FLAXSEED (*Linum usitatissimum* L.) GUM AND ITS DERIVATIVES: PHYSICOCHEMICAL PROPERTIES AND POTENTIAL INTERACTIONS WITH FOOD MACROMOLECULES

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ABSTRACT

Flaxseed (*Linum usitatissimum* L.) gum (FG) is a material with many potential food and non-food applications. Consistent performance is critical for FG utilization and this is possible through selection of genotype, characterization and optimization of constituents, and chemical modification. Physico-chemical and functional properties of FG aqueous solutions from six Canadian flaxseed cultivars were investigated. FG yield, carbohydrate composition, protein content, and zeta potential (ζ) varied among these cultivars. FG solution properties were also affected by temperature, solution pH, NaCl concentration, and sucrose concentration. Detailed studies were conducted on CDC Bethune FG (FGB) proteins that were separated by 2D-gel electrophoresis. Conlinin was identified as the major protein. Protease treatment decreased FGB solution emulsification properties suggesting that conlinin might enhance emulsification. Formation of BSA-FGB coacervates was monitored by turbidimetric analysis as a function of solution pH, biopolymer mixing ratio, NaCl and urea. Coacervates were stabilized primarily by attractive electrostatic forces and secondarily by hydrogen bonds. Further, anionic carboxymethyl ether moieties were introduced to FGB structure through ether forming reactions using monochloroacetic acid (MCA) to produce products with uniform properties. The highest degree of substitution (DS) was obtained at 70 °C, 7.0 M NaOH, and a molar ratio of MCA to FGB of 10:1 over 3 h. Carboxymethylated FGB (CMFG) exhibited both modified surface morphology and thermal behaviour. Solutions of CMFG demonstrated shear-thinning behaviour and apparent viscosity decreased with increased DS. A more liquid-like flow behaviour was observed for CMFG as DS increased. Findings here will introduce and expand FG applications in food or related fields with targeted performance.

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LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
CMFG	Carboxymethyl flaxseed gum
DDA	Data dependent acquisition
DLS	Dynamic light scattering
DS	Degree of substitution
DSC	Differential scanning calorimetry
DTT	Dithiothreitol
EAI	Emulsion activity index
ES	Emulsion stability
FG	Flaxseed gum
FG_B	Flaxseed gum from CDC Bethune
FTIR	Fourier transform infrared
GA	Gum Arabic
HMF	High molecular weight fraction
IAA	Iodoacetamide
IEP	Isoelectric point
MALDI	Matrix-assisted laser ionization
MCA	Monochloroacetic acid
MS	Mass spectrometry
MW	Molecular weight
NCBI	National Center for Biotechnology Information
NMR	Nuclear magnetic resonance
NPFG	Non-protein flaxseed gum
SDG	Secoisolariciresinol diglucoside
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
TEMED	*N,N,N′,N′*-tetramethylethylenediamine
TFA	Trifluoroacetic acid
TFMS	Trifluoromethanesulfonic acid
TOF	Time-of-flight

CHAPTER 1
INTRODUCTION

Natural polysaccharide gums, which represent one of the most abundant industrial materials, are used extensively in food, fine chemicals, pharmaceutical engineering, biomedical engineering, and artificial sensors etc. (Mirhosseini & Amid, 2012). These natural polymers have advantages over synthetic polymers, including their environmental sustainability, low cost, biocompatibility, biodegradability, biosafety, as well as their versatile physical and chemical properties (Prajapati, Jani, Moradiya, & Randeria, 2013). As examples they have excellent suspension, viscosity, rheological properties, flocculation, and adsorption (Jani, Shah, Prajapati, & Jain, 2009).

