg Unit
SDS	Sodium dodecyl sulfate
SPI	Soy protein isolate
T_d	Denaturation temperature
TG	Transglutaminase
T_{gel}	Gelation temperature
T_o	Onset temperature
U	Units/mL
U_E	Electrophoretic mobility
w/w	Weight to weight
ΔH	Enthalpy
ΔT	Difference in temperature
ε	Permittivity
ζ	Zeta potential
η	Viscosity
λ_ε	Lacunarity
σ	Standard deviation

1. INTRODUCTION

1.1 Overview

Canola is an economically important crop for both Saskatchewan and Canada, and is the third largest source of vegetable oil, next to soybean and palm oil (USDA, 2010). After oil extraction, the resulting meal is considered a rich source of both protein and fibre. Despite the protein having an excellent balance of essential amino acids (Ohlson and Anjou, 1979), the meal is typically given to animals as a low value feedstock (Canola Council of Canada, 2011). However, market shifts within the protein ingredient market towards plant-based alternatives from animal-derived proteins (e.g., gelatin, whey, casein and ovalbumin) are driving consumer and industry interest in canola, especially since soy represents a major allergen concern and has a distinct beany flavour. Various extraction technologies for canola protein from defatted meal have been developed spanning a range of processes including micellar precipitation (Logie and Mianova, 2010), alkaline extraction – acid precipitation (Xu and Diosady, 2002), and membrane filtration (Ismond and Welsh, 1992; Gruener and Ismond, 1997). Only recently, Burcon NutraScience Corporation (Vancouver, BC) and BioExx Specialty Proteins (Toronto, ON) applied for and received GRAS (Generally Recognized as Safe) status in 2011 for their canola protein ingredients (Schnarr and Koyich, 2011). Depending on the canola variety, oil extraction method and process used to produce the protein isolate, functionality can vary considerably (Aluko and McIntosh, 2001; Khattab and Arntfield, 2009; Can Karaca et al., 2011).

The overall goal of this research was to examine the gelation mechanism of canola protein isolate (CPI) intended for use in food applications, and to compare it to that of a commercial soy protein isolate (SPI). Canola proteins are dominated by two main proteins, a salt-soluble globulin protein (cruciferin) and a water-soluble albumin protein (napin). The two proteins differ in terms of size, amino acid composition and surface characteristics (e.g., charge and hydrophobicity), which can impact protein functionality. Gelation studies involving canola proteins have typically involved the use of cross-linking agents (Pinterits and Arntfield, 2007; Sun and Arntfield, 2011) alone or in combination with polysaccharides (Uruakpa and Arntfield 2004, 2006a,b; Klassen et

al., 2011), or involved the use of chemically modified canola proteins (Paulson and Tung, 1988; Schwenke et al. 1998). In the present work, mechanisms of gelation were elucidated for canola protein as a function of temperature, protein concentration, pH, NaCl concentration and destabilizing agents (e.g., urea and 2-mercaptoethanol) using rheology and calorimetry, and compared with that of soy protein. A fractal model was used to examine the aggregation behaviour of both proteins as a function of protein concentration and pH.

1.2 Objectives

Objectives of this research were follows:

- to examine the rheological properties of canola and soy protein dispersions as a function of temperature, protein concentration and pH;
- to examine the nature of interactions within the canola and soy protein gels in response to NaCl concentration, and destabilizing agents (e.g., urea and mercaptoethanol); and
- to examine and model the fractal aggregation behaviour of canola and soy proteins during gelation.

1.3 Hypotheses

As part of this study, the following hypotheses were tested:

- canola protein gel networks will be stronger at higher protein concentrations (*due to increased macromolecular packing*), and at pHs near their isoelectric point (*such that they are slightly charged*), and require heating to high temperatures followed by cooling to form a gel. Soy gels will follow a similar pattern as a function of concentration, pH and temperature;
- canola and soy protein gels will be stabilized by disulfide bridging, hydrophobic interactions and hydrogen bonding; and
- canola and soy proteins will aggregate in a fractal manner resembling the growth of self-similar clusters.

2. LITERATURE REVIEW

Canola meal remains a relatively underutilized source of protein beyond that of animal feed, which has potential as an ingredient in higher value food and non-food applications. In this review, canola proteins are reviewed in relation to their structure, methods of extraction, and functionality. In particular, emphasis is placed on protein gelation (*in general*), rheological and fractal analysis, and the gelling behaviour of both canola and soy proteins.

2.1 Canola and canola meal

Canola was originally bred in Canada from rapeseed varieties (*Brassica napus* L. and *Brassica rapa* L.*)* to have low levels of erucic acid (<2%) and glucosinolates (<30 μmol/g) for use mainly as an edible healthy oil, but also for use in margarines and biofuels (Newkirk, 2009, Canola Council of Canada, 2011; Wanasundara, 2011). Canola seed itself comprises of 40% oil and 17-26% protein (Aider and Barbana, 2011). After oil extraction, the remaining meal tends to be rich in protein (36- 39%, wet basis) and fibre (~12%, wet basis); used mainly as a low cost feed for dairy and beef cattle, poultry, swine, sheep and farmed fish based on its nutritional value (Khattab and Arntfield, 2009; Newkirk, 2009). The meal is also high in phenolic compounds and phytic acid which can lead to poor protein functionality and digestibility (Wu and Muir, 2008; Aider and Barbana, 2011). The proteins within the meal are considered to be highly nutritious, offering a well balance of essential amino acids for both animal and human nutrition (Ohlson and Anjou, 1979). Canola proteins have high levels of glutamine, glutamic acid, arginine and leucine, however the method of oil extraction can reduce the levels of sulfur-containing amino acids, such as cysteine and methionine in canola protein (Aider and Barbana, 2011). Protein efficiency ratios also tend to be higher for canola (2.64) than for soybean protein (2.19) indicating that is the protein is better digested (Delisle et al., 1985; Aider and Barbana, 2011). Also, the albumin fraction within canola contains higher levels of histidine, cysteine, lysine, methionine, and arginine compared to egg albumin (Aider and Barbana, 2011).

2.2 Canola Proteins

Proteins arising from the meal are primarily comprised of the storage proteins napin and cruciferin found within the embryo of the seed, accounting for ~20% and ~60% of the total protein, respectively (Aider and Barbana, 2011). However another structural protein, known as oleosin can also be found in the oil fraction (i.e., oil bodies within the seed) and accounts for ~2-8% of the total proteins (Höglund et al., 1992; Salleh et al., 2002; Aider and Barbana, 2011). Cruciferin (11/12 S, S is a Svedberg Unit) is a salt soluble globulin protein with a molecular mass of 300-310 kDa, has an isoelectric point (pI) of 7.25, and has secondary structures comprised of β-sheets (~50%) and α-helices (~10%) (Zirwer et al. 1985; Wanasundara, 2011). The cruciferin molecule is a hexameric protein comprised of six subunits, each having an acidic α-chain (~30 kDa) and basic β-chain (~20 kDa) held together by one disulfide linkage (Aluko and Mclntosh, 2001; Wanasundara, 2011). Wu and Muir (2008) reported non-covalent linkages such as hydrophobic interactions, hydrogen bonding and van der Waals interactions also played a signficant role in stabilizing the native conformation. The hexamer can experience pH- and/or ionic strength dependent reversible association or disassociation. For instance, at medium to high ionic strengths, cruriferin exists as a 11S/12S protein, however as ionic strength is reduced, it can disassociate to form a 7S trimer (Folawiyo and Apenten, 1996). Cruciferin is also known to unfold between pHs 3.0 and 5.0, but can refold below pHs 3.0 due to a hydrophobic effect (Wanasundara, 2011).

Napin is a water-soluble albumin protein (2S) with a molecular mass of ~12-17 kDa. Napin is considered to have more of an open structure than cruciferin, with higher levels of α-helices (~45%) and lower levels of β-sheets (~12%) (Crouch et al., 1983; Schwenke et al., 1998). Napin is comprised of only 2 polypeptides of ~4 and ~10 kDa linked together by a disulfide bond (Salleh et al., 2002) and has a calculated pI value of ~11 depending on its amino acid sequence (Wanasundara, 2011). Schwenke et al. (1988) also reported that napin secondary and tertiary structures are stabilized by inter- and intra- chain where it could be dissociated at high temperature and alkaline conditions. On the other hand, at high temperatures, and acidic or neutral pH the helix content of napin remains the same, which indicated that the napin structure stayed unchanged (Schwenke et al., 1988). Typically, the napin molecule is very hydrophilic, carries a positive net charge at neutral pH and displays low surface hydrophobicity (Wanasundara, 2011). Oleosins proteins are alkaline in nature and are of low molecular mass (15-

26 kDa). The meal also may contain minor amounts of proteins such as thionins, trypsin inhibitors and lipid transfer proteins (Aider and Barbana, 2011).

2.3 Protein extraction

Protein concentrate and isolate products typically contain <65% and 90% protein on a moisture-free basis, respectively (Wanasundara, 2011). Depending on the extraction process, significant quantities of the 7S fraction (i.e., dissociated 11S/12S proteins) may arise, and the relative proportion of 11S/12S, 7S and 2S proteins may vary (Aider and Barbana, 2011). Furthermore, extraction processes also act to reduce the levels of anti-nutritional factors, such as, phenolics, glucosinsolates and phytic acid further for use in foods (Ismond and Welsh, 1992). The presence of phenolics can lead to the production of dark coloured protein powder and unpleasant flavour profiles (Xu and Diosady, 2002). In all cases, protein extraction is carried out on defatted meal, which typically involves an organic solvent such as hexane, and high temperatures. Since canola is processed primarily for its oil, the quality of the meal by-product may be quite variable depending on the process. In most cases, some level of protein denaturation or damage occurs, which reduces extraction yields.

The scientific and patent literature is filled with extraction protocols for producing canola protein products with slight variances. For instance, protein isolates have been produced using a membrane-based process, whereby proteins are extracted under alkaline conditions (pH 12.0) and followed by isoelectric precipitation (pH 4.5-5.5, typically giving the maximum yield) (Aider and Baranana, 2011). The precipitation pH may vary depending on the process. Some researchers precipitate at 7.5 and others at 3.5, giving completely different protein profiles once the extraction is complete. Once precipitated, the pellet is then re-suspended and filtered through ultrafiltration (50 molecular weight cutoff) and diafiltration system to concentrate and purify the soluble proteins, followed by drying (Xu and Diosady, 2002; Ghodsvali et al., 2005). The extraction at high pHs helps reduce the levels of phytic acid and phenolics within the protein product leading products that are bland tasting and pale in colour (Xu and Diosady, 2002). Protein levels can be increased greatly using membrane filtration, which helped reduce the levels of glucosinolates, soluble sugars, and fibre further (Wanasundara, 2011).

In another instance, micellular precipitation was used to produce a CPI (Ismond and Welsh, 1992; Gruener and Ismond, 1997). In general the method involves solubilizing defatted

canola meal within a Tris/HCl buffer containing 0.1 M NaCl to solubilize the proteins. The extract buffer pH varies between research groups, but can influence the levels of anti-nutritional factors in the final product and protein quality. For instance, Ismond and Welsh (1992) extracted at pH 5.5 and found lower levels of anti-nutritional factors in the protein powder than did Gruener and Ismond (1997) who extracted at pH 7.0. Upon centrifugation to remove insoluble particles, the supernatant is typically diluted 10-fold with cold (4ºC) distilled deionized water to help facilitate micelle formation via hydrophobic interactions between neighboring proteins. The system remains static, allowing formed micelles to flocculate and sediment to form an amorphous, sticky, gelatinous gluten-like protein micellular mass. This mass is then collected and dried. Ismond and Welsh (1992) indicated that the PMM method was able to remove 75.5% of phytic acid, 85.3% of phenolic compounds, and 92.4% of glucosinolates from canola protein. In addition, PMM was noted to be a milder treatment where proteins are not denatured during the process. Yang et al. (2014) modified the aforementioned method by Ismond and Welsh (1992) to concentrate water-soluble and salt-soluble protein fractions using molecular weight cut-offs by dialysis tubing for both the supernatants and precipitates. Yang et al. (2014) was able to produce protein isolates high in cruciferin and napin, each showing gelling and thermal properties.

2.4 Functionality of canola proteins

The majority of the functionality studies in the literature have focused on using a canola protein concentrates or isolates comprised of a mixture of proteins of unknown ratios. Typically, high protein solubility is desired by industry, especially in applications such as beverage fortification. High protein solubility also tends to be positively correlated with better emulsion and gel forming properties (Can Karaca et al., 2011). However, depending on the food application, poor solubility may be desired, especially if strong water or oil holding properties is desired (Aider and Barbana, 2011).

Water (or oil) holding capacities describe the ability for the protein to absorb and retain water (or oil) allowing for food products to retain flavours, water (or fats) and to improve mouth-feel (Aider and Barbana, 2011). Sosulski et al. (1976) measured both the water and oil holding capacities for a canola and soy protein concentrates to find values for canola of 398% and 389%, respectively, whereas soy had lower reported values at 331% and 202%, respectively. Can Karaca et al. (2011) investigated the emulsifying properties and solubility of CPI produced by

isoelectric precipitation and salt extraction. The authors reported creaming stability for isoelectric precipitated canola protein stabilized emulsions to be 86.1% at pH 7.0, whereas salt extracted proteins under the emulsifying conditions used did not form a stable emulsion. The authors also found that emulsion capacity was found to be ~500-550 g oil/g protein regardless of the extraction protocol used. Creaming stability provides an estimate of the ability for the emulsion to resist gravitational separation (Bury et al., 1995); whereas emulsion capacity refers to the amount of oil one gram of protein can hold (Can Karaca et al., 2011). Solubility was found to be higher for salt extracted canola proteins (~80%) than isolates prepared by isoelectric precipitation (~3%) at pH 7.0 (Can Karaca et al., 2011). Yoshie-Stark et al. (2008) measured solubility of ultrafiltered CPI over the pH range of 3.0 and 9.0, where it was found solubility to range between 52.5% and 97.2%. Also, the authors reported that ultrafiltrated CPI had higher emulsification capacity and stability than whole egg. Foaming capacity is the ability to produce foam at given concentration of protein (Liu et al., 2010). Aluko and Mclntosh (2001) investigated the emulsion stability and foaming capacity of defatted canola meal and CPI. The authors reported that meals with high foaming capacity had higher protein solubility values. Also, the methods of CPI extraction also influenced the foaming capacity. Overall, acid-precipitated CPI (189.15%-192.93%) had higher foaming capacity than calcium-precipitated CPI (170.24%-185.37%) (Aluko and Mclntosh, 2001).

The majority of earlier functionality studies involving canola proteins have focused on the use of chemical modification to alter the protein's surface chemistry and performance. Paulson and Tung (1987) studied solubility and physicochemical properties of a succinylated CPI with 5.2% and 14.2% degrees of succinylation. Solubility of the modified proteins was found to increase as the degree of succinylation increased due to a rise in charge repulsion and reduced surface hydrophobicity. Paulson and Tung (1989) also studied the use of succinylation of CPI to enhance gelation. Unmodified canola protein gelled at pHs only above 9.5, but succinylated canola protein was able to gel over a much wider pH range (pH 5.0-11.0). The presence of insoluble particulates in the unmodified canola resulted in the formation of opaque gel, whereas the succinylated CPI produced translucent gel except at pH 5.0. The authors reported that opaque gels had the characteristic of having a pasty precipitate with weak elasticity, whereas the translucent gel was firm and springy. Gruener and Ismond (1997) investigated the functional properties of acetylated and succinylated micellar masses produced canola 12S globulin. The

authors reported that acetylation (16%, 26%, 42%, 62%) of 12S proteins significantly reduced the foam stability however increased the foaming capacity and emulsifying activity. Also, the authors indicated that acetylation lighten the colour of the gel compared to untreated canola 12S globulin. Succinylation (3%, 48%, 53%, 61%) of canola 12S globulin also improved the functionality. Succinylation increased the foaming capacity and fat absorption capacity. However, like acetylated canola 12S globulin protein, succinylation of the 12S decrease the foam stability. Schwenke et al. (1998) also studied the acetylated CPI on gelation properties. The authors reported that the acetylated CPI consisting of 70% cruciferin and 30% napin showed strong pH dependence for gelation temperature due to an increase in the number of negatively charged carboxyl groups. Acetylated CPI (12.5%) showed the highest dynamic storage modulus (G′) values at pH 6-6.3, whereas napin showed highest the G′ at ~ pH 9. Further, enzymatic modification has also been shown to improve the functional properties of CPI. Through use of food grade enzymes such as trypsin, alcalase, pepsin and chymotrypsin, Alashi et al. (2011) reported that the emulsifying properties were improved due to increased hydrolysis that allowed greater access to buried hydrophobic amino acids.

2.5 Basics of rheology

The field of rheology has been discussed in many review articles, book chapters and scientific literature over the years (Ferry, 1980; Weijermars and Schmeling, 1986; Steffe, 1992; Malkin and Isayev, 2012; Rao, 2014). In the food industry, rheology is use to understand the deformation or flow of materials or products, where deformation refers to the change in distance between different sites within the material in response to an imposed stress (Malkin and Isayev, 2012). Most foods can be described as fluids, displaying Newtonian or non-Newtonian flow, or as viscoelastic solids or structured fluids. Newtonian fluids are liquids whose viscosity remains independent of the shear rate (e.g., water, oil and honey), where viscosity is defined as the internal resistance to shear (Rao, 2014). In contrast, non-Newtonian fluids depend on the shear rate, may be time-independent (e.g., shear thinning or thickening) or time-dependent, and may or may not display a yield stress (Rao, 2014). Most fluids containing large biopolymers (e.g., proteins or polysaccharides) are considered to be non-Newtonian in nature. Changes to fluid properties are often measured using rotational or capillary type viscometers or rheometers.

The properties of solid materials can be described by Hooke's law, which suggests a direct proportionality between strain and stress through a modulus (G) parameter (i.e., a proportionality constant) (Gunasekaran and Ak, 2000; Rao, 2014). For an ideal fluid, the material will remained deformed when the applied stress is removed, whereas an ideal solid material will fully recover from the deformation once the stress removed (Gunasekaran and Ak, 2000). Foods are considered to be viscoelastic materials, displaying properties of both viscous and elastic materials, meaning after an opposed stress is removed that deformed material will only partially recover to its original state (Gunasekaran and Ak, 2000). Viscoelastic materials are often described using oscillatory shear rheometry in oscillation or creep relaxation modes.

2.6 Gelation in proteins

A gel is defined as a 3-dimensional network comprised of an 'infinitely branched polymer or aggregate' that spans the dimensions of the container. Gelation requires aggregation or association of protein particles, which is formed from partial protein denaturation or change in conformation (Matsumura and Mori, 1996). Depending on the type of protein, solvent and gelling conditions various categories of gels can develop. Physical-type gels may be either weak or strong in nature. Strong physical gels involve protein junction zones in the form of lamellar microcrystals, glassy nodules and double helices, and require elevated temperatures to induce melting of the gel network. In contrast, weak physical gels are more reversible in nature, and comprised of temporary linkages between proteins such as those from hydrogen or ionic bonding, or block copolymer micelles (Renard et al., 2006). Chemical-type gels are much stronger in nature due to the presence of point cross-links between protein molecules, such as from disulfide bridging or through the addition of fixatives (Renard et al., 2006). Globular proteins are typically considered to be heat-setting, meaning they require high temperatures to induce unfolding of the proteins and protein-protein association via hydrophobic interactions and disulfide bridging. As temperatures cool, hydrogen bonds develop to help strengthen the network structure (Renard et al., 2006). However, gel networks can vary considerably in strength, structure and opacity depending on the temperature used in the gelling process, the heating and cooling rates used, pH, protein concentration and the presence of salts resulting in a coagulate-type network comprised of random aggregates or a more fibrous type network resembling 'strings of beads' (Matsumura and Mori, 1996).

2.6.1 Rheological examination of proteins gels

The gelation properties of protein solutions are typically evaluated by small deformation oscillatory rheology involving temperature ramps, strain sweeps and frequency sweeps. Temperature ramps during heating enable the monitoring of network development to occur as evident by a rise in the dynamic storage moduli (G′) with temperature as proteins aggregate after denaturation is induced (Renard et al., 2006, Lamsal et al., 2007). Aggregation is facilitated by hydrophobic interactions, leading to the formation of a 'string of beads' fibrous or coagulum structure (depending on solvent pH and salt concentrations). Gel temperature can be denoted by various methods, but typically it involves extrapolating the tangent associated with the steepest part of the rise of G′ to x-axis. At this temperature, the solution transitions from a sol to a gel (Lamsal et al., 2007). As the network cools, an increase in the amount of van der Waals forces and hydrogen bonding occurs leading to further strengthening of the gel network. A frequency sweep at a constant strain provides information on the level of interactions within the system. For instance, if the dynamic loss moduli (G″) is greater than G′ then the system is behaving as a fluid under low frequency conditions, however, if G′>G″, then the material is more structured. A relative moduli-frequency independence may give an indication of a solid-like gel structure, whereas, if the moduli are frequency dependent at relative low frequency, the material may behaving like an entanglement polymer solution (Ferry, 1980).

2.6.2 Gelation properties of canola proteins

The gelation properties of canola proteins have typically involved the addition of fixatives (e.g., transglutaminase), the use of chemically modified canola proteins and mixtures involving anionic polysaccharides. Léger and Arntfield (1993) studied gel formation involving 6% of 12S CPI that was extracted using a protein micellar mass method. The authors investigated the gelling properties of CPI as a function of pH, and with the addition of different concentration of salts, dithiothreitol, and guanidine hydrochloride. As pH range varied from pH 4.0 to 11.0, the authors found that stronger gels formed under alkaline conditions relative to acidic ones. The authors reported that at pHs close to isoelectric point of CPI, showed the highest G′ (describes the elastic component of the gel). The addition of salt was found to contribute to the thermal denaturation properties of 6% 12S canola globulin. At pH 9.0, the 12S canola globulin thermal denaturation

was 81ºC, however with 0.1M sodium salt such as sodium sulfate, sodium acetate, sodium chloride, and sodium thiocyanate increased the thermal denaturation to 85.75ºC~87.4ºC. Moreover, Léger and Arntfield (1993) reported that the addition of the aforementioned sodium salts to the 12S canola globulin had similar cooling curves when a temperature ramp was performed from 90ºC - 25ºC at 2ºC/min. The authors indicated this could be due to similar gelation mechanism. Also, the study showed that addition of guanidine hydrochloride altered the protein conformation that interfered with the early stage of development of 12S canola globulin by disrupting the covalent bonds (Léger and Arntfield, 1993).