Flaxseed (*Linum usitatissimum* L.) gum (FG) is by-product of flax oil industry. FG occurs mainly at the outermost layer of flaxseed hulls. FG is water-soluble and, therefore, easily released when seed is soaked in water (Cui, Mazza, & Biliaderis, 1994). FG constitutes approximately 8% of seed dry mass (Oomah, Kenaschuk, Cui, & Mazza, 1995). Structure of FG has been widely investigated with D-xylose, L-arabinose, D-glucose, L-galactose, D-galacturonic acid, and L-rhamnose being major monomer of the polysaccharide polymer (Cui, Mazza, & Biliaderis, 1994; Cui, Mazza, Oomah, & Biliaderis, 1994; Qian, Cui, Nikiforuk, & Goff, 2012; Qian, Cui, Wu, & Goff, 2012). It contains at least two polysaccharide fractions: a neutral fraction (arabinoxylans, 75%, w/w) and an acidic fraction (rhamnogalacturonans, 25%, w/w) (Cui & Mazza, 1996). The neutral arabinoxylans are comprised of β-D-(1,4)-xylan backbones with a molecular weight (MW) of 1,200 kDa. However, Qian, Cui, Wu, and Goff (2012) reported that a fraction of FG with a MW of 1,470 kDa that was thought to be neutral contained a small amount (1.8%) of uronic acid. The acidic fraction was proposed as a rhamnogalacturonan-I (RG-I) backbone that features a diglycosyl repeating unit of [→2)-α-L-Rha*p*-(1→4)-α-D-Gal*p*A-(1→]. Protein has also been observed in extracted FG (Qian, Cui, Nikiforuk, & Goff, 2012) where protein content varies from 4 to 20% of gum dry mass

dependent upon flaxseed cultivar and extraction conditions (Cui, Mazza, Oomah, & Biliaderis, 1994).

As a hydrocolloid, FG shows marked water-holding capacity and rheological properties, as well as weak gel properties. When combined these properties make it possible to consider FG as replacer for most of the non-gelling gums for food and non-food applications (Cui & Mazza, 1996; Chen, Xu, & Wang, 2006). FG can also stabilize oil-in-water emulsions, and proteins in FG are thought to play an important role in its emulsification properties (Bhatty, 1993; Wang et al., 2010b). Moreover, FG also has potential utility as a functional food ingredient that could be used to reduce risks associated with diabetes and heart disease (Cunnane et al., 1993; Oomah & Mazza, 1998). FG rheological properties are dependent on extraction conditions, processing of drying, high-pressure homogenization, extrusion, as well as flaxseed genotype (Cui, Kenaschuk, & Mazza, 1996; Wang et al., 2009b; Wang et al., 2010b; Wang, Li, Wang, & Xue, 2011). However, very little is understood about genotype dependent functional properties of FG and the correlation between FG composition and physicochemical properties. The role of protein in FG in determining functional properties has not been fully elucidated but it's presence may lead to intrinsic disadvantages of FG, such as low dissolution rate in cold water, dull colour of gum solution, and low storage stability. Such properties might limit FG utilization. In the present study, six Canadian flaxseed cultivars (CDC Bethune, CDC Sorrel, CDC Arras, CDC Glas, Vimy, and Flanders) were selected for preparation of FG. The physicochemical properties of FG solutions prepared from each cultivar were investigated. Detailed studies of FG from the most common Canadian cultivar CDC Bethune were performed. Potential interaction of FG with food proteins based on its specific structure features was investigated.

1.1 Objectives

The specific objectives of this research are to: (1) characterize physicochemical properties and functional properties of FG to help to understand the correlation between them; (2) purify and identify FG protein fractions to determine how FG proteins influence solution functional properties; (3) investigate interactions between FG and food proteins; and (4) prepare FG derivatives through chemical modification and examine the potential for using modified FG as a novel food ingredient based on structural characteristics of FG for predicting the structure and property of food system with FG be involved.