Rubino et al. (1996) also studied the gelation properties of canola proteins isolate that primarily consisted of 12S canola protein. The study showed that 10% CPI did not form a gel at pH 4.5 due to strong repulsive forces. Also, the addition of sinapic acid or thomasidioic acid caused weakening of canola protein gel. Interaction between CPI and phenolic compounds (sinapic acid and thomasidioic acid) varied depending on the pH ranges; at pH 4.5 sinapic acid interact electrostatically with CPI whereas at pH 7.0 and 8.5, hydrophobic interaction occurs between the canola proteins and thomasidioci acid. However, at pH 7.2, 10% CPI did form an opaque gel. Also, Rubino et al. (1996) reported that replacing the solvent from water to 0.1 M NaCl solution increased the elasticity and lowered the gel strength. Furthermore, the addition of sinapic acid or thomasidioic acid was found to reduce the G′ and elasticity of the canola protein network at pH 7.0. Schwenke et al. (1998) reported that gelation temperature of salt extracted CPI that was comprised of 70% cruciferin and 30% napin is 69ºC at pH 9.0 with 15% CPI. Also, the author reported that 12.5% purified cruciferin protein isolate-formed stronger gels with higher shear modulus than 12.5% canola proteins isolate between pH 6.0 and 8.0. Understanding the control of protein gels is important in industry for application purposes and product formulation and design.

2.6.3 Gelation properties of soy proteins

The gelation properties of oilseed proteins found in the literature have primarily focused on soy (Gennadios et al., 1993; Ker and Chen, 1998; Renkema et al., 2000; Hu et al., 2013). Soy proteins are dominated by an 11S glycinin and 7S β-conglycinin protein. The former is a hexameric protein comprised of acidic and basic polypeptide chains linked together by disulfide bonds. In contrast, the 7S protein is a trimer composed of three subunits (α′, α and β) with no

disulfide linkages (Chen et al., 2013). Based on their structure, the thermal stability of the 7S protein is much less than the 11S protein allowing it to unravel at much lower temperatures. Salleh et al. (2002) produced heat set gel networks at pH 7.6 and 0.42 M NaCl using both soy glycinin and canola cruciferin to find that the soy gel was more transparent and elastic than the cruciferin network. The authors also reported that gel hardness increased with increasing in temperature, protein concentration, pH, and a decrease in ionic strength. Renkema et al. (2000) investigated the effect of pH on gel properties of purified glycinin and SPI (97% protein content) gels. Both glycinin and SPI gels formed find-stranded gels that had low G′ values at pH 7.6, however at pH 3.8 both soy proteins formed coarse gels that had higher G′ values. This also correlated with the solubility, where at pH 7.6 there was higher solubility compared to pH 3.8. The authors also stated the β-conglycinin role in SPI depends on the pH. At pH 7.6, β-conglycinin plays a minor role, however at pH 3.8 the onsets of heat denaturation cause early formation of the gel of SPI.

Renkema et al. (2001) researched glycinin, β-conglycinin and a 1:1 mixture of glycinin and β-conglycinin gels. The mixture of glycinin and β-conglycinin (1:1) formed gels with fracture stress and strain values that are between glycinin and β-conglycinin gels at pH 3.8. The authors reported that glycinin had a higher gelation temperature at the crossover point of G′ and G″ (G″; describes the viscous component of the gel) than β-conglycinin at pH 7.6. Where, at acidic pH the increase in glycine concentration did not increase gelation temperature, however at pH 7.6, the increase in concentrations decreased the gelation temperature (Renkema et al., 2001). The gelation temperature was high influenced by the ratio of glycine and β-conglycinin (Renkema et al., 2001). Similarly, Renkema and Van Vliet (2004) also reported that the critical concentration for gelation also depends on the factors such as ionic strength and pH. Where, at pH 7.6 and 0.2M NaCl SPI was able to gel at concentrations between 3 and 5%, and at pH 7.0 and 0.0 M NaCl higher concentrations (6.5% and 8.0%) was needed to gel (Renkema and Van Vliet, 2004). In addition, Renkema and Van Vliet (2002) indicated that after the heating stage, cooling of the soy protein gel caused increases in G′ however this was thermo-reversible. The authors reported that rearrangements and disulfide bonds do not form during cooling stage of soy protein. Utsumi and Kinsella (1985) reported bonds involved with the development of 11S gels are primarily disulfide bonds and electrostatic interactions. This was also supported by Nagano (2013) where the addition of 2-mercaptoethanol significantly reduced the G′ of 11S gel. In

contrast, hydrogen bonds and hydrophobic interactions play more of a major role in the formation of 7S gels (Utsumi and Kinsella, 1985).

2.7 Differential scanning calorimetry

Differential scanning calorimetry (DSC) measures the thermal properties of materials as a function of temperature (Renkema and Van Vliet, 2002; Gill et al., 2010). DSC can be used to determine the protein denaturation temperature, as well as its associated enthalpy, which describes the total heat energy uptake by the substance (Gill et al., 2010). There are a wide number of variations of the DSC instrument, including infrared-heated DSC, modulated-temperature DSC, microelectromechanical DSC, differential scanning microcalorimetry and conventional DSC (Gill et al., 2010). Conventional DSC measures the thermal properties of sample of interest by measuring the heat flow, which is determined by Ohm's law (Danley, 2002):

$$q = \Delta T/R \qquad \text{(eq. 2.1)}$$

where q is the heat flow of the sample, ΔT is the difference in temperature between reference and sample, and R is the thermoelectric disk resistance.

Many studies have determined the thermal properties of canola and soy protein (Hermansson, 1986; Renkema and van Vliet, 2002; Sallah et al., 2002; Wu and Muir, 2008), using micro-DSC. Renkema and van Vliet (2002) investigated the denaturation temperature of 10% SPI at pH 7 using micro-DSC. The authors reported that denaturation peak temperature of glycinin was 88ºC and β-conglycinin was 68ºC. In addition, Hermansson (1986) indicated that with a higher concentration of NaCl, the denaturation temperature of the soy protein solution increased at pH 7 from 80ºC (0 M NaCl) to >103ºC (2 M NaCl). The presence of NaCl acts to screen charges on the protein to reduce the electric double layer to promote protein-protein aggregation. As the degree of aggregation increases, higher temperatures are needed to promote denaturation (Keowmaneechai and McClements, 2002). Wu and Muir (2008) reported that napin and cruciferin denaturation temperatures (T_d) were 109.9ºC and 90.7ºC, respectively. Similarly, Yang et al. (2014) reported that CPI mainly consist of napin had higher denaturation temperature (pH 7; T_d = 104.71ºC) than CPI mainly consist of cruciferin (pH 7; T_d =88.95ºC). The main

reason for higher T_d in napin is presumed due to inter- and intra- disulfide bonds that stabilize napin structure (Schwenke et al., 1988).

2.8 Fractal aggregation

Fractal aggregates, unlike simple aggregation models includes the characteristic of being vulnerable to fragmentation and alteration with environmental changes (e.g., temperature and shear) (Meakin and Jullien, 1988). In contrast, simple aggregation models assume that cluster permanently and firmly attach to each other when they first interact (Meakin and Jullien, 1988). When considering the fractal nature of proteins, two types of aggregation dominate: diffusion-limited and rate-limited cluster-cluster aggregation. Cluster-cluster aggregation refers to scattering proteins within solution that randomly orient to form a larger cluster (Jullien and Kolb, 1984; Ikeda et al., 1999). Aggregates form via a 2-step process involving weak and strong linkages. In the initial step, proteins (small clusters) interact with each other via weak bonds, such as van der Waals interactions. Where, diffusing particles stick to other particles in a random way with probability *p* (Ikeda et al., 1999). In the second step, stronger bonds form via ionic interactions, covalent bonds and hydrophobic interactions to hold the larger clusters together (Meakin and Jullien, 1988). Diffusion-limited cluster-cluster aggregation (DLCA) forms aggregates rapidly and it is limited by the diffusion of aggregate particles (Vreeker et al., 1992). DLCA grows linear or 'string of beads' in structure ($p \sim 1$) where reaction-limited cluster-cluster aggregation (RLCA) grows coarse and open in structure ($p < 1$) (Ikeda et al., 1999). RLCA forms aggregates slowly due to electrostatic repulsion (Vreeker et al., 1992). This favors the particulate type aggregation where particles weakly interact with more than one particle at a time. The D_f values that correlate with DLCA and RLCA are 1.7 - 1.8 and 2.0 –2.2, respectively (Ikeda et al, 1999; Meakin and Jullien, 1988). Ikeda et al. (1999) indicated that with increasing in NaCl concentration from 25 to 500 mM NaCl, D_f was reduced from ~2.2 to ~1.8. Figure 2.1 depicts the theoretical growth of protein forming diffusion-limited cluster-cluster aggregation, where Figure 2.1A gives the protein molecules in a solvent randomly moving. The black protein molecules in Figure 2.1A show the initial stage of protein aggregation, where formation of clusters occurs by protein particles interacting and orienting with each other to form aggregate (Meakin and Jullien, 1988). The Figure 2.1B indicates the branching of the network as the protein aggregates become larger. The clusters stick to the starting aggregates and this creates larger flocs (Doi, 1993;

Marangoni et al., 2000). The gradual change in colour in Figure 2.1C indicates that the network grows in a similar manner as it grows in size, where it can be characterized by D_f (Hagiwara et al., 1998). The Figure 2.2 show the RLCA, which shows greater number of particles weakly interacting with neighboring particles compared to DLCA. Like Figure 2.1C, gradual change in colour indicates the fractal growth; as fractal flocs aggregate it produce the gel network.

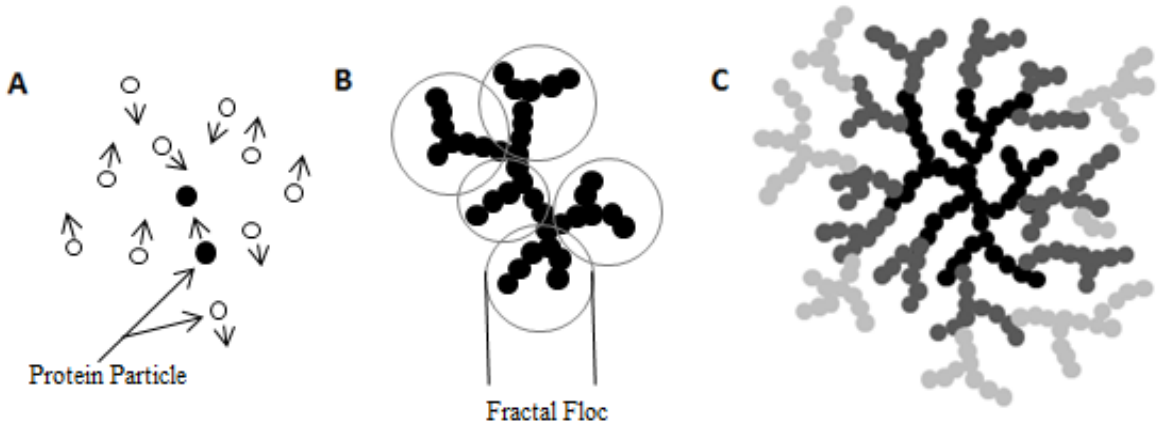


Figure 2.1. Growth of Diffusion-limited cluster-cluster aggregations (Marangoni et al., 2000; Markossian et al., 2009)

The 'string of beads' growth of the protein give arises to DLCA structure of the protein. The 'string of beads' polymer can be seen in heat-set protein gels, where factors such as pH, ionic strength and temperature play important role in development of ordered linear polymer (Doi, 1993). For instance, if the solution is away from pI and/or is low in ionic strength the protein repulse each other due to high surface charge and forms ordered linear and branched-type aggregates (Doi, 1993). In contrast, proteins that are close to isoelectric point and/or has high ionic strength in the solution the partly denatured protein forms random or 'particulate-type' aggregates (Doi, 1993). Thus, relating the D_f values to the type of aggregate aids the understanding of gel structure.

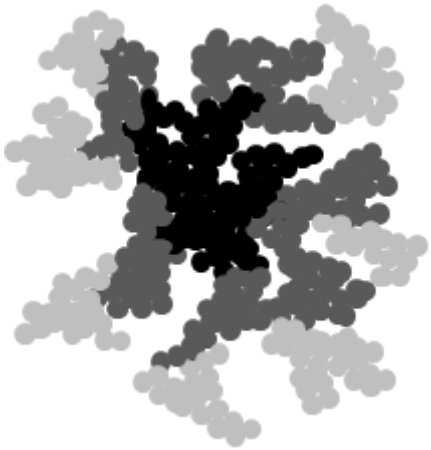


Figure 2.2. Reaction-limited cluster-cluster aggregations (Markossian et al., 2009)

2.8.1 Fractal analysis

Fractal dimension (D_f) of protein gels can be measured using a wide range of techniques such as light scattering, x-ray/neutron scattering, gel permeability, rheology and microscopy (Feder and Jossnag, 1984; Hagiwara et al., 1997 Marangoni et al., 2000; Enright and Leitner, 2005; Dàvila and Parés, 2007). Although, light scattering is the most widely used analysis, it is limiting in concentrated systems where gelation occurs (Ikeda et al., 1999). The most direct way for evaluating the D_f in more concentrated systems/gels is through microscopic image analysis, however sample preparation can become a limiting factor (Ikeda et al., 1999; Bi et al., 2013). In self-supporting gels, large deformation testing is also often performed using gels prepared from multiple protein concentrations and then scaling rules are applied in determining the D_f (Hagiwara et al., 1997; Marangoni et al., 2000; Özkan et al., 2006).

The D_f value varies from protein types and conditions, but generally ranges between 1.5 and 2.8 (Hagiwara et al., 1997; Ikeda et al., 1999; Eleya et al,, 2004; Nagano and Tokita, 2011). Hagiwara et al. (1997) used both confocal laser scanning microscopy (CLSM) and rheology methods to determine the D_f of BSA and β-lactoglobulin gels to range between 2.00- 2.82 depending on the salt conditions. The addition of 30 mM $CaCl_2$ was found to increase the D_f from ~2.00 to 2.82 for BSA, and from ~2.14 to 2.69 for β-lactoglobulin gels. Hagiwara et al. (1997) concluded both methods yielded similar results when determining D_f. Bi et al. (2013) determined the D_f of acid-induced soy protein isolate gel as a function of ionic strength (0 – 800 mM NaCl)

using the CLSM box counting method and large deformation testing to find values to range from 2.53 to 2.73. Vreeker et al. (1992) studied the D_f of whey protein isolate gels with higher concentration of NaCl, where values for D_f decreased from 2.2 to 1.7 as the NaCl levels increased from 0.16 to 0.29 M. The authors concluded that protein aggregates shifted from reaction-limited cluster-cluster aggregation to diffusion-limited cluster-cluster aggregation due to a reduction in electrostatic repulsion within the gel. Thus, fractal analysis is an important tool that allows understanding of gel structures and growth.

In addition to D_f values for gels, microscopy images can also be used to measure the gels lacunarity which describes the distributions of pores within the network (Dàvila and Parés, 2007; Karperien, 2012). Dàvila and Parés (2007) reported that with increasing values of D_f there was a decrease in heterogeneity of void spaces within the network for blood plasma gels. The authors reported that increase D_f associated with higher packing of the gels resulted in reduced pore size which increased texture property such as hardness and springiness (Dàvila and Parés, 2007).

2.9 Summary

Overall, canola proteins have tremendous potential as an alternative plant protein ingredient to rival soy because of its nutritional value and functionality. And although it has not been launched into the food industry, research surrounding its use is on the rise. However, in order to support its increased utilization, a greater understanding of structure-function relationships is needed under different environmental and solvent conditions. In the present study, mechanisms driving structure-function relationships involved with canola and soy protein gelation will be investigated.

3. MATERIALS AND METHODS

3.1 Materials

Defatted canola meal produced from *Brassica napus* L. (2012 crop year) was kindly donated by Agriculture and Agri-Food Canada (Saskatoon, SK, Canada) after being processed by POS BioSciences Corp. (Saskatoon, SK, Canada). The meal served as the starting material for protein extraction. A commercial soy protein isolate product was kindly donated by Archer Daniels Midland Company (PRO-FAM 974, Lot 13020412, Decatur, IL, USA) for this project. All chemicals used in this study, unless otherwise stated were purchased from Sigma-Aldrich (Oakville, ON, Canada). Water used in this study was Milli-Q^{TM} water (EMD Millipore, Billerica, MA, USA).

3.2 Preparation of canola protein isolates

Canola protein isolate (CPI) was prepared from defatted meal using slightly modified methods of Folawiyo and Apenten (1996), and Klassen et al. (2011). In brief, 0.05 M Tris-NaCl buffer (Lot 103470, Fisher Scientific, Fair Lawn, New Jersey, USA) containing 0.1M NaCl was prepared and adjusted to pH 7.0 using 1.0 N (HCl). The prepared buffer was then used to dissolve the defatted meal at a meal-to-buffer ratio of 1:10 for 2 h at room temperature (22-23ºC) under constant stirring (500 rpm) using a mechanical stir plate. The dispersion was then centrifuged (Sorvall RC Plus Superspeed Centrifuge, Thermo Fisher Scientific, Asheville NC, USA) at 3000 × *g* for 1 h to collect the supernatant, followed by a second centrifuge step after removal of the pellet (3000 × *g* for 1 h) to further clarification. The supernatant was then vacuum filtered using a #1 Whatman filter paper (Whatman International Ltd., Maidston, UK), dialyzed (Spectro/Por tubing, 6-8 kDa cut off, Spectrum Medical Industries, Inc, Rancho Dominguez, CA USA) at 4ºC where Milli-Q^{TM} water was changed 3 times a day for 72 h to remove the salt, and then freeze-dried (Labconco Corporation, Kansas City, MO, USA) to produce a dry CPI powder. The powder was stored at 4ºC for later usage.

3.3 Proximate composition

AOAC method 960.39 (1990) was used to measure the crude fat levels within the canola meal, CPI and SPI. About 3.0 g of defatted canola meal, CPI and SPI samples were used for the extraction using a Labconco Goldfisch fat extractor apparatus with petroleum ether. AOAC method 925.10 (2003) was used to measure the moisture content of the defatted canola meal, CPI and SPI, where ~2-3 g of sample was weighed using an analytical balance (Sartorius, USA) into pre-weighted aluminum pans. These were then dried within an oven at 102ºC for 18 h. After heating, the samples were placed in the desiccator to cool, and then re-weighed to determine the moisture lost. Ash content was determined according to AOAC Method 923.03 (2003), whereby 0.5 g of sample was weighed into pre-dried crucibles, and then placed within a muffle furnace (Fisher Scientific Isotemp Basic Muffle Furnace, Iowa, USA) at 600ºC for 18 h. After heating, the samples were placed in the desiccator to cool, and then reweighed to determine the ash content. Protein content determined by AOAC method 920.87 (2003). In brief, 0.05 g of sample was used to perform micro-Kjeldahl digestion and distillation (Labconco®65000 Rapid distillation apparatus, Kansas City, MO, USA), where glycine was used as a standard. In brief, the samples were digested using sulfuric acid, a catalyst (e.g., K_2SO_4 and $CuSO_4$) and heat. After digestion was complete, the samples were distilled, followed by a back titration using 0.02N HCl and N-indicator. A nitrogen conversion factor of 6.25 was used to determine the percent protein for all samples. The proximate analysis was conducted in triplicate.

3.4 Amino acid composition

The amino acids profile was determined by POS (POS BioSciences Crop., Saskatoon, SK, Canada). High performance chromatography (HPLC) and pico-tab amino acid analysis system (Waters Corporation, Milford, MA, USA) was used to analyze the amino acid profile of CPI and SPI. In brief, it followed method developed by Bidlingmeyer et al. (1987), where 15 mL of 6 N HCl was added to the CPI and SPI samples to hydrolyze the protein before HPLC separation. AOAC official methods 985.28 (AOAC, 2003) was used to determine the sulfur-containing amino acid where cysteine and methionine was oxidize with 10 mL of cold performic acid before hydrolysis of protein. AOAC method 988.15 (AOAC, 2003) was used to examine the amount of tryptophan where tryptophan was hydrolyzed with 10.0 mL of 4.2 M NaOH prior to HPLC analysis.

3.5 Differential scanning calorimetry

The thermodynamic properties of a 9.0% (w/w) CPI gel network were investigated using differential scanning calorimetry (DSC) as a function of pH (5.0, 7.0 and 9.0). CPI solutions that contain 9.0% (w/w) CPI were used instead of 7.0% CPI as the enthalpy of transition was greater providing more accurate analysis. Gel samples of approximately 10 mg were weighted into Tzero Alodined pans and hermetically sealed (TA Instruments, New Castle, DE, USA). Samples were heated at 5ºC/min from 25 to 110ºC using a Q2000 DSC (TA Instruments, New Castle, DE, USA). The instrument was calibrated using indium. From the heating curve, the onset temperature, denaturation temperature and the enthalpy associated with the denaturation were determined. Samples were measured in triplicate and reported as a mean ± one standard deviation. CPI at pH 3.0 was non-gelling and therefore was not tested, whereas exothermic events associated with soy proteins could not be detected by the instrument.

3.6 Surface charge (zeta potential)

Overall surface charge of CPI and SPI was determined by measuring the electrophoretic mobility (U_E) of 0.05% (w/w) protein solutions as a function of pH (2.0-9.5) using a Zetasizer Nano-ZS90 (Malvern Instruments, Westborough, MA, USA). Zeta potential (ζ) is calculated by applying U_E to the Henry's equation:

$$U_E = \frac{2\varepsilon\zeta f(\kappa\alpha)}{3\eta} \qquad \text{(eq. 3.1)}$$

where ε is permittivity, f(κα) is a function related to the ratio of particle radius (α) and Debye length (κ), and η is the dispersion viscosity. A Smoluchowski approximation f(κα) of 1.5 was assumed for this study, as is convention when using a folded capillary cell, and with samples of particles sizes larger than 0.2 μm dispersed in a moderate electrolyte solution (> 1mM). The Smoluchowski approximation assumes that: a) the concentration of particles (proteins) is sufficiently high such that such thickness of the electric double layer (Debye length) is small relative to the particle size (κα>>1); and b) ζ is linear related to U_E. All measurements were reported as the mean ± on standard deviation (n = 3).