1.2 Hypotheses

The following hypotheses will be tested in this project: (1) the chemical composition of FG is determined, in part, by the flaxseed genotype. Therefore the physicochemical, and functional properties of FG solutions prepared from the whole seeds of different flax cultivars will be different; (2) proteins present in FG play an important role in determining emulsification properties of FG solutions; (3) FG could interact with food proteins and even form coacervates when used in food products; and (4) FG with altered functional properties could be used as new food ingredients based on new structural features through chemical modification.

CHAPTER 2
LITERATURE REVIEW

2.1 Flaxseed

Flaxseed or linseed is of Mesopotamic origin. The species name of flax, *ustitatissimum*, means "very useful". Flaxseed has been cultivated for 5,000 years (Carraro et al., 2012). Seed structure includes the embryo or germ, endosperm (inner hull), two cotyledons, spermoderm (Outer hull), and epiderm (Figure 2.1). Flaxseed is flat and oval with a pointed tip. Seed colour varies from dark to yellow while seed texture is smooth and shiny with commercial seed measuring approximately 2.5 × 5.0 × 1.5 mm (Bhatty, 1995). There is substantial variation among all these traits in the species. Flaxseed is grown for oil and fibre in India, Canada, China, United States, Russia, Kazakhstan, and Ethiopia. The oil has been traditionally used for industrial oils but the use in food and feed is increasing (Oomah & Mazza, 1998). The world production of flaxseed is fairly stable during the last decade, averaged 2.3 million metric tons in 2002−2012 and Canada is the world's largest producer of flaxseed which accounts for nearly 80% of the global trade in flaxseed (Rabetafika et al., 2011).

2.2 Composition and Health-Benefits of Flaxseed

Flaxseed products are emerging as a key source of phytochemicals for functional food applications. The seed has high energy density (4.5 kcal/g) and protein content (20%) in addition to being the richest source of dietary α-linolenic acid and lignan. Flaxseed is also a source of soluble fibre, and orbitides and has a considerable potential as a source of phenolic compounds (Oomah, 2001).

2.2.1 Oil in Flaxseed

Flaxseed is cultivated as an oilseed crop and its oil is traditionally utilized as an ingredient in paints, varnishes, linoleum, inks, PVC plastics, and personal care products