3.7 Rheological properties of canola protein isolate and soy protein isolate solutions

All rheological measurements were made using an AR-1000 rheometer (TA Instrument, New Castle, DE, USA) equipped with a peltier plate temperature control, and a 40 mm diameter - 2º cone and plate geometry (with a gap of 51 μm). Each protein solution (~630 μL) was transferred onto the geometry, and allowed to equilibrate for 5 min prior to analysis. To prevent sample drying during heating, a light application of mineral oil was placed on the fringe of the geometry. The viscoelastic storage (G′) and loss (G″) moduli was initially followed during a heating-cooling cycle for each sample. Temperature was ramped upwards from 25ºC to 95ºC on a continuous basis at a rate of 1ºC/min, a frequency of 0.1 Hz and strain amplitude of 1%. The sample was then allowed to equilibrate at 95ºC for 5 min, and then ramped downwards from 95ºC to 25ºC at the same rate. The G′ was plotted vs. temperature on arithmetic coordinate to determine the heat setting temperature (or sol-gel transition temperature; or gelation temperature), taken by extending the tangent from the steepest part of the rise in G′ to the x-axis in the heating curve (Winter & Chambon, 1986; Rogers and Kim, 2011). Following the temperature cooling ramp, the sample was allowed to equilibrate at 25ºC for 1 min, followed by a time sweep measurement of G′ for 1 h at a frequency of 0.1 Hz and strain amplitude of 1% to evaluate the level of structure formation over time. Once completed, both G′ and G″ was measured as a function of frequency over the range of 0.01 and 100 Hz at strain amplitude of 1%, and plotted on log-log coordinates to give an indication of whether the sample is behaving as a viscous fluid, entangled solution or semi-solid gel. The magnitude of moduli was also given an indication of the relative strength of the structures being formed (or the level of order within the network. After the frequency scans, a strain sweep was performed over a strain range of 0.014% to 500% at a frequency of 5 Hz. The strain sweep provided information relating to the relative strength of junction zones formed within the material, and their relative resistance to flow. The strain break was measured by extending the tangents for data before and after the break. The intersection point was taken as the % strain at break. All measurements were made within the linear viscoelastic regime. All samples were prepared in duplicate.

The rheological properties of CPI and SPI solutions were examined under the following sample conditions. (a) Initially, the rheological properties of SPI solutions were examined as a function protein concentration (5.0, 6.0, 7.0, 8.0 and 9.0% w/w) at pH 7.0, followed by CPI at

protein levels of 5.0, 7.0 and 9.0% (w/w) at the same pH. Canola and SPI was prepared by dispersing their respective powders (adjusted for protein levels) into 0.1 M NaCl prepared with Milli-Q water (Millipore Corporation, MA, USA), and was then stirred using a mechanical stir plate at 500 rpm for 1 h at room temperature (22-23ºC). The pH of the solution was adjusted to 7.0 using 0.5 M NaOH or HCl, and periodically checked during stirring. (b) Secondly, the rheological properties for a 7% (w/w) CPI or SPI solution at pH 7.0 were examined as a function of NaCl (0.1 and 0.5 M NaCl), urea (0.1. 0.5, 1.0 and 5.0 M) and mercaptoethanol (0.1% and 1%) levels to test the nature of interactions within during gel formation.

In study 2, the rheological properties of CPI and SPI solutions were examined using a 7.0% (w/w) CPI or SPI protein concentration at pH 3.0, 5.0, 7.0 and 9.0. The pH of the solutions were adjusted to appropriate pH using 0.5 M NaOH or HCl, and periodically checked during stirring.

3.8 Confocal laser scanning microscopy of canola protein network

The morphology of CPI and SPI networks was examined using a Nikon Eclipse LV100 Confocal Laser Scanning Microscopy (Nikon, Tokyo, Japan). CPI and SPI gels were prepared as a function protein concentration (5.0, 7.0 and 9.0%, w/w) at pH 7.0. The gels were made by dispersing their respective powders (adjusted for protein levels) into 0.1 M NaCl prepared with Milli-Q water (Millipore Corporation, MA, USA), and then stirred using a mechanical stir plate at 500 rpm for 1 h at room temperature (22-23ºC). After 1 h of stirring, 10 μL of 1% Rhodamine B Isothyocyanate (RITC) in methanol solution was added to the CPI solutions, followed by stirring for an additional 1 h using a mechanical stirrer (500 rpm) at room temperature. The solution was then covered with aluminum foil to prevent light from reacting with the RITC dye. The solution was transferred to 0.5 mm-deep well concavity slide and was closed with a cover slip. The slides were carefully transferred to either an AR-1000 or AR-G2 rheometer (TA Instrument, New Castle, DE, USA), where they were placed on top of the peltier plate temperature control. The slides were also covered with aluminum foil. Temperature was ramped upwards from 25ºC to 95ºC at a rate of 1ºC/min, allowed to equilibrate at 95ºC for 5 min, and then ramped downwards from 95ºC to 25ºC and then held at 25ºC for 1 h to mimic the rheological heating/cooling profile Excitation and emission wavelengths were at 543 and 573 nm, respectively. Gel morphology images were captured from a depth close to the midpoint of the

concave slide. All gels were prepared in triplicate and 3 images per slide were taken. A representative image from each slide was used for further analysis.

In study 2, the morphology of CPI and SPI gel networks at 7.0% (w/w) were examined as a function of pH 3.0, 5.0, 7.0 and 9.0, as previously described.

3.8.1 Image analysis

Fractal dimension and lacunarity was measured using Image J v1.48 (http://imagej.nih.gov/ij/) software. The FracLac V2.5 plug-in for Image J was used to convert the images from the confocal laser scanning microscopy to binary images. The white pixels represented the gel network whereas the dark areas represented aqueous solution. Furthermore, FracLac V2.5 was used for a box counting method to measure both the fractal dimension and lacunarity. The box counting method places a series of grids of decreasing in size over an image and counting the boxes that contain foreground pixels (e.g., white pixels) for each grid size. Fractal dimension (D_f) was calculated as $D_f = -d+1$, where d is the slope of the line from a plot of log ($N\varepsilon$) versus log (ε) (Hagiwara et al., 1997; Dàvila and Parés, 2007). Where in FracLac, ε is the corresponding scale (ε = box size / image size) and $N\varepsilon$ is the number of boxes containing foreground pixels in the grid at a certain scale. Lacunarity (λ_ε) is the variation of the number of foreground pixels at each grid box. This indicates distribution of the heterogeneity or a gap in the gel network. FraLac calculated lacunarity by the equation:

$$\lambda_\varepsilon = (\sigma/\mu)^2 \qquad \text{(eq. 3.2)}$$

where σ is the standard deviation in pixel density within all box sizes ε and the average number μ of foreground pixels per box for the same grid size.

3.9 Statistics

In study 1, a one-way analysis of variance (one-way ANOVA) was used to test for statistical differences between concentration in terms of sol-gel transition temperatures, the magnitude of G′ and G″ (at the end of the time sweep) and % strain at break for both CPI and SPI. A Tukey's honest significant difference Post-Hoc test was used to test for differences among the aforementioned parameters for each of CPI and SPI as a function of concentration. A student

T-test was also used to test for differences in the aforementioned parameters for gels in the absence and presence of urea, NaCl and mercaptoethanol. Finally, a one-way ANOVA with a Tukey's honest significant difference Post-Hoc test was used to test for significance for CPI (only) as a function of protein concentration for its thermal characteristics (e.g., onset and denaturation temperatures, and enthalpy), fractal dimension and lacunarity. The latter was not tested in the case of SPI since data was not collected (*See Results and Discussion*). In study 2, similar statistics were applied except as a function of pH rather than concentration. All experiment data was reported as the mean ± one standard deviation. Data was analyzed by R program software (Version 2.15.2, R Foundation, Vienna, Austria).

4. RESULTS AND DISCUSSION

4.1 The effect of protein concentration and the nature of interactions on the gelling properties of canola and soy protein isolates

4.1.1 Characterization of the canola meal, canola protein isolate and the commercial soy protein isolates

The proximate composition of the defatted canola meal obtained from AAFC/POS BioSciences indicated that residual crude fat levels were at ~3.1% (d.b), which is typical for industrial processes after oil extraction (Table 4.1). Protein levels were ~42% (d.b.) (Table 4.1). Similar protein content was reported by Klockeman et al. (1997) for defatted hexane canola meal from CanAmera Foods, Inc. (Oakville, ON, Canada), and by Bell & Keith (1991) for another commercial canola meal product. Canola protein isolates were prepared using a salt extraction process to obtain protein levels of ~98% (d.b.) using the micro-Kjeldhal digestion-distillation setup (Table 4.1). Using the conversion factor of 6.25 to convert % nitrogen to % protein is presumed to be an overestimation of the true protein content. Kjeldhal measures the total nitrogen in the sample, which also includes nitrogen from protein, peptides and free amino acids (McKenzie and Wallace, 1954). The amino acid compositions of CPI and SPI are shown in Table 4.2. Both CPI and SPI had high levels of glutamic acid + glutamine (19.2% in CPI; 16.4% in SPI), arginine (6.00% in CPI; 6.95% in SPI), leucine (6.68% in CPI; 7.04% in SPI), and lysine (4.99% in CPI; 5.56% in SPI) (Table 4.2). Aider and Barbana (2011) also indicated that CPI had high levels of glutamic acid, arginine and leucine. However, there were some noticeable differences between CPI and SPI. The CPI (2.28%) had higher cysteine levels than SPI (0.90%); on the other hand SPI (9.60%) had higher aspartic acid + asparagine levels than CPI (5.21%) (Table 4.2). The cysteine content in CPI has been previously shown to vary depending on the oil extraction method (Aider and Barbana, 2011). In protein, cysteine plays an important role in the formation of disulfide bonds between neighbouring cysteine (Doi, 1993; Léger and Arntfield, 1993). Amino acids such as arginine, glutamic acid and lysine are charged at neutral pH which

gives rise to hydrogen bonding. Protein characteristics are highly influenced by amino acid profile, protein orientation, and solvent conditions.

Residual fat was removed prior during the hexane extraction process, allowing for better protein extraction. Moisture, ash and crude fat levels were also significantly reduced relative to the meal. The majority of proteins are presumed to be cruciferin proteins since they are the

Table 4.1 Proximate composition of canola meal, canola protein isolates (CPI) and a commercial soy protein isolates (SPI). Data represent the mean values ± one standard deviation (n = 3).

Material	Moisture (%)	Protein (%, d.b.)	Ash (%, d.b.)	Crude Fat (%, d.b.)
Canola meal	6.19 ± 0.04	42.43 ± 0.37	9.42 ± 0.06	3.08 ± 0.04
CPI	1.38 ± 0.06	98.23 ± 0.25	4.18 ± 0.04	1.12 ± 0.01
SPI	3.62 ± 0.01	95.20 ± 0.68	4.31 ± 0.04	0.41 ± 0.00

Table 4.2 Amino acid profiles of canola protein isolate and soy protein isolate.

	Canola protein isolate	Soy protein isolate
Amino acids	Amino acid content (%)	
Alanine	3.36	3.50
Arginine	6.00	6.95
Aspartic acid + Asparagine	5.21	9.60
Glutamic acid + Glutamine	19.2	16.4
Glycine	4.12	3.52
Proline	6.00	4.49
Serine	3.50	4.65
Histidine	3.14	2.67
Isoleucine	3.40	4.07
Leucine	6.68	7.04
Lysine	4.99	5.56
Methionine	1.81	1.10
Cysteine	2.28	0.90
Phenylalanine	3.86	4.90
Tyrosine	1.95	3.29
Threonine	2.97	3.44
Tryptophan	1.30	1.24
Valine	3.85	3.76

dominant salt-soluble globulin proteins; however contamination by napin (water soluble albumin) is thought to be present. Schatzki et al. (2014) reported cruciferin to napin ratios to range from 0.13 to 1.05, respectively, with variations arising from variety differences, growing conditions and processing. The extraction process gave an average isolate yield of 9.8 ± 0.6 (relative to the original raw material). Protein levels were similar to those reported by Chang and Nickerson (2013) and Cheung et al. (2014) who used a salt extraction procedure for extraction. The proximate composition of the commercial SPI product sample showed protein levels of ~95% (d.b.) with low levels of moisture, ash, and crude fat (Table 4.1).

Surface charge or zeta potential for CPI and SPI was determined with and without 0.1 M NaCl at pH 7.0. In the absence of NaCl, CPI and SPI were found to both carry a net negative charge of -20.2 ± 0.98 mV and -43.9 ± 2.62 mV, respectively. The more highly charged SPI may result in increased electrostatic repulsion between neighbouring proteins relative to CPI at the same protein concentration resulting in weaker networks once formed. For both proteins, the addition of 0.1 M NaCl resulted in a reduction in charge to -4.1 ± 0.21 mV and -13.2 ± 0.28 mV for CPI and SPI, respectively. The addition of NaCl acted to significantly reduce the magnitude of the protein's surface charge most likely due to a charge screening effect, where Na^+ and Cl^- ions acted to screen the negatively and positively charged sites on the protein's surface, effectively reducing the thickness of the electric double layer in the process (Keowmaneechai and McClements, 2002).

A differential scanning calorimeter (DSC) was used to measure the thermodynamic properties of CPI and SPI at pH 7.0 and a 9.0% concentration. The onset of denaturation (T_o), the denaturation temperature (T_d) (point where maximal denaturation occurs) and associated enthalpy was determined to be 78.6 ± 0.4ºC, 87.1 ± 0.8ºC and 0.51 ± 0.06 J/g, respectively for CPI. Salleh et al. (2002) and Wu and Muir (2008) reported denaturation temperatures of 86.6ºC and 83.9ºC associated with a cruciferin-rich isolates. During denaturation, hydrogen bonding becomes disrupted causing the quaternary and tertiary structures of the proteins to disassociate and unravel into their secondary structures. Above these temperatures, hydrophobic interactions can begin to dominate in part due to previously exposed hydrophobic sites and the formation of covalent disulfide bonds between neighboring cysteine residues (Doi, 1993). Enthalpy is equal to the energy released in a reaction, which in this case it is lower than other studies that studied DSC on CPI (Wu and Muir, 2008; Yang et al., 2014). In Wu and Muir (2008) study, CPI enthalpy was 1.5

J/g, where higher enthalpy values were noticed for cruciferin (12.5 J/g) and napin (15.9 J/g). The low thermal stability might be due to presence of protein and non-protein components, which could affect the thermal stability (Marcone et al., 1998). Diluted protein solutions are difficult to perform DSC measurement because of low energy and wide range of denaturation temperature, therefore using enthalpy to evaluate the molecular breakage is difficult.

In contrast, denaturation could not be measured using the DSC in the case of the SPI most likely since the values were below the sensitivity limits of the instrument. In the future, a micro-DSC should be explored as an alternative measuring system. Arntfield and Murray (1981) also reported that if denaturation has occurred previously, the no exothermic dips in the thermogram would be evident. It is possible that the commercial product may have undergone some level of denaturation during the production process. When comparing CPI and SPI, the lack of measurable values in SPI may indicate that the CPI proteins are more thermally stable.

4.1.2 Rheological properties of canola protein isolate during gelation

The rheological properties of CPI and SPI were followed first as a function of temperature, time, frequency and then strain as a function of protein concentration. In the case of CPI, little evidence of an elastic structure was observed until ~87-90ºC, in which a slight rise in G′ was evident (Figure 4.1A), becoming greater than G″ (not shown). Before this rise, CPI solutions behaved as a viscous liquid where G″ was found to be greater than G′ (not shown). This rise in G′, corresponded to CPI denaturation temperature (87ºC) where proteins began to unravel to expose hydrophobic moieties, followed by protein aggregation driven by hydrophobic interactions and the formation of disulfide bonds between neighbouring cysteine residues. The rise is also denoted as the gelation temperature (T_{gel}) and was found to be similar regardless of the protein concentration ($p>0.05$) (Table 4.3). G′ was greater at the 7.0% (w/w) concentration because due to higher protein packing and protein-protein association which lead to reduction of G″ and increase in G′ (Figure 4.1A). Upon cooling, formed CPI-CPI aggregate further associated as hydrogen bonds began to reform and the gel network became stronger (Léger & Arntfield, 1993). As temperatures lowered from 95ºC to 25ºC, the elastic component saw an exponential increase in magnitude (Figure 4.1B). This similar pattern was also seen in Léger & Arntfield who evaluated CPI rheological properties during a temperature ramp (1993). In the present study, the

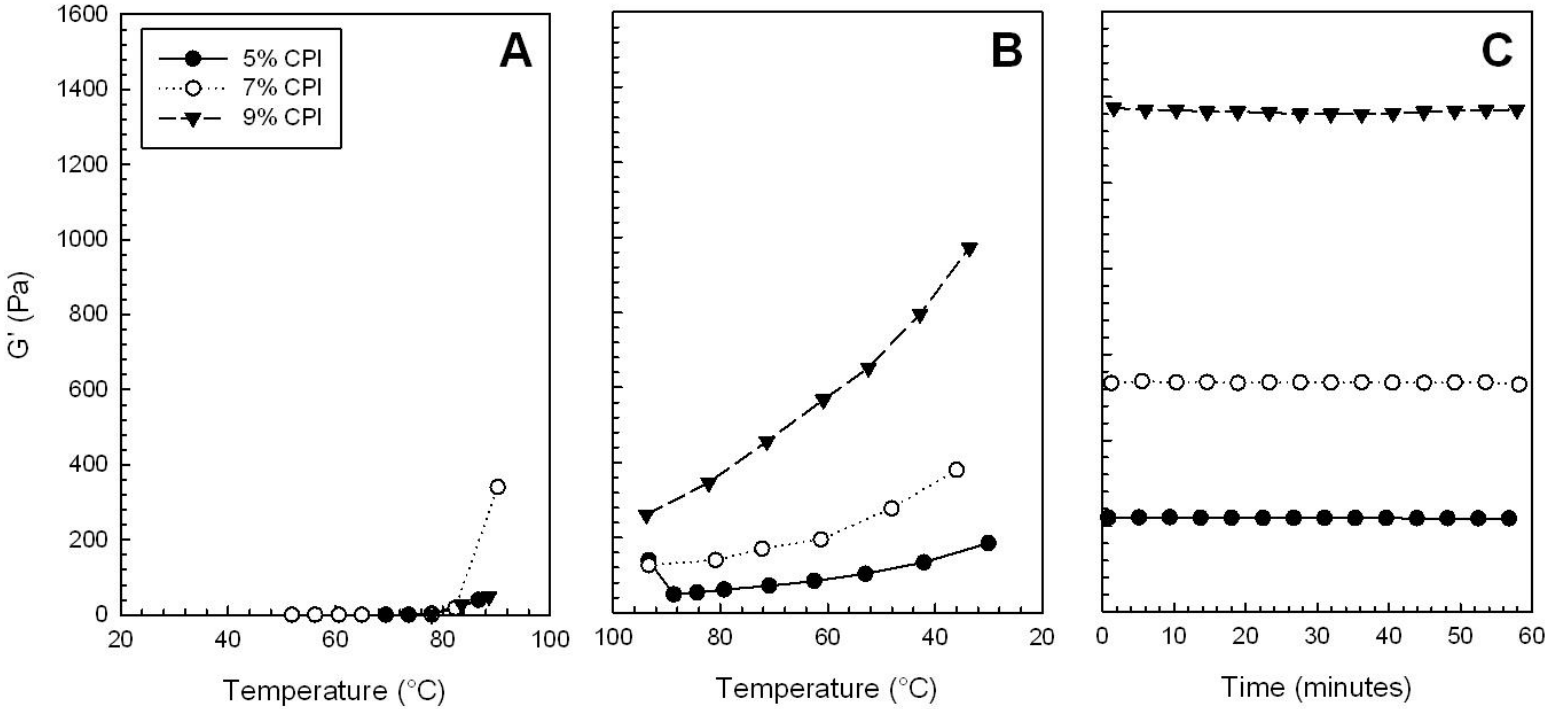


Figure 4.1 Dynamic storage (G′) modulus as a function of temperature and time for a canola protein isolate concentrations (5.0%, 7.0%, 9.0%) at 1% strain, 0.1 Hz and pH 7.0. a) temperature ramp from 25˚C to 95˚C; b) temperature ramp from 95˚C to 25˚C; c) 1 hour time sweep at 25˚C.

Table 4.3 The gelation temperature during heating (T_{gel}), log viscoelastic storage (G′) and loss (G″) moduli after the 1 h time sweep at 25ºC and pH 7.0, and the log % strain at break for canola and soy protein isolates as a function of protein concentration. Data represent the mean and standard deviation of duplicate samples. The abbreviation of n.g. denotes a material that is non-gelling.

Concentration (%, w/w)	**T_{gel} (^oC)**	**G′ (Pa)**	**G″ (Pa)**	**log % Strain at break**
a) Canola protein isolate				
5.0	90.0 ± 0.0^a	210.8 ± 10.0^c	26.8 ± 1.1^b	1.70 ± 0.0^b
7.0	87.0 ± 3.5^a	508.4 ± 31.2^b	61.8 ± 5.5^b	1.80 ± 0.0^a
9.0	87.4 ± 0.8^a	1222.0 ± 69.3^a	191.4 ± 23.8^a	1.78 ± 0.0^a
b) Soy protein isolate				
5.0	n.g.	n.g.	n.g.	n.g.
6.0	78.0 ± 2.8^a	8.6 ± 0.4^b	1.2 ± 0.0^b	1.60 ± 0.2^a
7.0	83.5 ± 4.9^a	29.1 ± 11.7^{a,b}	3.5 ± 1.3^{a,b}	1.55 ± 0.0^a
8.0	78.8 ± 2.3^a	43.5 ± 0.1^{a,b}	5.2 ± 1.3^a	1.51 ± 0.0^a
9.0	76.7 ± 6.6^a	48.6 ± 8.8^a	6.0 ± 1.0^a	1.54 ± 0.0^a

G′ was found to be greatest for the 9.0 % (w/w) concentration, followed by the 7.0% (w/w) and 5.0% (w/w) at the start of the time sweep upon the completion of the heating/cooling ramps, and remained relatively constant over the 1 h period suggesting no further ordering within the network structure was occurring (Figure 4.1C; Table 4.3). Gaps in magnitude between the end of the heating run and start of the cooling rate (Figure 4.1A, B) and the end of the cooling run and the start of the time sweep (Figure 4.1B, C) reflect protein ordering during the short rest period within the experimental protocol.

At the end of the time sweep, networks were found to increase in magnitude from 211 Pa to 1222 Pa as the CPI concentrations increased from 5.0 to 9.0 % (w/w) (Table 4.3). In all cases, G′ was greater than G″ (Table 4.3). The rise in network strength was thought to be caused by increased protein aggregation, compaction and junction zone formation within the network as the void volume decreased. It is also thought that the rate of hydrogen bond formation and break down was similar over time, as moduli remained constant.

Following 1 h time sweep, frequency sweeps of viscoelastic moduli on double logarithmic coordinates indicate characteristic gel-like material behavior where G′>G″ and the G′ is relatively independent of frequency (also known as the rubbery plateau of the viscoelastic spectrum (Ferry, 1980) (Figure 4.2). The crossover point of viscoelastic moduli at higher frequencies indicates that the material is entering the rubber-glass transition region of the viscoelastic spectrum. Within this region, mobility of proteins within the network is severely restricted to protein side chains or smaller molecules re-conforming to relieve stress by dissipating energy (Ferry, 1980). Frequency sweeps followed similar profiles, except the magnitude of moduli increased with increasing protein concentration as the material was presumed to have a greater amount of protein ordering and compaction (less free volume).

Following frequency sweep, a strain sweep was performed on all gels after to measure the relative strength of junction zones formed within the CPI and their resistance to flow. As shown in Figure 4.3, there was a sharp break in the log G′ versus log % strain suggesting the gel network was quite brittle in nature. For all CPI concentrations, G′ stayed relatively constant until it rapidly decrease, this area where sudden break occurs is where gel network breaks due to breaking of bonds within network (Eleya et al., 2004). The log % strain at break increased slightly from 1.70 to 1.80 (or 50 to 63 anti-logged) as CPI concentration increased from 5.0 to 7.0 % (w/w), then remained constant (Table 4.3, Figure 4.3). At the higher protein concentrations it was presumed that the network was stronger and capable of withstanding a higher amount of strain before a break in the network structure occurred, dissipating applied stress.

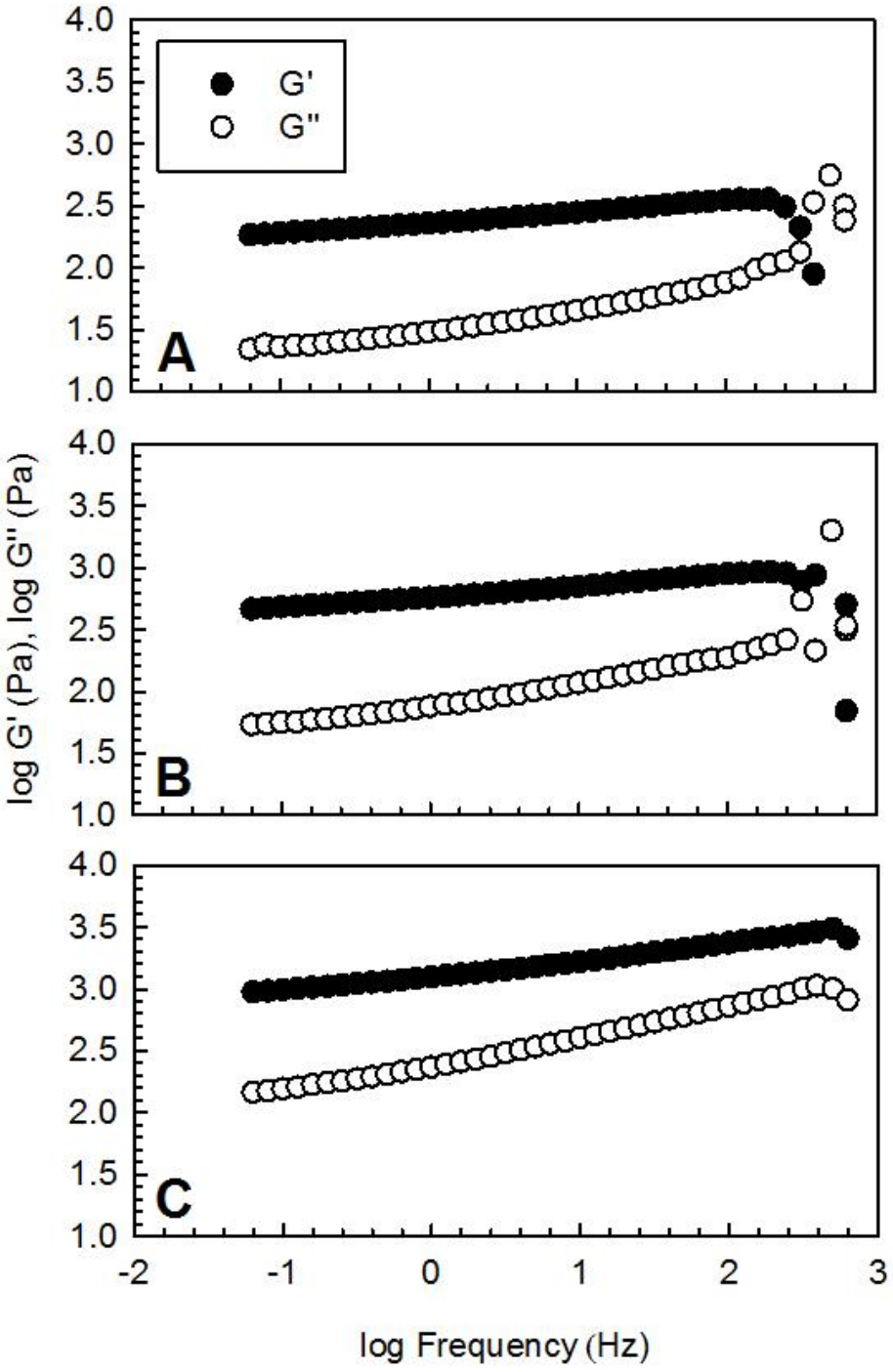


Figure 4.2 Dynamic storage (G′) and loss (G″) moduli as a function of frequency for a canola protein isolates at 5.0% (A), 7.0% (B) and 9.0% (C) protein concentrations at 1% strain. Frequency sweeps are continuation from temperature ramps and time sweep.

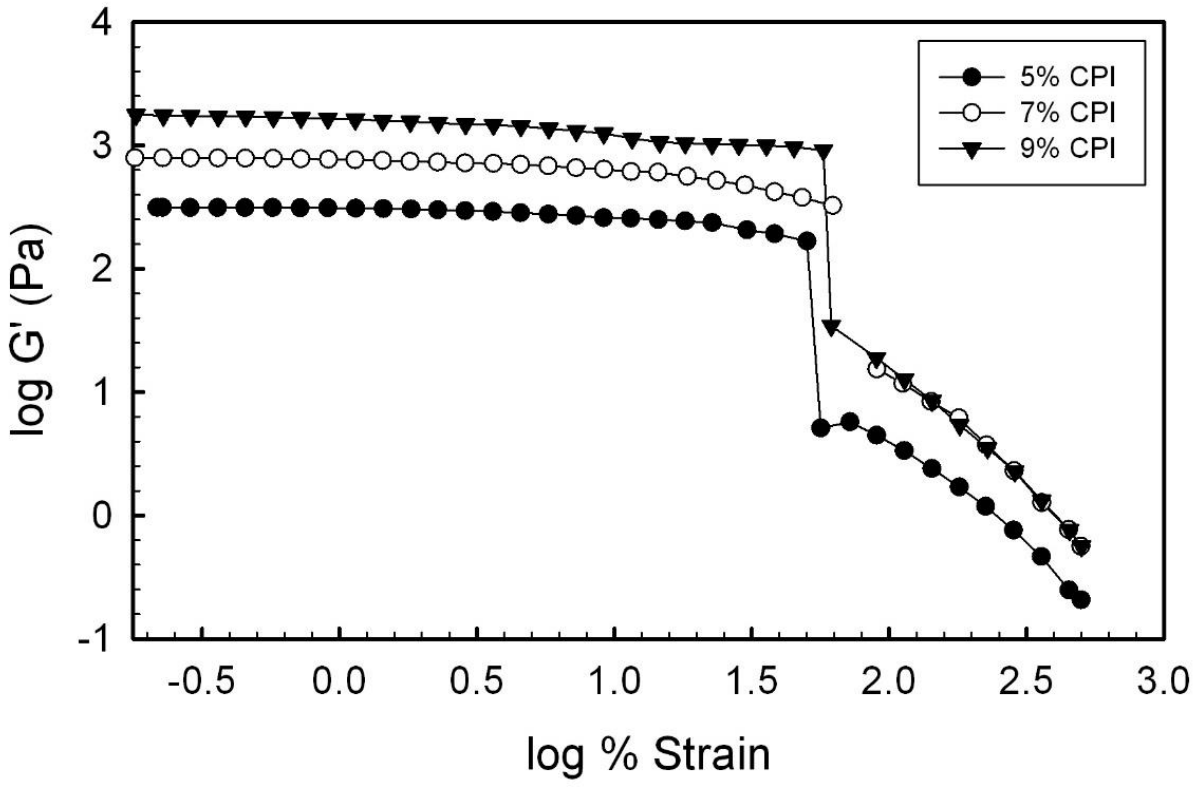


Figure 4.3 Dynamic storage (G′) modulus as a function of % strain for canola protein isolates at 5.0%, 7.0% and 9.0% (w/w) protein concentrations at 5 Hz. Strain sweeps are continuation from temperature ramps, time sweep and frequency sweep.

4.1.3 Rheological properties of soy protein isolate during gelation

The rheological properties of SPI were also followed first as a function of temperature, time, frequency and then strain as a function of protein concentration. Similar to the CPI, elastic-like behaviour was not seen until higher temperatures (> ~75ºC). The loss moduli were not shown, however at T_{gel}, G′ was greater than G″. The gelling temperature for SPI was all found to be similar in magnitude ranging between ~77 and 83ºC, which was typical for a heat setting protein network (Table 4.3). The 5.0% (w/w) SPI level did not result in network formation- due to insufficient protein concentration to form a solid three dimensional network that could retain liquid and to act as elastic material. Globular protein gels can be categorized into fine-stranded, mixed or particulate gel (Renard et al., 2006). Fine-stranded globular proteins have high electrostatic repulsion and formation of elementary subunits is low, due to low reactivity of the sulfhydryl groups (Renard et al., 2006). Where, mixed or particulate gel are formed by small

globular aggregate interact with other aggregate to establish fractal structures (Renard et al., 2006). Although the denaturation temperatures of the commercial SPI could not be measured in this study due instrument sensitivity, others have reported the denaturation of pure soy glycinin and conglycinin to be near 88ºC and 68ºC, respectively using micro-DSC (Renkema et al., 2000; Renkema and Vliet, 2002). The denaturation of mixed soy protein isolates have been shown to have two endothermic transitions, representing soy glycinin and conglycinin (Renkema et al., 2000). Depending on the pH, denaturation temperatures shift to lower temperature as pH becomes acidic (Renkema et al., 2000). After T_{gel}, G′ continued to rise at similar rates (independent of protein concentration), as the soy proteins unravelled on heating and then aggregated via hydrophobic interaction and then the formation of disulfide bridges (Figure 4.4A). Contrast to CPI, SPI further aggregated as temperatures were above 80ºC during the cooling scan (Figure 4.4B), showing greater structure formation (higher G′) than seen at the end of the heating scan. The greater magnitude possibly could be the result of a time delay to allow for proteins to re-orient being in a better orientation for form disulfide bridges. A similar profile was not found at higher temperatures during the cooling scan of CPI (Figure 4.1B) presumed to less covalent bonds being formed. Above cooling, a loss in strength occurred, followed by slight rise in G′ starting at temperatures <60ºC due to the reformation of hydrogen bonds (Figure 4.4B). In contrast to CPI, which saw significant increases in structure upon cooling, SPI remained relatively unchanged suggesting that the gel network formed was less dependent upon hydrogen bonding for stability. Similar to CPI, SPI gels remained relatively constant over a 1 h duration at 25ºC suggesting the gel structures were not changing (Figure 4.4C). G′ at the end of the time sweep was found to increase from ~8.6 Pa to ~48.6 Pa as the concentration increased from 6.0% (w/w) to 9.0% (w/w) (Table 4.3). In all cases, G′>G″ except for the 5.0% (w/w) protein concentration where G′<G″ (Table 4.3). SPI networks were also found to have significantly reduced gel strength relative to the CPI networks (Table 4.3).

Following time sweeps, frequency sweeps of viscoelastic moduli for a 5.0% and 9.0% (w/w) SPI material after the time sweep is shown in Figure 4.5. The 5.0% (w/w) plot indicates that the SPI is behaving as a liquid within the flow region of the viscoelastic spectrum where moduli change rapidly as a function of frequency, and G′<G″ (Figure 4.5A). Within this region, protein mobility is great, and protein-protein interactions are not sufficient for

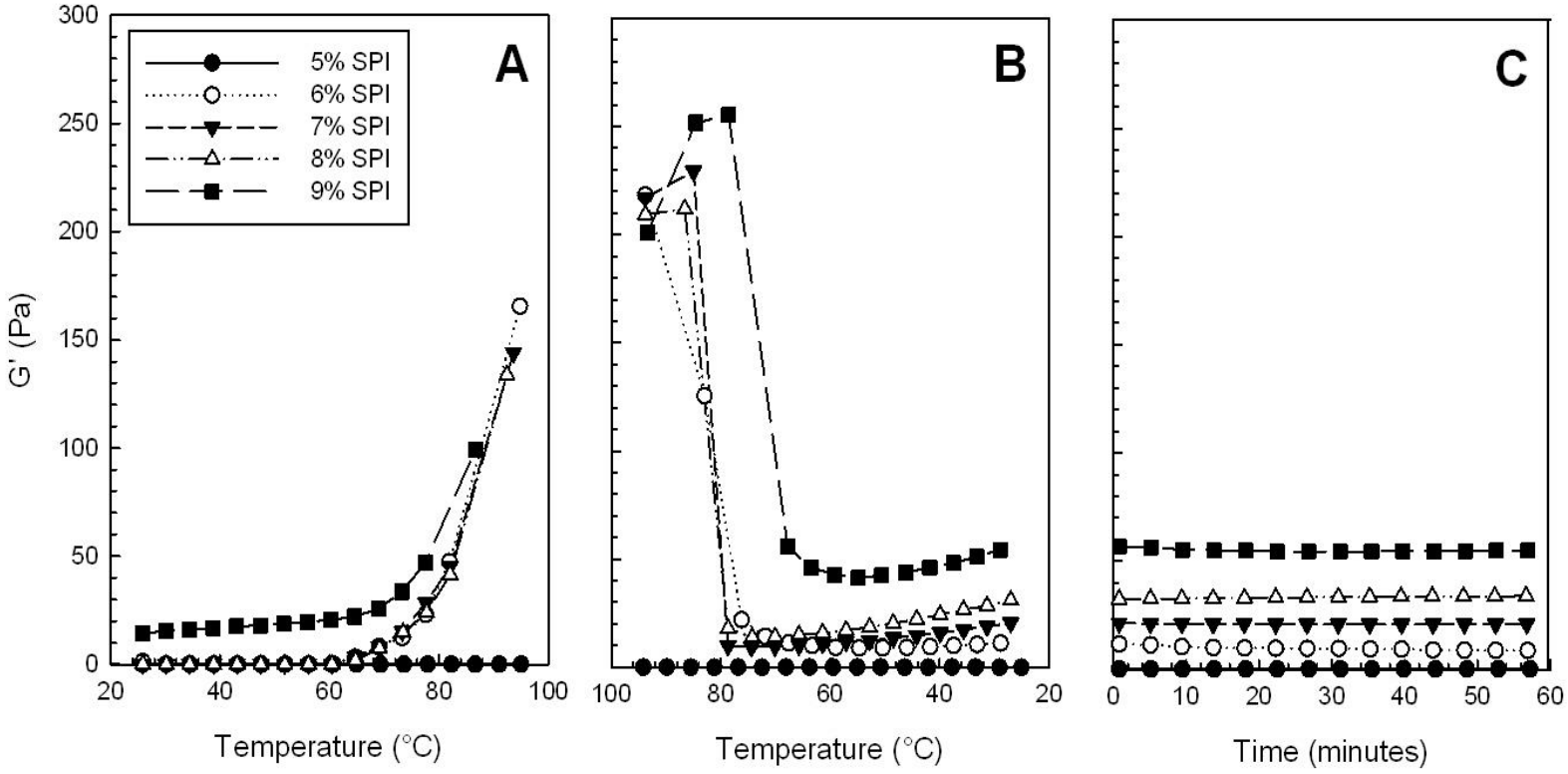


Figure 4.4 Dynamic storage (G′) modulus as a function of temperature and time for a soy protein isolate concentrations (5.0%, 6.0%, 7.0%, 8.0% and 9.0%) at 1% strain, 0.1 Hz and pH 7.0. a) temperature ramp from 25ºC to 95ºC; b) temperature ramp from 95ºC to 25ºC; c) 1 hour time sweep at 25ºC.

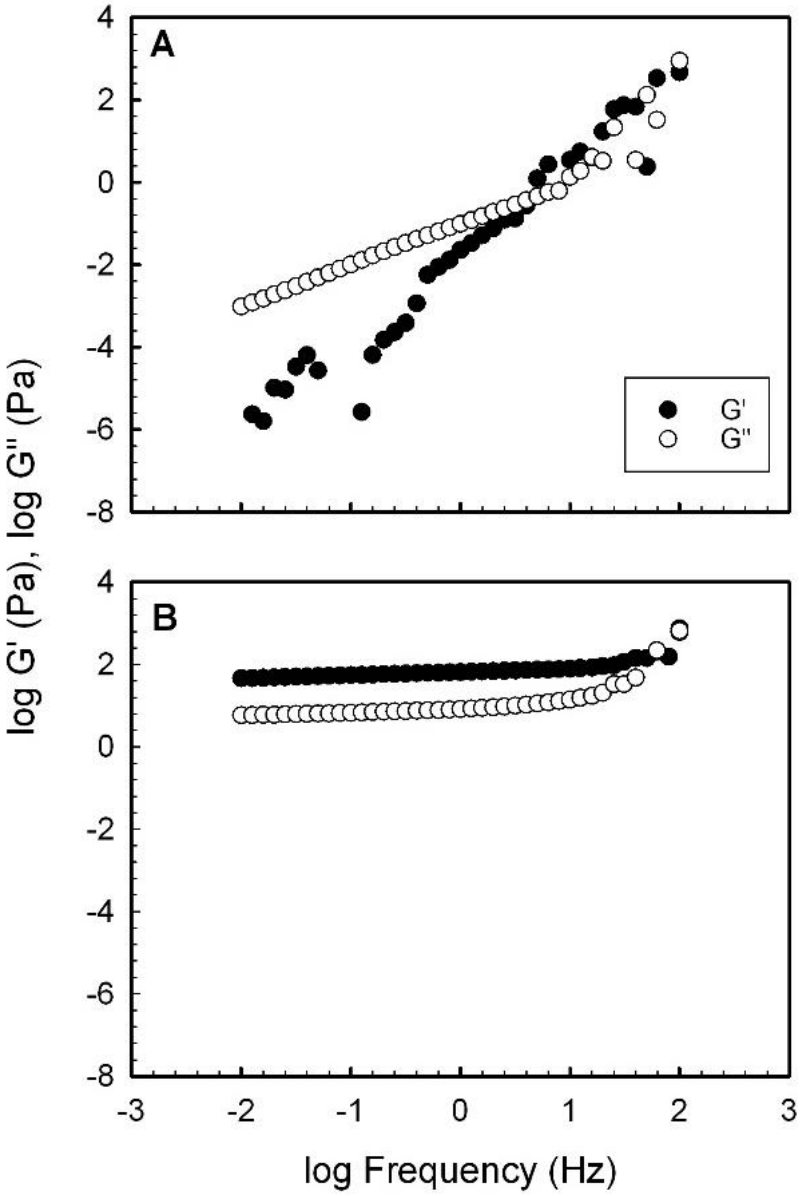


Figure 4.5 Dynamic storage (G′) and loss (G″) moduli as a function of frequency for soy protein isolates at 5.0% (A) and 9.0% (B) protein concentrations at 1% strain. Frequency sweeps are continuation from temperature ramps and time sweep.

start forming network structures. Profiles were similar for concentrations between 6.0 and 9.0% (w/w) with only minor differences in magnitude. Therefore only the frequency sweep for the 9.0% (w/w) SPI concentration was given (Figure 4.5B). The profile suggest a gel network is formed, as evident by frequency independence of moduli within the rubber plateau region of the

viscoelastic spectrum and G′>G″ (Figure 4.5B). Similar to CPI, moduli entered the rubber-glass transition region at higher frequencies.

Following frequency sweeps, strain sweeps were also carried out at all SPI concentration to determine the % strain at break (Figure 4.6). In contrast, to CPI a more gradual break was evident suggesting the network was more rubbery in nature than brittle, and that junction zones within the SPI network were most likely weaker than the CPI gels. For all concentrations, the % strain at break was similar at 1.55 (35.5 anti-log) (Table 4.3).