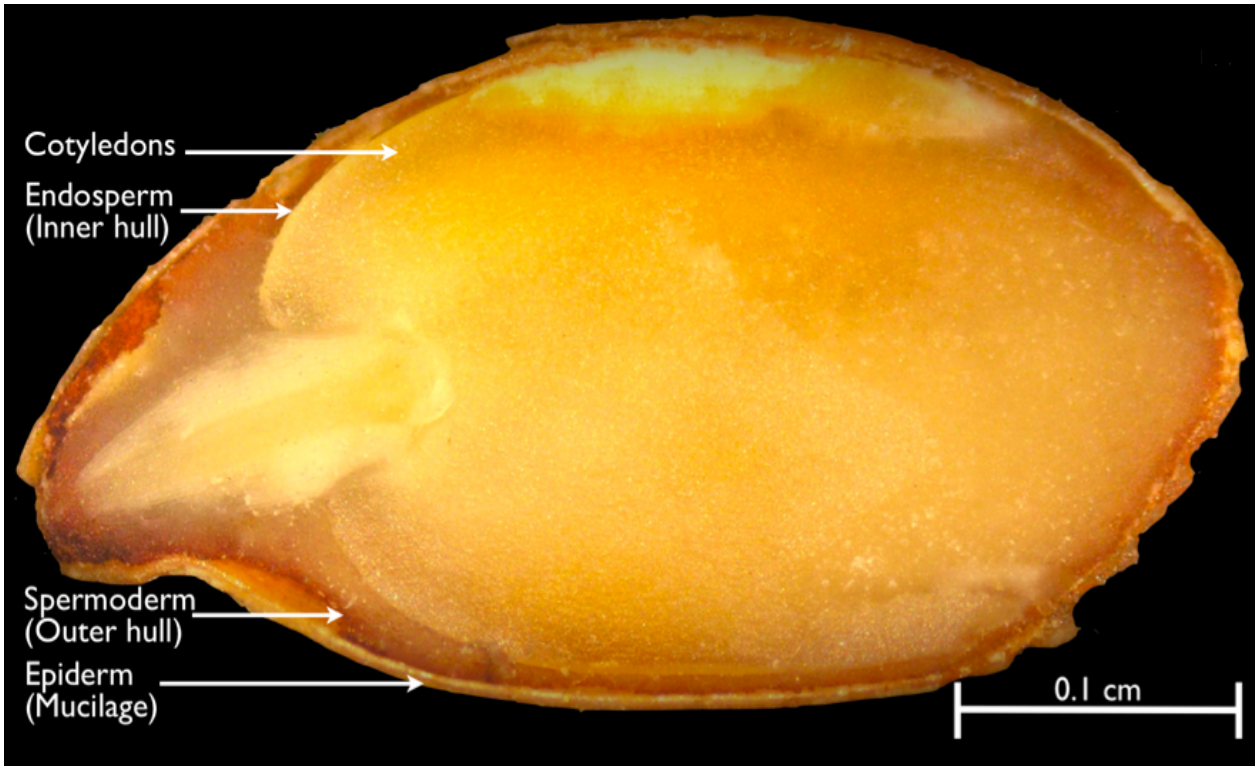

Figure 2.1 Anatomical structure of hand cut flaxseed (*Linum usitatissimum* L., var. CDC Bethune) (Shim, Gui, Wang, & Reaney, 2015).

(Berglund, 2002). Consumption in these applications accounts approximately 70% of global flaxseed oil markets though more recently it is reported to be used in foods including salad dressings and as a partial substitute for milk fat in ice cream desserts (Nykter & Kymäläinen, 2006; Goh, Ye, & Dale, 2006; Lim, Norziah, & Lu, 2010).

Flaxseed lipids include 5 to 6% palmitic acid (16:0), 3 to 6% stearic acid (18:0), 19 to 29% oleic acid (18:1, n-9), 14 to 18% linoleic acid (18:2, n-6), and 45 to 52% alpha-linolenic acid (ALA, 18:3, n-3) (Bhatty, 1995). Though varieties exist with substantially different composition. Flaxseed oil is rich in polyunsaturated fat (73%) and possesses excellent n-6: n-3 fatty acid ratio of approximately 0.3:1. This high ratio makes flaxseed and its oil a useful nutritional supplement to diets with low n-3 fatty acid content (Cunnane et al., 1993). Consumption of flaxseed oil could be utilized to lower the ratio of dietary omega-6 (ω−6) to omega-3 (ω−3). Improved ω-3 content in the diet might mitigate risks of non-communicable chronic diseases (Singh, Mridula, Rehal, & Barnwal, 2011). However, ω-3 fatty acids are sensitive to heat, oxygen, and light and such fatty acids are readily oxidized even though flaxseed oil is rich in anti-oxidants such as beta-carotene. Therefore, flaxseed oil is not recommended as cooking oil and heating should be avoided during flaxseed oil processing (Holstun & Zetocha, 1994). Screw pressing has been widely used in flaxseed oil extraction. When pressing temperature was kept low during oil extraction it is said to be cold pressed. Supercritical carbon dioxide extraction and ultrasonic assisted extraction have also been investigated as methods for extracting flaxseed oil (Zheng, Wiesenborn, Tostenson, & Kangas, 2003; Zhang et al., 2008).

2.2.2 Protein in Flaxseed

Flaxseed contains about 20 to 30% protein by mass, which are primarily albumins (1.6S and 2S, water-soluble) and globulins (11S and 12S, salt soluble). The legumin-like globulin fraction is rich in sulphur amino acids but the 1.6S fraction was reported to act as a sulphur reserve for seed germination (Bhatty, 1995). Flaxseed protein contains a relatively higher proportion of arginine and glutamic acid (Table 2.1) while lysine is the most limiting amino acid followed by methionine and cysteine (Rabetafika et al., 2011). Environmental conditions and genotypic variation are thought to have significant effects on flaxseed protein amino acid composition (Daun & Przybylski, 2000).