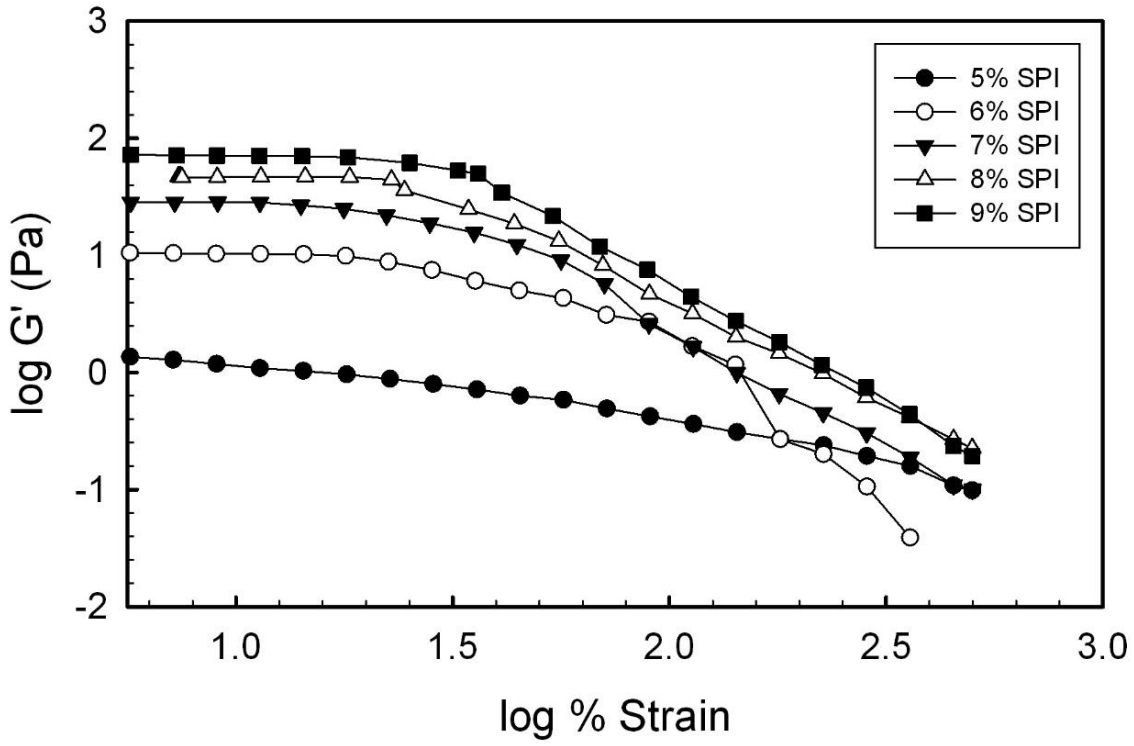


Figure 4.6 Dynamic storage (G′) modulus as a function of % strain for soy protein isolates at 5.0%, 6.0%, 7.0%, 8.0% and 9.0% (w/w) protein concentrations at 5 Hz. Strain sweeps are continuation from temperature ramps, time sweep and frequency sweep.

4.1.4 The nature of interactions within canola and soy protein gel networks

Rheological testing was done for CPI and SPI as a function of temperature, time, frequency and strain at a protein concentration of 7.0% (w/w) in the presence of NaCl (0.1 and 0.5 M), urea (0.1, 0.5, 1 and 5 M) and 2-mercaptoethanol (0.1 and 2%). In the case of CPI, a

similar temperature profile was evident (not shown) as to Figure 4.1 for samples with NaCl and Urea (0.1 – 1 M), with some minor reduction in magnitude. T_{gel} values were also similar to those reported earlier (~86.0 - 90.2ºC). The addition of 5 M urea resulted in no gel formation, whereas the addition of 2-mercaptoethanol reduced the strength of formed networks considerably. Figure 4.7 gives the G′ values after the 1 h time sweep. The addition of NaCl at the levels used (<0.5 M) had little effect on network strength, despite its ability to reduce the electrostatic double layer and

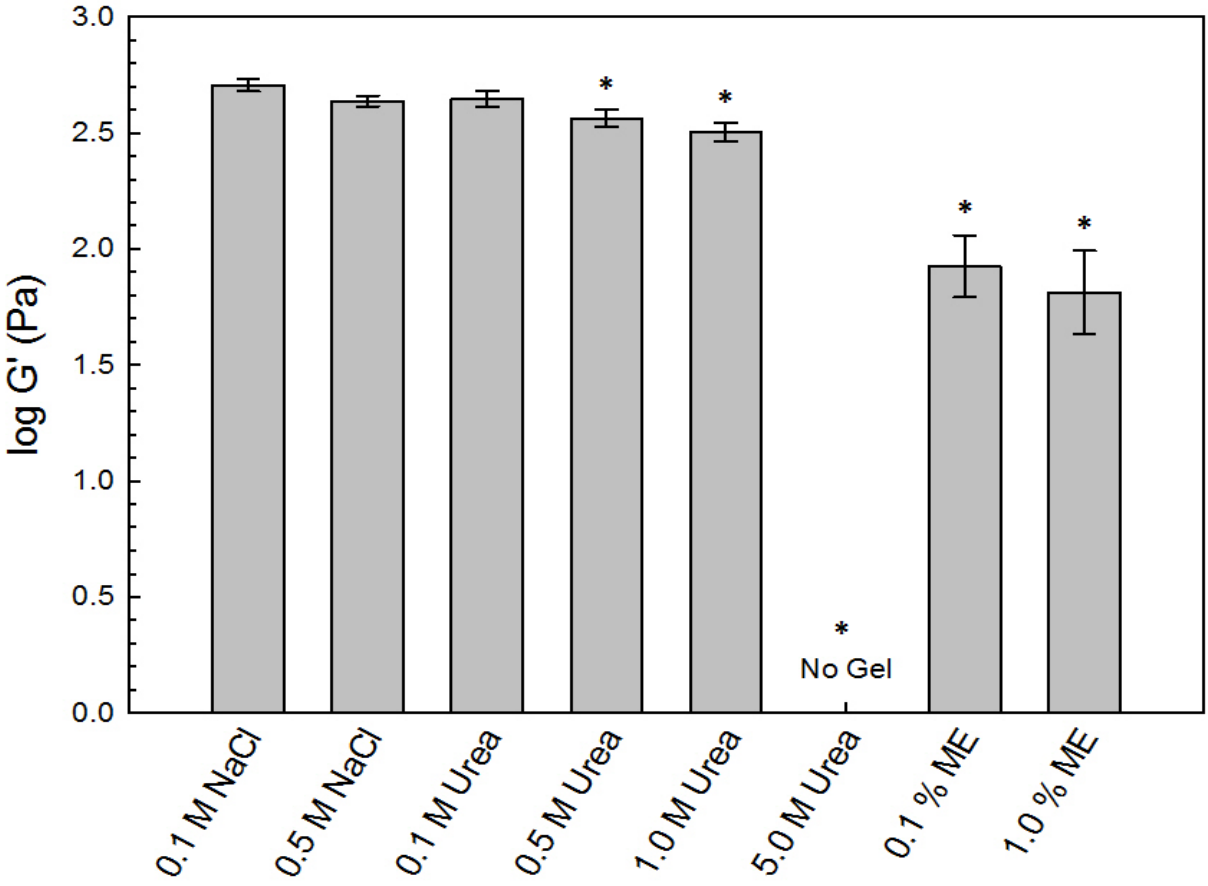


Figure 4.7 Dynamic storage (G′) modulus at the end of 1 h time sweep at 25ºC for canola protein isolate networks (7.0% w/w) as a function of NaCl (0.1 and 0.5 M), urea (0.1, 0.5, 1 and 5 M) and 2-mercaptoethanol (ME) (0.1 and 1%) concentrations at pH 7.0. The asterisk (*) symbol denote that they were significantly different than the control (0.1 M NaCl) ($p<0.05$).

surface charge (zeta potential). CPI is high in aspartic acid + asparagine, glutamic acid + glutamine, arginine, and lysine (Table 4.2) that contain polar and charged side chain that can form inter and intra hydrogen bonding that stabilize the protein as it cools. The addition of increasing concentration of urea also had an impact as it disrupted primarily hydrogen bonding, but also hydrophobic interactions resulting in a progress reduction on G′ (Cho et al., 2006) (Figure 4.7). The disruption of hydrophobic interaction within the protein destabilizes the protein aggregation that weakens the CPI gel. At the 5.0 M urea concentration, sufficient disruption of hydrogen bonding was evident to prevent network formation suggesting that hydrogen bonding plays a significant role in gelation.

The addition of 2-mercaptoethanol resulted in a reduction in disulfide bonds between neighbouring cysteine residues on the canola proteins, however 0.1% and 1.0% of 2-mercaptoethanol concentrations wasn't sufficient to prevent CPI network formation. However, similar to Léger and Arntfield (1993) who added dithiothreitol to reduce disulphide crosslinks within CPI gels, the addition of 2-mercaptoethanol produced an inferior gel. As indicated by Table 4.2, CPI had higher cysteine content than SPI. The higher amount of cysteine allows more disulfide bridging to occur for CPI than SPI. The increase in 2-mercaptoethanol not only cleaves disulfide bonds but also unfolds the proteins to affect the gel stability due to alteration of secondary, tertiary and quaternary formation (Anfinsen, 1973; Xiang and Arntfield, 2012). The folding of the protein also play significant role in determination of stability of the structure.

Following temperature ramps, time sweeps and frequency, strain sweeps were performed and it showed similar pattern as those seen in Figure 4.3 for samples with NaCl and urea (0.1 – 1.0 M) in which a rapid break point was evident. The findings suggest that these destabilizing salts had no major impact on the brittleness of the network (Figure 4.8A, B). However the addition of 5.0 M urea prevented gel formation, giving a strain profile characteristic of an entangled protein solution (Figure 4.8B). As noted from Table 4.3, the addition of 2-mercaptoethanol resulted in a switch from a more brittle gel to one with weaker junction zones as the disulfide bonds were reduced. The break point was more gradual in nature as the concentration of 2-mercaptoethanol increased (Figure 4.8C). Overall, it is believed that CPI gels are stabilized primarily through hydrophobic interactions and hydrogen bonding with some stabilization and strength from disulfide bridging. The addition of 2-mercaptoethanol significantly ($p<0.001$) reduced the % strain at break compared to 0.1 M NaCl CPI gel. In

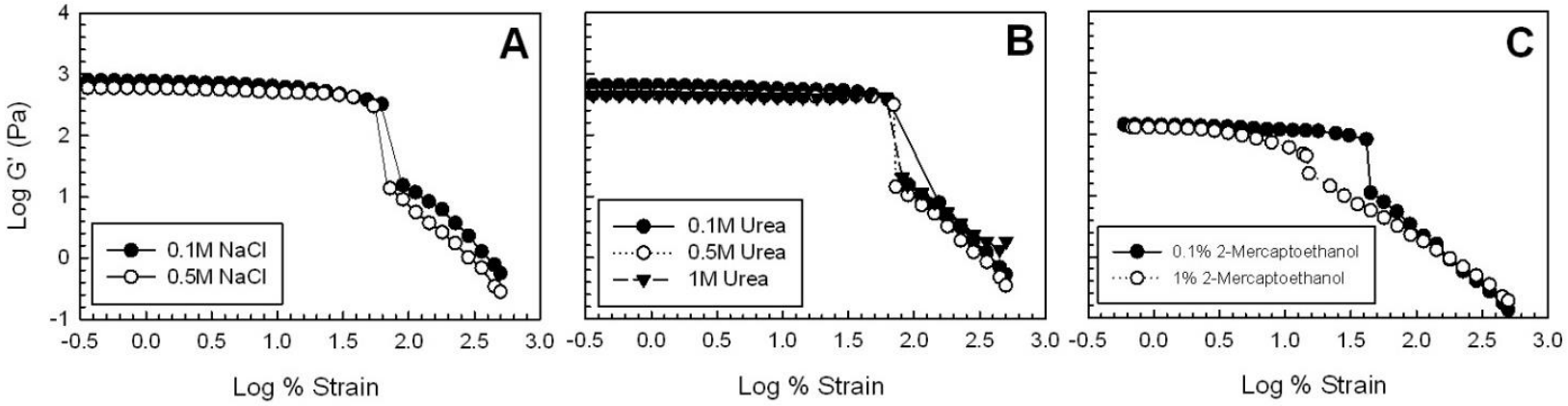


Figure 4.8 Dynamic storage (G′) modulus as a function of strain for a canola protein isolate as a function of NaCl (0.1 and 0.5 M) (A), Urea (0.1, 0.5 and 1 M) (B), and 2-mercaptoethanol (0.1 and 1 %) (C) at 5 Hz. Strain sweeps are continuation from temperature ramps, time sweep and frequency sweep.

addition, at higher percentage of 2-mercaptoethanol from 0.1% to 1%, there was significant ($p<0.001$) reduction in the % strain at break from 1.78 ± 0.04 to 1.34 ± 0.06. Unlike CPI, SPI had lower cysteine content than CPI, where CPI had about 2.5 times more cysteine (Table 4.2). The lower amount of cysteine indicates that the SPI will have less disulfide bonds present relative to CPI. No gel SPI gels were formed under 0.1% 2-mercaptoethanol, which supports the above statement where low amounts of 2-mercaptoethanol is sufficient to cleave significant amounts of disulfide bonds to disturb gel formation. Rheological measurements of SPI as a function of temperature and frequency in the presence of destabilizing additives were similar to those without for samples with NaCl (0.1 and 0.5 M) and urea (0.1, 0.5 and 1 M) with the exception of magnitude differences, whereas SPI solutions with urea (1.0 and 5.0 M) and 2-mercaptoethanol (0.1 and 1%) were all non-gelling (results not shown). Gelling temperatures for SPI with the addition of 0.5 M NaCl was found to significantly decrease from 83.5±5.0 to 66.7 ± 1.0˚C indicating the structure formation was happening much earlier than when denaturation was expected ($p<0.05$). The addition of excess NaCl is thought to promote protein-protein aggregation earlier. The addition of urea (0.1 and 0.5 M) has little effect on T_{gel}, which was 73.3 ± 2.1 and 80.0 ± 2.8ºC, respectively relative to the control (0.1 M NaCl) most likely since hydrogen bonds are mostly disrupted at higher temperatures.

G′ at the end of the time sweep is given in Figure 4.9 for all materials. In contrast to CPI, the addition of 0.5 M NaCl to SPI caused enhanced ordering of the protein structure resulting in significantly stronger gel networks forming. NaCl is thought to screen charges on the SPI to reduce the amount of electrostatic repulsion and the thickness of the electric double layer on proteins, to allow a greater amount of protein-protein interactions. SPI is thought to be more sensitive to the NaCl (0.5 M) than CPI, since the SPI carried a much stronger negative charge (-43.9 mV) than did CPI (-20.2 mV) at pH 7.0. Unlike CPI, the addition of 2-mercaptoethanol prevented network formation in SPI completely suggesting that disulfide bonding was essential for the formation of the network structure. Earlier, it was hypothesized that a greater amount of disulfide bonds were forming based on differences in the heating-cooling profiles for both systems (Figure 4.1 and 3.4). Further, SPI were also more sensitive to hydrogen bonding than the CPI, where networks were unable to form at both the 1.0 and 5.0 M concentrations.

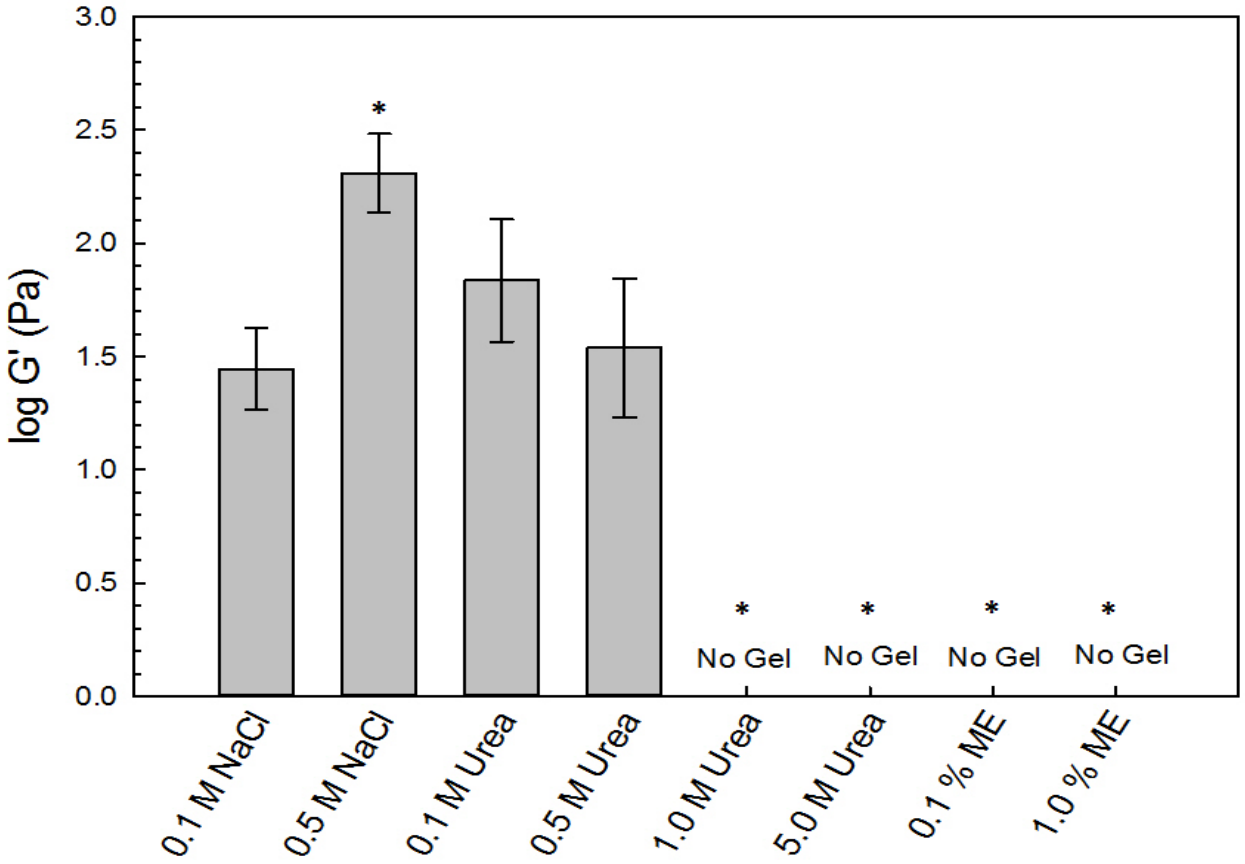


Figure 4.9 Dynamic storage (G′) modulus at the end of 1 h time sweep at 25ºC for soy protein isolate networks (7.0% w/w) as a function of NaCl (0.1 and 0.5 M), urea (0.1, 0.5, 1.0 and 5.0M) and 2-mercaptoethanol (ME) (0.1 and 1%) concentrations at pH 7.0. The asterisk (*) symbol denotes that they were significantly different than the control ($p<0.05$).

Strain sweep data of the gel networks which was collected after temperature ramps, time sweeps and frequency sweep indicated that the 0.5 M NaCl SPI gel became more brittle, whereas the other gel networks containing (0.1 and 0.5 M) urea were similar (Figure 4.10). The % strain at break was similar in values for SPI gels that contained 0.1 M and 0.5 M urea (2.63 ± 0.04 and 2.70 ± 0.00). At low concentration of urea, SPI gels were able to form a gel without changing in gel structure. In contrast, CPI gels had significant decrease in % strain at break when 0.5 M urea was added to the gel. This indicate that hydrogen bonding play more important role in CPI than SPI.

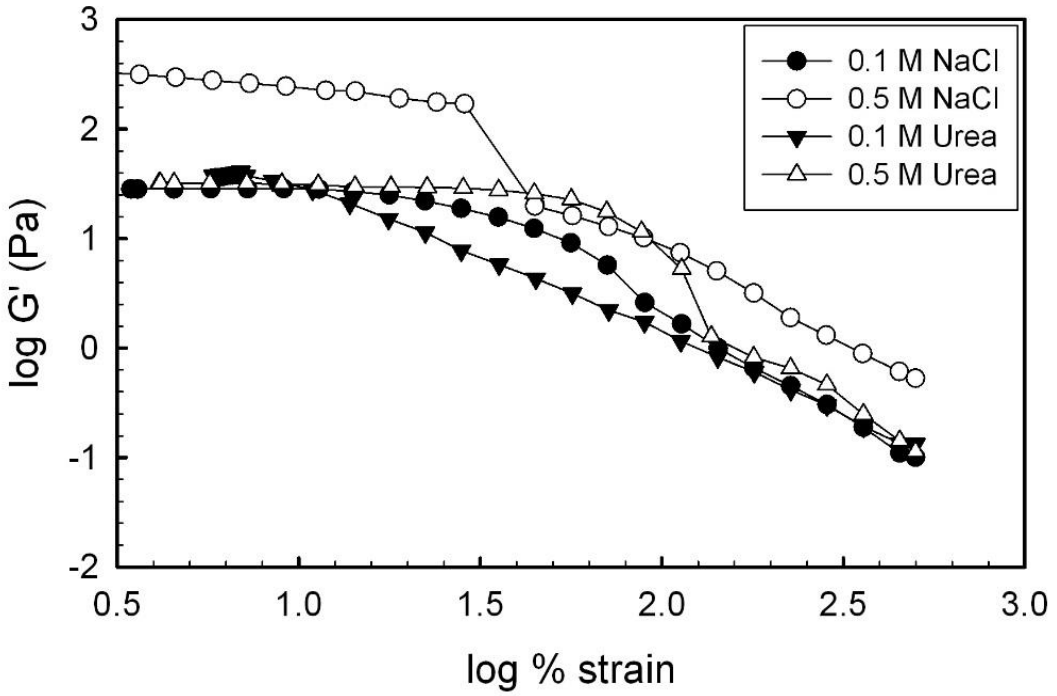


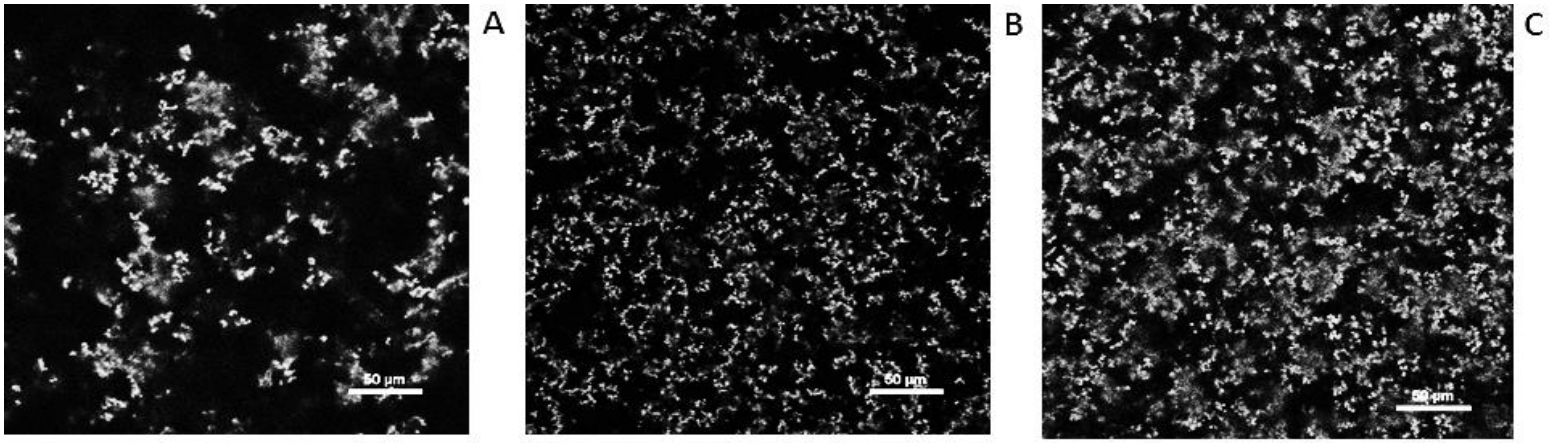
Figure 4.10 Dynamic storage (G′) modulus as a function of strain for a soy protein isolate as a function of NaCl (0.1 and 0.5 M) and urea (0.1 and 0.5 M) concentration at 5 Hz. Strain sweeps are continuation from temperature ramps, time sweep and frequency sweep.

4.1.5 Fractal analysis of canola and soy protein gel networks

Confocal scanning laser microscopy (CSLM) was used to image the morphology of the CPI and SPI as a function of protein concentration, and then used to determine the fractal dimension and lacunarity of the gel network. Figure 4.11 gives CSLM images of CPI as a function of concentration, showing that the level of aggregation increases as the protein concentration was raised. After applying the box count method on CSLM images, data was fitted using a power-law model where the slope was used to calculate fractal dimensionality. The fractal dimension was found to be similar for all concentrations ($p<0.05$), having values of 1.52 ± 0.08, 1.53 ± 0.03 and 1.59 ± 0.04 for the 5.0, 7.0 and 9.0% (w/w) concentrations, respectively. The CPI fractal dimension values fall within the range of other protein gels, where values of 1.9-2.4, 2.6-2.7, 1.5-2.2, and 1.73-2.82 were reported for albumen (Eleya et al., 2004); β-lactoglobulin, 11S soybean globulin, caseinate (Hagiwara et al., 1997); whey protein isolate

(Ikeda et al., 1999); and colloidal gels (Wu and Morbidelli, 2001), respectively. The fractal dimension value stay relatively constant as concentration increased, indicating that small aggregates grow into self-similar larger ones in a fractal manner, and close to value that is expected for the diffusion-limited cluster-cluster aggregation model (D_f = ~1.8; as determined by rheological small-strain rheometry and large-strain torsional testing) (Ikeda et al., 1999; Weitz and Lin, 1986). When the scatter particles in solvent stick to one another in random orientation it forms a cluster. The cluster further associate with other clusters in the system to form larger cluster that gives rise to the cluster-cluster aggregation (Ikeda et al., 1999; Jullien and Kolb, 1984). The diffusion-limited cluster-cluster aggregation correlate to protein growth of 'string of beads', where the diffusing particles attached to each other in random way with the probability of ~1 giving order growth (Ikeda et al., 1999; Doi, 1993). The fractal dimension of CPI was slightly lower than that for bovine serum albumin (D_f = ~2.6-2.8; as determined by large deformation testing) (Hagiwara et al., 1998) and whey protein isolate (D_f = 1.73 – 2.82; as determined by microscopic structural parameters) (Wu and Morbidelli, 2001), but were similar to that of pure soy glycinin (D_f = 1.64) and a mixed soy protein isolate (D_f = 1.81; as determined by image analysis of CLSM images) which contained $MgCl_2$ (Nagano and Tokita, 2011). Thus, the fractal dimension might imply CPI grows in order manner. However, the alternation of solvent conditions (eg. pH and NaCl) could change the formation of the network to be more random aggregation (Ikeda et al., 1999).

Fractal dimension looks at the complexity of the gel structure; however a better understanding of the gel network occurs when lacunarity is also evaluated (Dàvila and Parés, 2007). As the CPI concentration increased from 5.0% to 7.0%, then lacunarity of the gel decreased from 0.62 ± 0.06 to 0.41 ± 0.02 ($p<0.01$), where it then became constant as the concentrations were raised to 9.0% (w/w) (lacunarity of 0.40 ± 0.03) ($p>0.05$). The reduction of lacunarity suggests that there is less void space within the network (or a dense gel is formed). This suggests that at the 5.0% CPI concentration, cavity sizes are larger and less protein is available to occupy a given space. In Figure 4.11A, there is larger gap than Figure 4.11B and C, however, the fractal dimension alone did not indicate a difference. High fractal dimension and lacunarity values indicate that there is noticeable heterogeneity in gel structure (Karperien, 2012). The lacunarity values furthered explain the morphology of the CPI as a function of protein concentration.



Figrue 4.11 Confocal micrographs of canola protein isolate gels (0.1M NaCl, pH = 7.0) as a function of concentration: A) 5.0%, B) 7.0% and C) 9.0% (w/w).

In the present study, clear images of the SPI could not be obtained using the CSLM. Several studies have reported issues with producing CSLM images of globular protein gels, due to differences in solvents, protein-type, and gelation method used to prepare the samples. Hagiwara et al. (1997) was unable to obtain clear images of either soy glycinin or caseinate gels using CSLM, however was able to image β-Lactoglobulin. The gels were produced using varies methods depending on protein-types, and contained different levels of NaCl. There are several studies that were successful at imaging SPI and/or pure soy glycinin networks by CSLM (Nagano and Tokita, 2011; Renkema, 2004; Lakemond et al., 2003), however the proteins were prepared differently and contained some NaCl. The SPI used in the present study was a commercial from Archer Daniels Midland Company process (PRO-FAM 974, Lot 13020412, Decatur, IL, USA).

4.1.6 Summary

The present study examined the rheological properties and morphology of CPI and SPI gels as a function of concentration (5.0 – 9.0%), ionic strength (0.1 and 0.5 M NaCl) and in the presence of destabilizing agents such as urea (0.1, 0.5, 1.0 and 5.0 M) and 2-mercaptoethanol (0.1% and 1%). Small-deformation oscillatory measurements showed that the CPI formed stronger gels than SPI, with less dependence on disulfide and hydrogen bonds relative to SPI. For both proteins, there was no significant difference (~77ºC - ~90ºC) in gelling temperature as the protein concentration increased. Fractal dimension and lacunarity was analyzed using CLSM imaging showing the microstructure of CPI gels became denser as the concentration increased from 5.0% to 9.0% and followed a cluster-cluster aggregate growth model during the formation of the gel network.

4.2 The effect of pH on the gelling properties of canola and soy protein isolates

4.2.1 Effect of pH on the surface charge and thermal characteristics of canola and soy protein isolates

Surface charge or zeta potential for CPI and SPI is given in Figure 4.12 as a function of pH with and without 0.1 M NaCl. In the absence of NaCl, CPI was highly negatively charged (-32.7 mV) at pH 9.5, and then increased in magnitude reaching its isoelectric point (i.e., zero net charge) at pH 5.6. Surface charge increased to a maximum (+20.9 mV) at pH 3.5, before

experiencing a slight decline to +6.4 mV at pH 2.0 (Figure 4.12A). A similar pattern was also noted in Cheung et al. (2014) for cruciferin protein-rich isolates. The dip at lower pHs may be attributed to changes in the conformation of canola proteins at low pH potentially causing less charged amino acids to become exposed at the protein's surface. The addition of 0.1 M NaCl acted to significantly reduce the magnitude of the CPI's surface charge where zeta potential values ranged between -8.27 mV at pH 9.5 to +8.16 mV at pH 2.0 (Figure 4.12A). The addition of NaCl also acted to shift the net neutrality from pH 5.6 to 4.2. The reduction in surface charge and shift in net neutrality was thought to be associated with charge screening from the Na^+ and Cl^- ions in solution, which screen the negatively and positively charged sites on the protein's surface, respectively. This screening effect causes a reduction in the thickness of the electric double layer surrounding the protein leading to greater instability of the particles in solution (Keowmaneechai and McClements, 2002). Cheung et al. (2014), Stone et al. (2013), Yang et al. (2014) reported pI values for CPI of 4.8, 5.8, and 7.0 respectively which was within the range of those in the present study. The extraction methods, genetics, varieties and environmental factors can all have an impact on the protein profile within the isolate product and its subsequent pI value.

In contrast, SPI behaved in much of a similar manner as CPI, except that it carried a higher charge. At pH 9.5, the zeta potential was found to be -51.0 mV, which then increased to net neutrality at pH 4.6, reached a maximum at pH 3.0 (+35.0 mV) before declining slightly to +25.1 at pH 2.0 (Figure 4.12B). Similar to CPI, the addition of 0.1 M NaCl resulted in a reduction in the overall surface charge of SPI. Zeta potential ranged from -14.9 mV at pH 9.5 to +15.4 mV at pH 2.0, and a shift in the pH corresponding to net neutrality from pH 4.7 to 4.2. Smith and Circle (1978) reported pI values for SPI between pH 4.0 and 5.0, respectively.

The thermodynamic properties of canola proteins as a function of pH (3.0, 5.0, 7.0 and 9.0) at a 9.0% (w/w) concentration were determined by the use of differential scanning calorimeter (DSC). Thermograms captured the thermal denaturation of canola proteins (Figure 4.13) where the onset of denaturation (T_o), denaturation temperature (T_d) (represented by the temperature where the maximal denaturation occurs), and the enthalpy for protein denaturation were determined, which is summarized in Table 4.4. At pH 3.0, no thermal transitions were observed by DSC. Gels at pH 5.0 were significantly lower T_o and T_d ($p<0.001$) than at pH 7.0 and 9.0, which were similar ($p>0.05$) (Table 4.4). Enthalpy data at pH 5.0 was also significantly

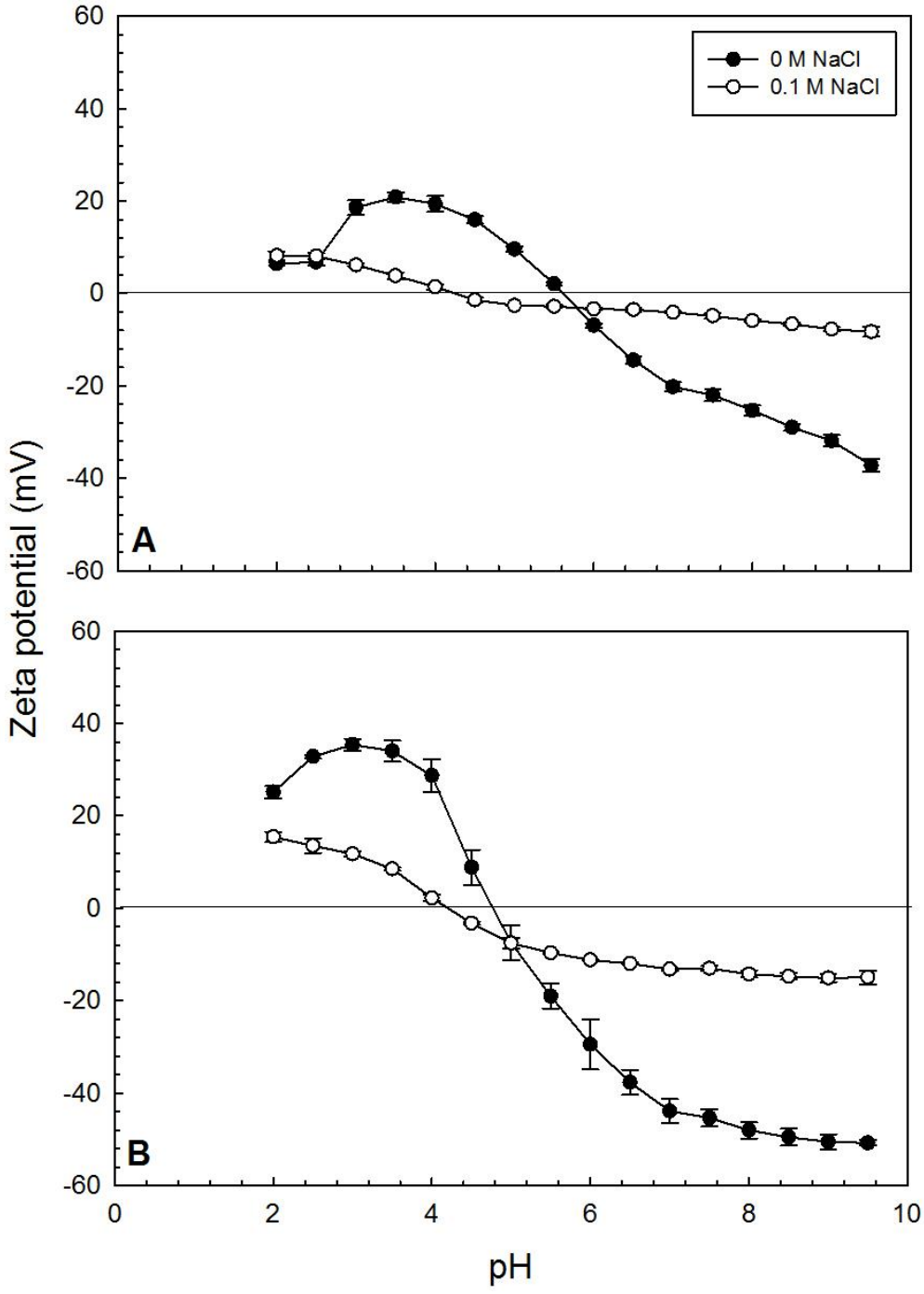


Figure 4.12 Zeta potential (mV) of canola (A) and soy (B) protein isolates as a function of pH in the absence and presence of 0.1 M NaCl. Data represent the mean ± one standard deviation (n = 3).

lower than at pH 7.0 ($p<0.01$) and pH 9.0 ($p<0.05$) (Table 4.3). No statistical significance was found between any of the thermal properties of gels formed at pH 7.0 and 9.0 ($p>0.05$) (Table 4.4). For CPI, the T_d shifted towards higher temperatures as pH increased from pH 5.0 (78.6ºC) to pH 7.0-9.0 (~87ºC). The earlier denaturation and onset temperatures at pH 5.0 may be attributed to its close proximity to its pI value (at pH 5.6) where protein-protein interactions is greatest due to a reduction in electrostatic repulsive forces between proteins. Similar findings were noted by Léger and Arntfield (1993) where at pH 5.0 the T_d (75.42ºC) occurred earlier than at pH 7.0 (T_d = 80.92ºC) and 9.0 (T_d = 81.01ºC) (1993). In contrast to the present study which observed enthalpy values increase from pH 5.0 to pH 7.0 and then remain constant as pH levels were again raise to pH 9.0, Léger and Arntfield (1993) and Yang et al. (2014) both reported an increase in enthalpy over the same pH range. It is presumed the difference may be associated with the napin and cruciferin ratio.

Table 4.4 Onset (T_o) and denaturation (T_d) temperatures, and enthalpy (ΔH) of a 9.0% (w/w) canola protein isolate (10 mg sample) solution as a function of pH (3.0, 5.0, 7.0 and 9.0). Data represent the mean ± on standard deviation.

pH	T_o (ºC)	T_d (ºC)	ΔH (J/g)
3.0	-	-	-
5.0	68.33 ± 1.16^b	78.59 ± 0.87^b	0.20 ± 0.02^b
7.0	78.56 ± 0.41^a	87.05 ± 0.80^a	0.51 ± 0.06^a
9.0	79.08 ± 0.39^a	86.91 ± 0.38^a	0.38 ± 0.10^a

The higher denaturation temperatures and enthalpy values at higher pHs (7.0 and 9.0) relative to that at pH 5.0 suggests the more energy is required to denature the canola proteins under neutral and alkaline conditions most likely related to an increasing amount of disulfide bonds and non-covalent bonding and interactions. Schwenke et al. (1988) reported that napin, which is stabilized by inter- and intra-chain disulfide bonds could be split at high temperature only under alkaline conditions; where under acidic or neutral pHs the protein remained stable.

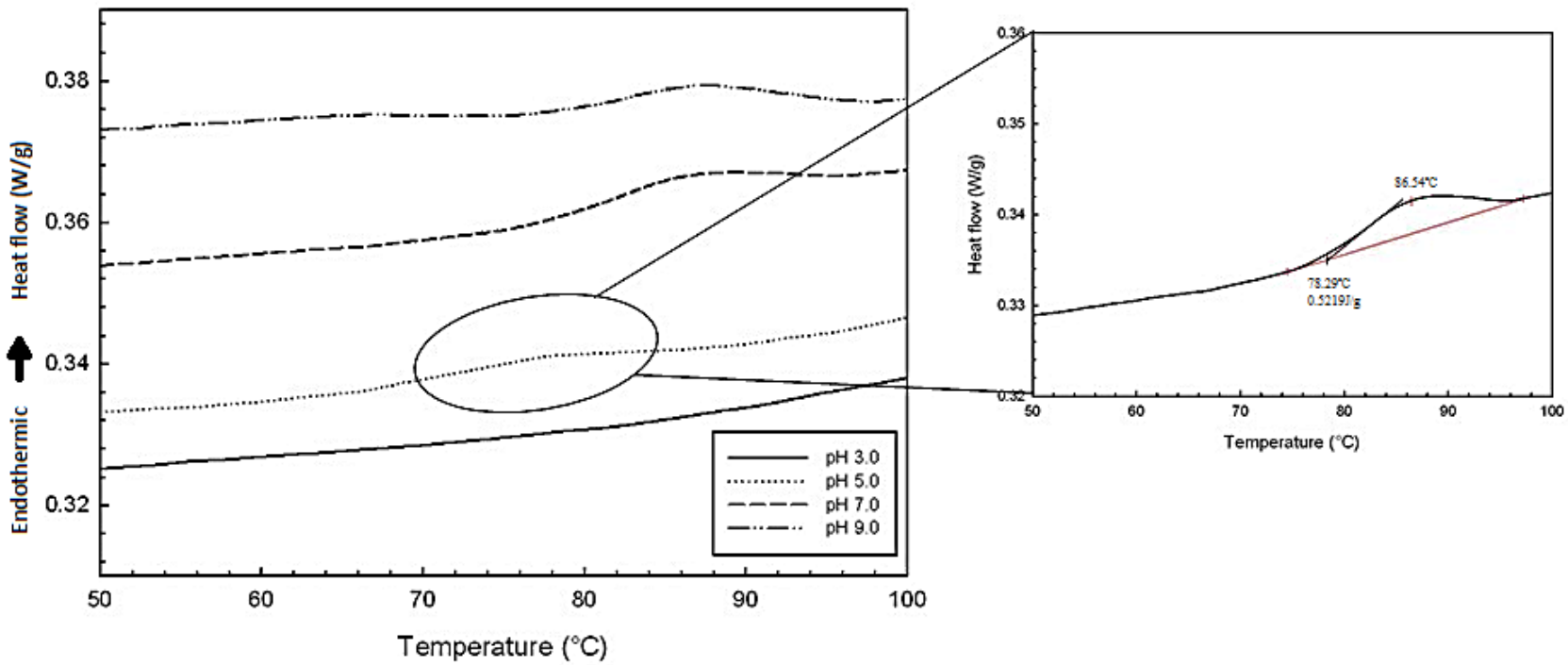


Figure 4.13 Conventional DSC thermograms of 9.0% (w/w) canola protein isolate (10 mg sample) solution as a function pH (3.0, 5.0, 7.0 and 9.0). The expanded thermogram gives an example of how the onset and denaturation temperatures were determined, whereas the enthalpy values were determined by integrating the area under the endothermic peak.

The authors reported at pH 9.0, hydrogen bonding, inter- and intra- disulfide bonds break to cause increases in both T_d and ΔH values. In present study, at pH 5.0, 7.0 and 9.0, enthalpy values from Table 4.4 were 0.20 ± 0.02 J/g, 0.51 ± 0.06 J/g and 0.38 ± 0.10 J/g, respectively. Yang et al. (2014) reported that cruciferin rich CPI enthalpy values at pH 5.0, 7.0, and 9.0 were 0.7720 ± 0.24 J/g, 1.4564 ± 0.10 J/g, and 1.8900 ± 0.11 J/g, whereas napin rich CPI enthalpy values at pH 5.0, 7.0, and 9.0 were 0.1072 ± 0.02 J/g, 0.2213 ± 0.04 J/g, and 1.2525 ± 0.06 J/g. The enthalpy values for the present study fall between the napin rich and cruciferin rich CPI when compared to Yang et al. (2014) study. This indicates that the cruciferin and napin ratio are in between the 2S and 11S rich CPI.

In the case of soy protein isolate (9.0% w/w), no thermal transitions were observed under the conditions examined. It was proposed that the transitions were weaker than that of canola protein, preventing transitions from being observed using the conventional DSC instrument.

4.2.2 Rheological properties of canola protein isolate during gelation

The rheological properties of CPI were studied first as a function of temperature, time, frequency and strain as a function of pH (3.0, 5.0, 7.0 and 9.0) at 7.0% (w/w). Above temperatures of ~82-89ºC there was a noticeable rise in G′ at pHs of 5.0, 7.0 and 9.0 upon heating indicating the formation of a heat induced gel network (Figure 4.14A). These temperatures corresponded to the gelation temperature (T_{gel}), which was found to be independent of pH, at pHs 5.0, 7.0 and 9.0 (~82-88ºC) ($p>0.05$) (Table 4.5). After T_{gel}, G′ became greater than G″ indicating the formation of an elastic-like structure, whereas below T_{gel} the opposite occurred indicating the solution was fluid-like in nature (not shown). Temperatures corresponding to T_{gel} also corresponded to denaturation temperatures (Table 4.4), suggesting that the canola proteins were unraveling to expose buried hydrophobic moieties followed by hydrophobic-induced aggregation and disulfide bridging of the proteins. Upon subsequently cooling, G′ experienced an initial drop in magnitude at temperatures >80ºC, before rising again as temperatures cooled hydrogen bonding became more prominent and lower temperatures (Léger & Arntfield, 1993) (Figure 4.14B). The magnitude of G′ increased as the solution pH behaves more alkaline during cooling and after the time sweep (Table 4.5). The rise in G′ is thought to be due to pH induced conformational changes of the protein, to promote more protein-protein interactions via

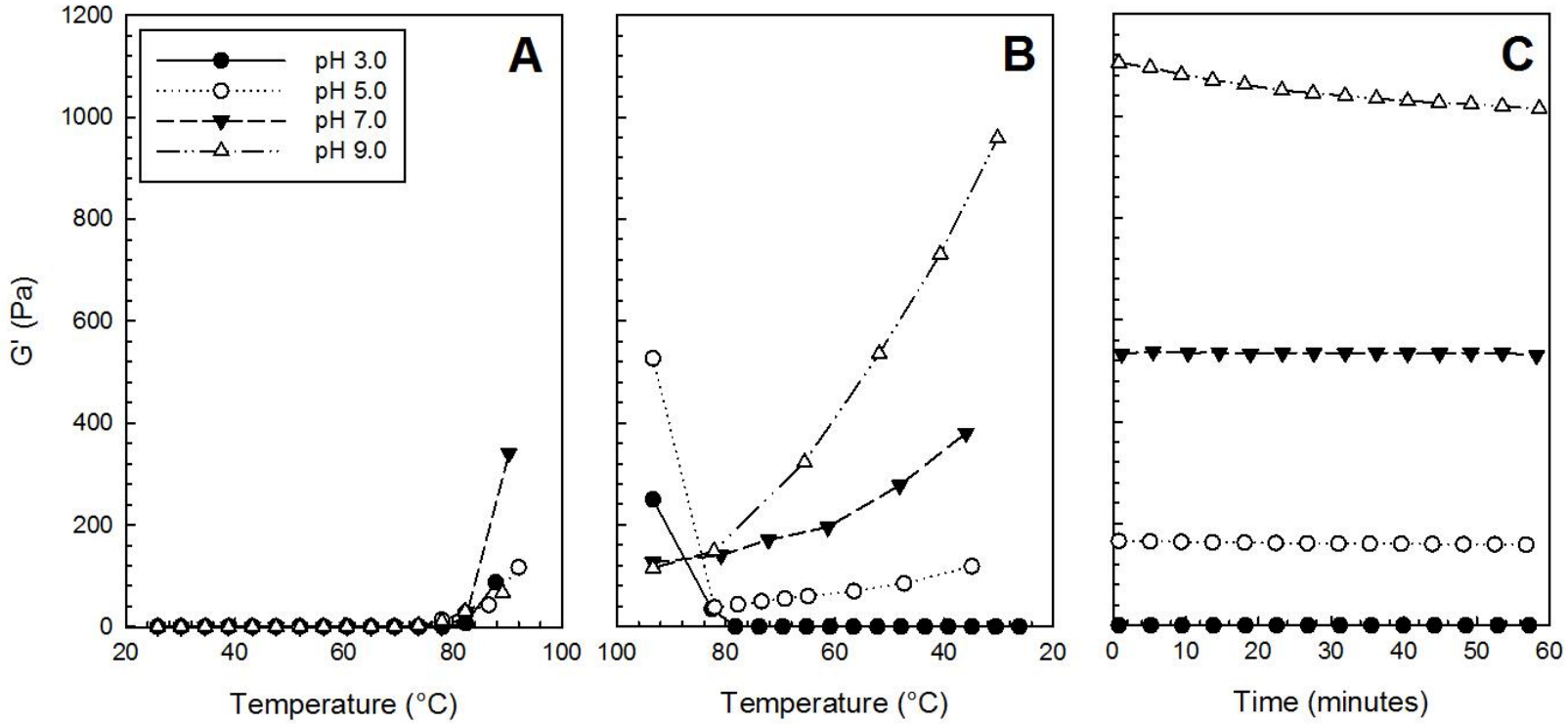


Figure 4.14 Dynamic storage (G′) modulus of a 7.0% (w/w) canola protein isolate solution as a function of pH (3.0, 5.0, 7.0 and 9.0) at 1% strain and 0.1 Hz during a: A) temperature ramp from 25ºC to 95ºC; B) temperature ramp from 95ºC to 25ºC; and C) 1 h time sweep at 25ºC.

Table 4.5 The gelation temperature during heating (T_{gel}), and dynamic storage G′) and loss (G″) moduli after the 1 h time sweep at 25°C, and the log % strain at break for canola and soy protein isolates as a function of pH at 7.0% (w/w) canola protein isolate solution. Data represent the mean and standard deviation of duplicate samples. The abbreviation of n.g. denotes a material that is non-gelling.

pH	T_{gel} (°C)	G' (Pa)	G″ (Pa)	log % Strain at break
Canola protein isolate				
3.0	n.g.	n.g.	n.g.	n.g.
5.0	88.5 ± 0.7^{a}	177.4 ± 27.4^{c}	30.3 ± 7.2^{c}	1.30 ± 0.2^{c}
7.0	87.0 ± 3.5^{a}	508.4 ± 31.2^{b}	61.8 ± 5.5^{b}	1.80 ± 0.0^{b}
9.0	81.6 ± 0.6^{a}	969.3 ± 63.2^{a}	102.7 ± 6.8^{a}	2.02 ± 0.0^{a}
Soy protein isolate				
3.0	85.6 ± 1.1^{a}	186.2 ± 4.7^{a}	25.0 ± 3.8^{a}	1.24 ± 0.1^{a}
5.0	45.7 ± 6.6^{b}	178.7 ± 8.3^{a}	26.0 ± 1.6^{a}	1.17 ± 0.1^{a}
7.0	83.5 ± 5.0^{a}	29.1 ± 11.7^{b}	3.5 ± 1.3^{b}	1.34 ± 0.0^{a}
9.0	n.g.	n.g.	n.g.	n.g.

hydrophobic associations and disulfide bridging. At pH 3.0, the CPI solution did not form a gel, where at end of 1 h time sweep G″>G′ (not shown). Léger and Arntfield (1993) reported a similar finding for canola protein isolates at pH 4.0. The lack of structure formation is believed to be caused by a higher amount of electrostatic repulsive forces arising between proteins, inhibiting protein-protein aggregation. During the 1 h time sweep, G′ remained relatively constant indicating no further polymer ordering or reordering was occurring within the network (Figure 4.14A). The magnitude of G′ was found to increase from 177 Pa to 508 Pa to 969 Pa as pH increased rose from 5.0 to 7.0 and then to pH 9.0 (Table 4.5). Continuation from temperature

ramps and time sweeps, frequency sweeps was performed to understand the effect of the interaction of the proteins in terms of network structure (Franak, 2013). The viscoelastic moduli on double-logarithmic plots show both fluid (G″>G′) and gel-like characteristic (G′>G″) (Figure 4.15). At pH 3.0 CPI behave as a fluid where G″>G′ (Figure 4.15A), the rapid change in moduli as a function of frequency indicates that CPI is within flow region of the viscoelastic spectrum. Material within this region has insufficient structure to entrap and influence the flow of solvent within the system. At pH 5.0, CPI behaved

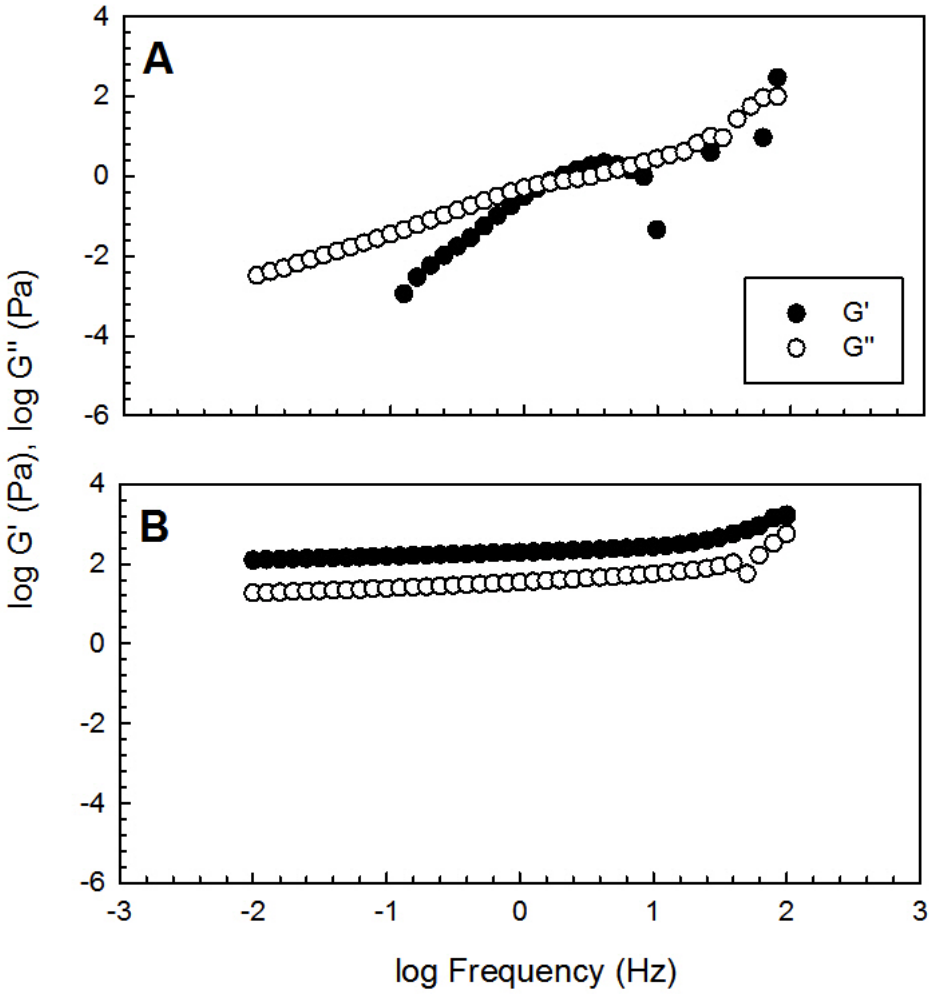


Figure 4.15 Dynamic storage (G′) and loss (G″) moduli as a function of frequency for a canola protein isolates (7.0% w/w) at pH 3.0 (A) and 5.0 (B) at 1% strain. Frequency sweeps are continuation from temperature ramps and time sweep.

as a gel-like material where moduli was independent of frequency at G′>G″(Figure 4.15B). Similar profiles (except different magnitude) were found at pH 7.0 and 9.0 (not shown).

Following frequency sweep, a strain sweep was performed on all gels to determine the material's linearity and relative strength of junction zones. As shown in Figure 4.16, there was a sharp break in the double-log storage modulus-strain profile suggesting the gel network was brittle in nature. For all CPI gels at pH 5.0, 7.0 and 9.0, G′ stayed relatively constant until it suddenly decreased due to the breaking of bonds within network (Eleya et al., 2004). The log % strain at break was found to decrease from 2.0 to 1.3 (or 104 to 20 anti-logged) as pH shifted from 9.0 to 5.0 (Table 4.5, Figure 4.16). As pH approach alkaline conditions, the network is thought to be highly compacted structure. The rise in % strain break value indicate that as network becomes stronger and higher strain is needed to break the gel system as gel pH approach alkaline condition.

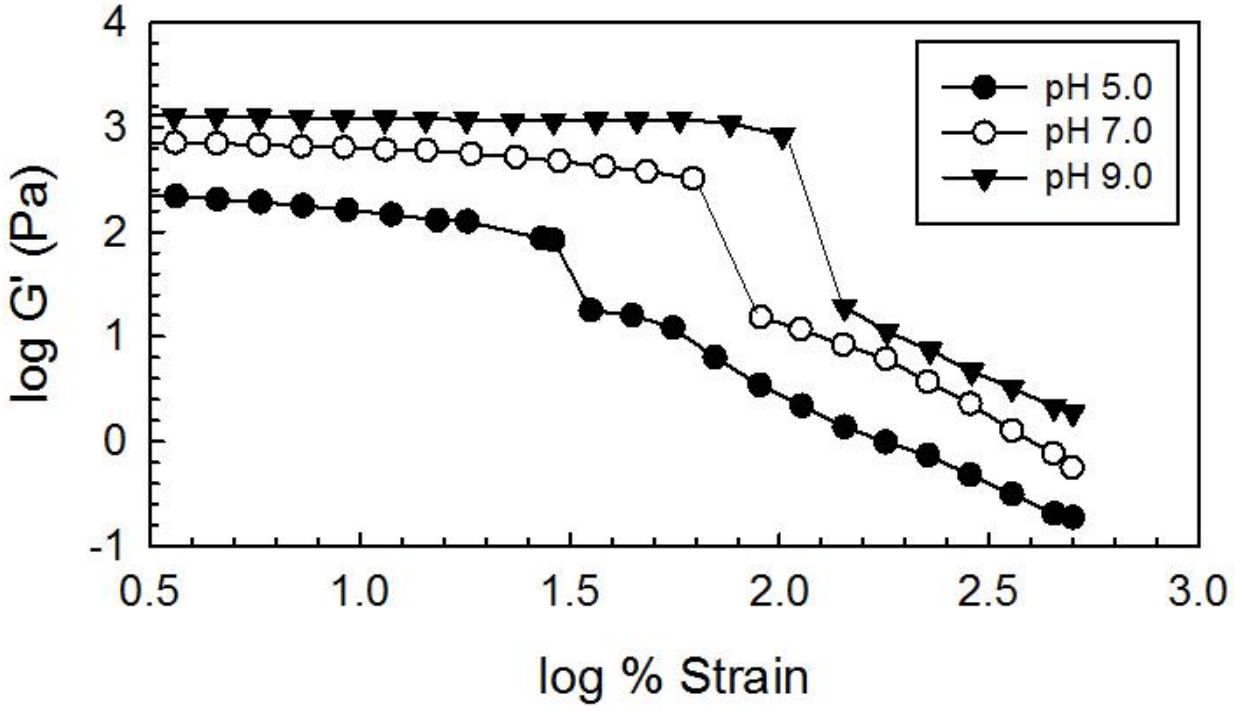


Figure 4.16 Dynamic storage (G′) modulus of a 7.0% (w/w) canola protein isolate network as a function of % strain and pH (5.0, 7.0 and 9.0) at 5 Hz. Strain sweeps are continuation from temperature ramps, time sweep and frequency sweep.

4.2.3 Rheological properties of soy protein isolate during gelation

The rheological properties of SPI were studied first as a function of temperature, time, frequency and strain as a function of pH (3.0, 5.0, 7.0 and 9.0) at 7.0% (w/w). T_{gel} were found to occur at ~86ºC, ~46ºC and ~83ºC for SPI solutions at pH 3.0, 5.0 and 7.0, whereas at pH 9.0 the systems were non-gelling. (Figure 4.17, Table 4.5). At pH 9.0, SPI did not result in network formation presumed due to strong repulsion force, which inhibited protein-protein interactions. Although network strength was similar for SPI at pH 3.0 and 5.0, gel formation occurred at much lower temperatures at pH 5.0 mostly likely since it was near its pI value (pH 4.2) were charge repulsion was less and protein-protein aggregation was greater (Puppo et al., 1995). At pH 7.0, network strength decreased significantly to ~29 Pa from 178 Pa at pH 5.0 (Table 4.5), and then became non-gelling at pH 9.0 due to an increase in the strength of the electrostatic repulsive forces between proteins. Although the present study could not determine specific denaturation temperatures, Renkema et al. (2000) reported them for soy glycinin and conglycinin to be near 88ºC and 68ºC, respectively. The authors also reported at more acidic pHs, the denaturation temperature occurred a lower temperatures (Renkema et al., 2000). For glycinine, as pH shifted from 7.6 to 3.8 there was two endothermic peaks at 68ºC and 82ºC at acidic pH whereas at pH 7.6 one endothermic peak was reported at 88ºC (Renkema et al., 2000).

After T_{gel}, G′ continued to increase as shown in Figure 4.17B, where denaturation of proteins expose hydrophobic patches to produce gel network and assists further association of the proteins (Renard et al., 2006, Lamsal et al., 2007). Unlike CPI, G′ continued to increase during the cooling phase until ~80-75ºC before declining (Figure 4.17B). It was hypothesized that the additional rise in G′ was caused by the time delay in-between the heating and cooling scans that allowed for additional protein rearrange to facilitate the formation of a greater number of disulfide linkages. In contrast, CPI did not show any increased during the time lapse. This difference in cooling scan may suggest that CPI networks may rely less on covalent bonds than SPI gels. Upon cooling, a loss in network strength was seen at pH 7.0 and 9.0, whereas at pH 3.0 and 5.0 G′ dropped sharply around 75-80ºC down to a plateau at ~60ºC (Figure 4.17B). It is proposed that the pH induced changes to protein structure occurred, particularly with the glycinin protein which is thought to dominate the isolate. Lakemond et al. (2000) reported that at pH 7.6 glycinin exist in hexameric form, whereas at pH 3.8 it exists in trimers. Thus it is presumed that

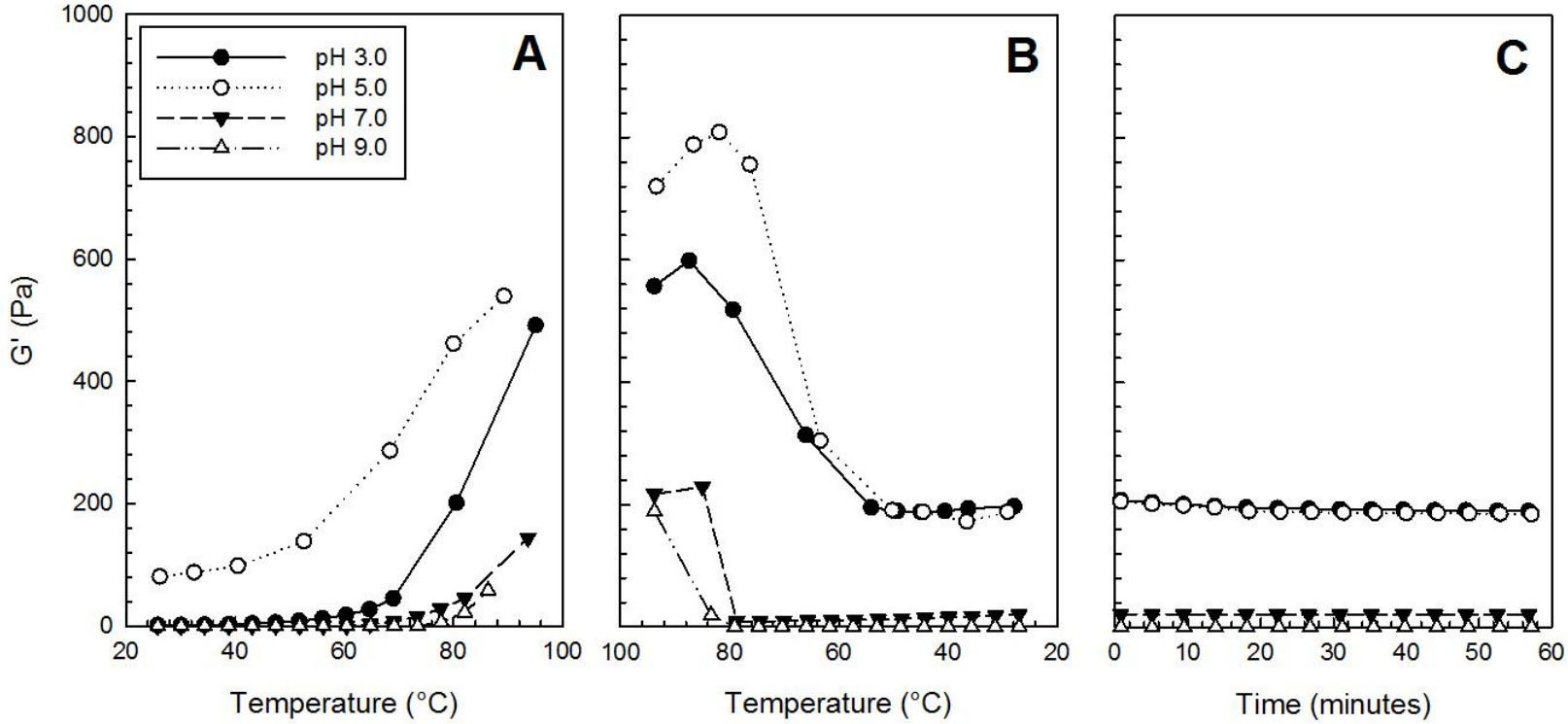


Figure 4.17 Dynamic storage (G′) modulus as a function of temperature and time for a soy protein isolate (7.0% w/w) solution as a function of pH (3.0, 5.0, 7.0 and 9.0) at 1% strain and 0.1 Hz. A) temperature ramp from 25ºC to 95ºC; B) temperature ramp from 95ºC to 25ºC; and C) 1 hour time sweep at 25ºC.

the soy glycinin in the present study at pH 3.0 is in its trimer form, a conformation possibly more conducive to gel formation. And as more hexameric structures reduce into trimmers, a greater number of cysteine moieties may become exposed and free to partake in disulfide bridging. In contrast to CPI, SPI did not experience an increase in G′ during cooling suggesting that hydrogen bonding may play less of a role in the stability of the network. G′ remained relatively stable over 1 h period for SPI, indicating that further ordering of the proteins was not occurring within the gel (Figure 4.17C).

In Figure 4.18A, SPI at pH 9.0 behaved as fluid-like material and not a gel during frequency sweeps, where G′<G″. Expect for minor differences in magnitude, similar frequency sweep profiles were noted at pH 3.0, 5.0 and 7.0; therefore only pH 3.0 was given as an example.

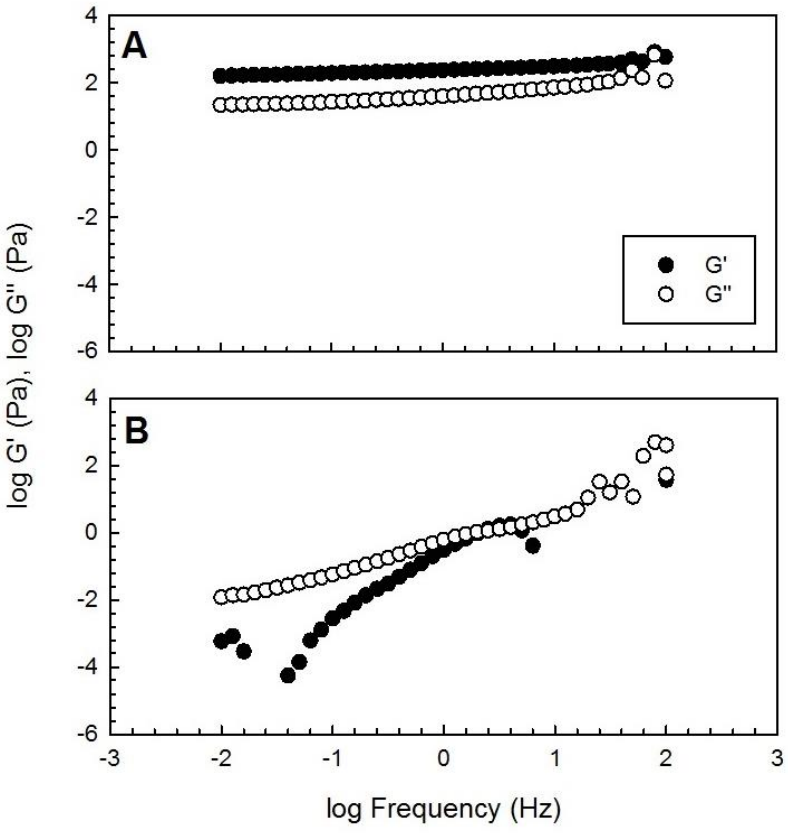


Figure 4.18 Dynamic storage (G′) and loss (G″) moduli as a function of frequency for a soy protein isolate (7.0% w/w) solution at pH 3.0 (A) and pH 9.0 (B) at 1% strain. Frequency sweeps are continuation from temperature ramps and time sweep.

At pH 3.0, a gel-like structure was evident where G′> G″ and moduli were relatively independent of frequency (Figure 4.18B). This type of frequency dependence of moduli falls within the rubbery plateau region of the viscoelastic spectrum. At high frequencies in Figure 4.18B, another crossover of moduli occurs where G′<G″, indicative of the rubber-glass transition region of the viscoelastic spectrum.

Following the frequency sweep, strain sweeps was performed at pH 3.0, 5.0, and 7.0 to determine the % strain at break (Figure 4.19). The strain sweep was conducted to measure the relative strength of junction zones formed within the SPI and their resistance to flow. Unlike CPI gels, SPI networks had more of a gradual transition from a linear to non-linear behavior suggesting that SPI networks were more flexible in nature, and stabilized by weaker junction

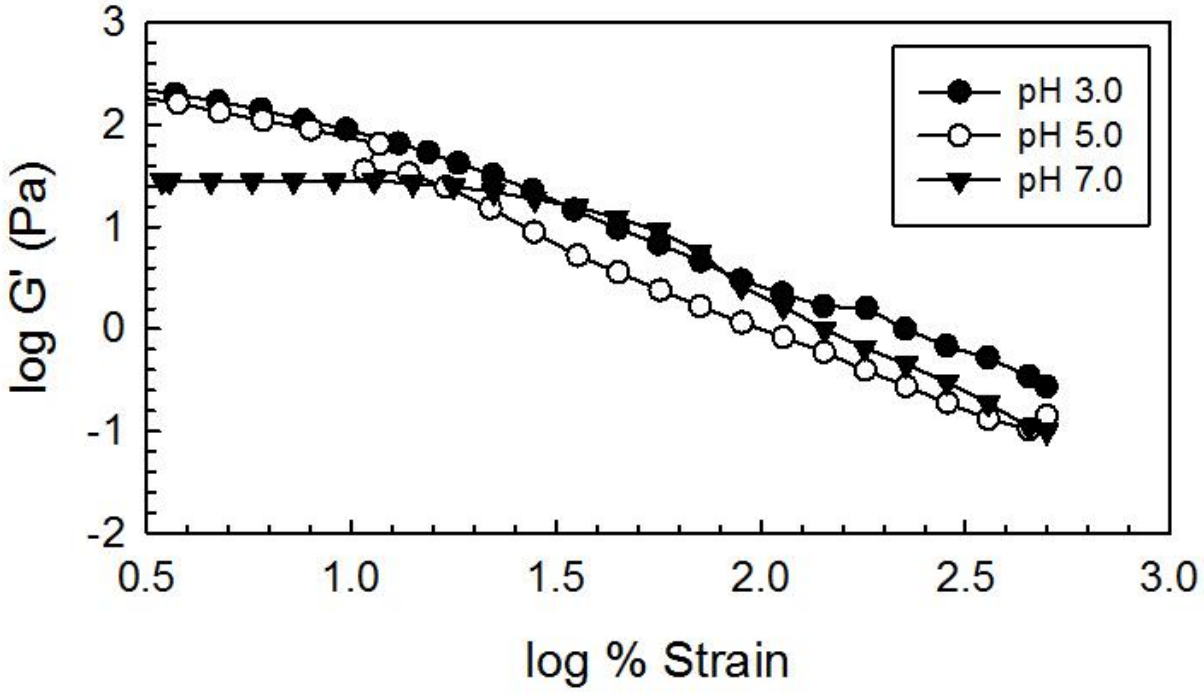


Figure 4.19 Dynamic storage (G′) (a) and loss (G″) (b) moduli as a function of strain for a soy protein isolate (7.0% w/w) as a function of pH (3.0, 5.0 and 7.0) at 5 Hz. Strain sweeps are continuation from temperature ramps, time sweep and frequency sweep.

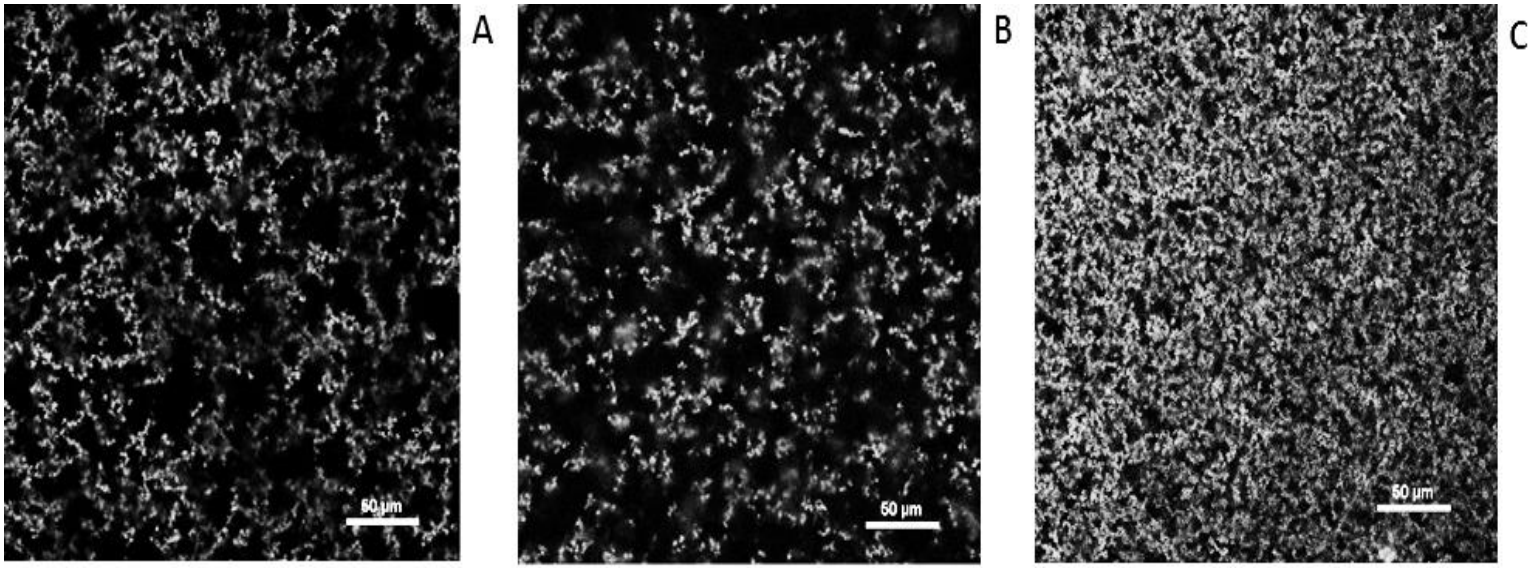
zones compared to CPI gels. At pH 3.0, 5.0 and 7.0, the % strain at break was similar regardless of the pH at ~1.25 (17.78 anti-logs) suggesting junction zones were similar in nature (Table 4.5).

The % strain at break values of SPI at pH 3.0, 5.0, and 7.0 was also similar to CPI gel formed at pH 5, where CPI produced the weakest network. This suggests that CPI forms stronger network compared to SPI.

4.2.4 Fractal analysis of canola protein gel networks

Confocal scanning laser microscopy was used to image the internal structure of the CPI network as a function of pH, and then the image was used to determine the D_f and lacunarity. CSLM images were analyzed using box count method, and fitted to the power-law model to calculate the D_f and lacunarity. Figure 4.20 gives CLSM images of CPI as a function of pH, where with increasing in pH there was an increase in level of aggregation. This also support the rheological properties, where increasing in pH increased the G′, resulting in stronger network. The D_f was found to be similar at pH 5.0 ($D_f = 1.59 \pm 0.07$) and 7.0 ($D_f = 1.54 \pm 0.03$) ($p>0.05$), however at pH 9.0 the D_f became significantly higher ($D_f = 1.82 \pm 0.01$) ($p<0.05$).

At higher temperatures and under alkaline pHs it is proposed that the canola proteins start to degrade allowing for a greater number of hydrophobic moieties to become exposed, leading to a greater amount of protein aggregation than other pH conditions. Ultimately this leads to a greater D_f in the gel network. Similarly, Eleya et al. (2004) indicated that egg white protein exhibited a similar trend where higher D_f values were noticed at basic pH ($D_{f.}$=2.2-2.4) compared to acidic and neutral pH ($D_{f.}$= 1.9-2.1). Like CPI, egg white protein contains other proteins such as lysozyme and ovotransferrin that have higher pI values which could contribute to higher D_f at higher pH (Eleya et al., 2004). Compared to other protein gels such as whey protein isolate ($D_{f.}$= 1.5-2.2), albumen ($D_{f.}$= 1.9-2.4) and plasma protein ($D_{f.}$= 2.63-2.83), CPI gels are within the same range of D_f of protein gels (Ikeda et al., 1999; Eleya et al, 2004; Dàvila and Parés, 2007). As well, the D_f values agrees with diffusion-limited cluster-cluster aggregation D_f value (~1.8), where aggregation takes the form of linear strand-like structure (Ikeda et al., 1999). Also, from Figure 4.20, there was a visual difference between pH 9.0 to pH 5.0 and pH 7.0. The change in pH had significant increase in D_f compared to increase in concentration, where the increase in concentration did not alter the formation of aggregation however, with increasing in pH gel morphology was altered. At pH 9.0, higher networking system can be seen compared to other pHs, and less void areas were notice.



Figrue 4.20 Confocal laser scanning micrographs of canola protein isolates (CPI) gels (7.0% CPI, 0.1M NaCl) as a function of pH: A) pH 5.0, B) pH 7.0, and C) pH 9.0.

The lacunarity values give additional information of the morphology of the CPI as a function of pH. By evaluating the lacunarity value, better understaning of gel network structure can be explained through how protein occupy a given space. Like D_f value, there was no significant difference in lacunarity value between pH 5.0 (0.41 ± 0.09) and pH 7.0 (0.41 ± 0.02) ($p>0.05$). However, at pH 9.0 the lacunarity was significantly lower (0.25 ± 0.02) ($p<0.05$) suggesting there was less free space within the network than at other pHs. In the present study, SPI CSLM images were not studied due to unclear CSLM image of SPI gels. It was also noted in Hagiwara et al. (1997) 11S glycinin CSLM images was unclear to be analyzed, thus in the future different method should be use to evaluate the D_f of SPI.

4.2.5 Summary

The present study investigated the rheological properties and morphology of CPI and SPI gels as function of pH (3.0, 5.0, 7.0 and 9.0). Small-deformation oscillatory measurements indicated that as pH approach alkaline conditions (pH 9.0) CPI formed strong gel networks whereas for SPI gels formed stronger network as pH approach isoelectric point (pH 5.0). Gelation temperatures for CPI and SPI were similar (~82ºC - ~86ºC) at all pH, except for SPI at pH 5.0 (~46ºC). CLSM images was used to determine the fractal dimension and lacunarity to examine the structural characterization of CPI gels, where CPI gel became denser as pH shifted to basic condition. Overall, there was an increase in aggregation as pH became more alkaline which formed denser CPI network that result in increased G′. It is also believed that covalent bonding played more of a role in SPI networks than CPI, however hydrogen bonding was more prevalent in the CPI networks. Hydrophobic interactions are believed to be important for both protein systems.

5. CONCLUSIONS

Canola protein isolate (CPI) contains a well-balanced amino acids profile making it highly nutritious for human health. To be utilized as a food ingredient, an understanding of the gelation properties is important to tailor its functionality to obtain the desired food microstructure and texture. The overall goal of this research was to investigate the gelation properties and morphology of canola protein isolate (CPI) as a function of protein concentration (5.0 – 9.0%), ionic strength (0.1 and 0.5M NaCl), destabilizing agents (urea 0.1, 0.5, 1.0 and 5.0 M; 2-mercaptoethanol 0.1% and 1%) and pH (3.0, 5.0, 7.0 and 9.0) and to be contrasted to that of commercial soy protein isolate (SPI). The current study showed that both protein concentration and pH influenced the gel strength; however, pH had a greater effect on CPI than did protein concentration. In general, SPI was more sensitive to changes in gelation conditions than was CPI, where SPI was unable to gel at 5.0% concentration, pH 9.0, with the addition of 2-mercaptoethanol (0.1 and 1.0%) and urea (1.0 and 5.0M). CPI was unable to gel only at pH 3.0 and with addition of 5.0 M urea.

An increase in protein concentration caused an increase in storage modulus (G′) for both CPI and SPI; however, gelation temperature (T_{gel}) remained constant. As the protein concentration increased, the degree of protein-protein aggregation also increased to make denser, more elastic networks. Confocal laser scanning microscopy images indicated that canola protein aggregates grew as self-similar clusters, where similar fractal dimension (D_f) values (1.52 - 1.59) were noticed with an increase in concentration from 5.0% to 9.0%. In contrast, the heterogeneity of void spaces was reduced as protein concentration increased. Based on the observed D_f values, CPI grew via diffusion-limited cluster-cluster aggregation, and formed strong junction zones that give arise to brittleness.

The proteins had similar amino acid profiles with minor differences in cysteine and aspartic acid + asparagine contents. The higher cysteine content in CPI was thought to allow for a greater number of inter- and intra-chain disulfide bridges to form and assist in the formation of the gel network. Also, this might account for the ability to form a gel network even with the

addition of 2-mercaptoethanol. Urea was found to reduce the gel strength of both networks. Hydrogen bonding was important during the cooling stage. The addition of NaCl increased the gel strength of SPI, but did not alter the gel strength of CPI. Zeta potential measurements indicated that with the addition of NaCl, the overall charge was reduced at all pHs. The screening of protein charges reduces the repulsion forces, which allows favourable protein-protein aggregation that in turn allows gel network formation. Thus, disulfide bridging, electrostatics and hydrogen bonding have minor to major roles in SPI and CPI gel formation.

SPI gel networks were stronger near the isoelectric point (pI), whereas CPI networks were strongest under alkaline conditions (away from its pI). The strength of SPI gel networks was attributed to protein aggregation near its pI, whereby proteins become more net neutrally charged as the pH approaches the pI favoring protein-protein aggregation. Furthermore, the onset of protein-protein aggregation was noticed for SPI near pH 5.0. The strength of CPI gel networks was found to be due to the arrangement of proteins depending on their pH. CPI fractal dimension values were similar as protein concentration increased; however, the fractal dimension significantly increased from pH 7.0 to 9.0 (~1.54 to ~1.82). The significant change suggests that CPI gels grow in a slightly different manner when it is at an alkaline pH. Also, the reduction in lacunarity value (~0.41 to ~0.25) for the same increase in pH indicated that the proteins are packing tightly. Thus, CPI increased in aggregation as the pH approach alkaline conditions, which increased the G′ and resulted in a strong gel network near pH 9.0.

6. FUTURE STUDIES

CPI is represents a potential alternative protein ingredient for use in food and non-food applications, providing value-added opportunities for the underutilized meal beyond that of feed. The current research mainly focused on canola protein isolates as a function of concentrations, ionic strength, pH and destabilizing agents as it related to protein gelation and their fractal behaviour.

There are many ways to determine fractal dimensions, such as rheology, light scattering, gel permeability and microscopy (Feder and Jossnag, 1984; Hagiwara et al., 1997 Marangoni et al., 2000; Enright and Leitner, 2005; Dàvila and Parés, 2007). To compare both SPI and CPI and to reduce complication, large or small deformation should be used in the future to determine D_f. In the current study, the D_f of SPI was unable to be determined due to issues that arose from slide preparation and solvent conditions that interact with protein. In the future, the use of large or small deformation testing will allow measuring of fractal dimensions without dealing with concentrations problem and complication that arise from preparation of slides (Ikeda et al., 1999; Bi et al., 2013). Nevertheless, microscopic images give the researcher the direct measurement of D_f to be evaluated and many studies were able to achieve clear image of SPI and/or soy glycinin gel (Nagano and Tokita, 2011; Renkema, 2004; Lakemond et al., 2003). However, large deformation testing will allow further investigation of the hardness of the self-supporting gels. These properties are important characteristics that should be investigated to predict the textural change when CPI is added as a food ingredient. However, a preliminary study within the current body of work indicated that achieving the same condition to form self-supporting gels for CPI and SPI was difficult. Thus, finding the processing conditions that suit both SPI and CPI will be the key factors that allow comparison of both proteins.

To further explore and improve the gelation properties of CPI, cross-linking agents (e.g. transglutaminase and genipin) and chemical modification (e.g. succinylation) should be studied. Paulson and Tung (1989) indicated that the use of succinylation on CPI enhance gelation, where the succinylated canola protein isolates produced translucent gel, whereas unmodified canola

protein isolate resulted in the formation of opaque gel. The exploration of protein's surface chemistry and performance as a function of similar parameters studied in current research will give further understanding of CPI. In addition, protein-based gels typically require some level of cross-linking agent or fixative to form a self-supporting rigid network. Microbial transglutaminase (TG) is a commonly used enzymatic cross-linker by the food industry to produce protein gels. Transglutaminase forms inter- or intramolecular cross-links involving the γ-carboxyamide group of a glutamine and the ε-(γ-glutamyl) group of lysine (Folk and Chung, 1973). Pinterits and Arntfield (2008) investigated the effect of TG on CPI gelation, and found that the addition of TG led to the formation of higher molecular weight aggregates, leading to increased protein-protein interactions and stronger gel networks. Tang et al. (2006) reported that when SPI were treated with TG, high molecular weight biopolymers were also produced, and that increasing the protein concentration in solution could reduce the levels of TG. Gelation onset time was reduced by 5 times at 37ºC with use of 0.8 U (units/mL) of TG by increasing the protein concentration from 3% to 8%. A natural chemical covalent fixative, known as genipin (GP) is also gaining importance as a potential alternative to TG. GP has been used to increase the strength of gels, films or microparticles involving various amino-containing polymers such as, chitosan (Butler et al., 2003; Mi et al., 2003), gelatin (Liang et al., 2003; Nickerson et al., 2006) and soy (González et al., 2011). The use of TG and GP to improve the gel strength to from self-supporting gels will allow further exploration of large deformation testing, swelling test and D_f of CPI and protein of interest. Also, the structure alteration of self-supporting CPI gels will allow collection of information about gel strength, pore size, and network formation to give further insight about CPI gel.

Many studies have evaluated the thermal properties of SPI and CPI (Hermansson, 1986; Renkema and van Vliet, 2002; Sallah et al., 2002; Wu and Muir, 2008). In current study, the thermal properties of SPI was not determined using DSC. In future, micro-DSC should be used to increase the sensitivity, in order to measure the protein better. The current study determined no exothermic dips in the thermogram for SPI, where there is possibility of SPI undergone denaturation. Arntfield and Murray (1981) study indicated that the denatured protein show no endothermic dips in thermogram. To strength the study in the future, with addition to commercial SPI, laboratory extracted SPI could be used to compare to CPI. The type of extraction method used to extract SPI in laboratory can further be compared to commercial SPI which gives better

comparison of proteins. Where, the understanding of commercial SPI to CPI is important to evaluate the characteristic of commonly used protein in food to that of less utilized protein. In addition, different extraction protocol can be utilized to extract CPI to have different napin to cruciferin ratio. Yang et al. (2014) extracted napin rich and cruciferin rich using slightly modified of Ismond and Welsh (1992) method. Where, there was noticeable difference in thermal and gelling properties due to their amino acid and molecular weight variation between napin rich and cruciferin rich CPI. The exploration extraction methods and cruciferin to napin ratio can further provide information regards to gelation mechanism and role of cruciferin and napin.

The understanding of ingredients interactions such as polysaccharide is important when CPI is added as food ingredient. It is noted by Uruakpa and Arntfield (2006a), the mixed systems between canola protein isolate and hydrocolloid behave differently when compared to CPI gel. Where, mixture of CPI and κ-carrageenan (κ-CAR) showed less susceptible to environmental change compared to CPI gel. Thus, the addition of other ingredients such as polysaccharide, lipid and sugar can be studied to further explore the CPI gelation mechanism and morphology.

In conclusion, CPI shows potential to be utilized by food industry as a plant protein ingredient. However, further studies should be performed to better understand gelation.

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