Table 2.1 Amino acid composition in flaxseed (Bhatty & Cherdkiatgumchai, 1990; Oomah & Mazza, 1993).

Amino acid	Brown flaxseed (NorLin) g/100 g protein	Yellow flaxseed (Omega) g/100 g protein	Flaxseed mg/g of N
Alanine	4.4	4.5	---
Arginine	9.2	9.4	560
Aspartic Acid	9.3	9.7	---
Cystine	1.1	1.1	120
Glycine	5.8	5.8	---
Histidine	2.2	2.3	120
Isoleucine	4.0	4.0	310
Leucine	5.8	5.9	360
Lysine	4.0	3.9	230
Methionine	1.5	1.4	100
Phenylalanine	4.6	4.7	270
Proline	3.5	3.5	---
Serine	4.5	4.6	---
Threonine	3.6	3.7	210
Tryptophan	1.8	NR	100
Tyrosine	2.3	2.3	180
Valine	4.6	4.7	320

The nutritional value and amino acid composition of flaxseed protein are comparable to those of soy proteins (Moure, Sineiro, Dominguez, & Parajo, 2006; Rabetafika et al., 2011). It has been reported that flaxseed protein can be an effective substitute for wheat flour without loss of texture and swelling reduction during cooking, and has been promoted as egg substitute due to its particular techno-functional properties (Shearer & Davies, 2005). There have also been studies showing potential health benefits of flaxseed protein (Marambe, Shand, & Wanasundara, 2008; Udenigwe et al., 2009). The high level of branched-chain amino acid and comparably lower aromatic amino acids found in flaxseed protein and the low ratio of lysine to arginine content is a potential indicator of anti-cholesterol and anti-tumour properties (Oomah, 2001). Flaxseed is also a source of bioactive orbitides including 11 compounds and their oxidized variants (Shim et al., 2014). In cell cultures and animal models these compounds exhibited immunosuppressive, antiproliferative (T-cell and osteoclast) and antimalarial activities (Möller, Scholz-Ahrens, Roos, & Schrezenmeir, 2008). Marambe, Shand, and Wanasundara (2008) showed that enzyme catalyzed hydrolysis generated flaxseed protein hydrolysates that inhibited angiotensin I-converting enzyme (ACE) inhibiting activity with an IC_{50} of 0.07 mg/mL *in vitro*. ACE is responsible for the regulation of blood pressure in human body and ACE inhibitors could potentially have this effect *in vivo*. Udenigwe et al. (2009) tested enzymatic hydrolysis of flaxseed proteins by various proteases and obtained bioactive peptides with antioxidant and anti-inflammatory properties. Recently, Udenigwe and Aluko (2010) obtained high Fischer ratio peptide mixture from flaxseed proteins with potential anti-hypertensive properties. However, protein hydrolysates are poorly defined materials and they are readily digested to more inert amino acids. It is questionable if this research can be translated into a useful application.

2.2.3 Other Bioactive Compounds in Flaxseed

Flaxseed is a rich source of bioactive phenolic compounds known as lignans. The lignan content in flaxseed was reported to be as high as 1,330 mg/100 g and secoisolariciresinol diglucoside (SDG) is the predominant lignan (Johnsson, Kamal-Eldin, Lundgren, & Aman, 2000). Intestinal microflora can convert SDG to enterodiol (ED) and enterolactone (EL). These compounds have been associated with SDG health benefits due to their antiestrogenic and antioxidant activities. The antioxidant potency of ED and EL are 5.02 and 4.35, respectively, compared to vitamin E (antioxidant potency of 1.00), and are 3.95 and 3.43 times more potent

than SDG (Prasad, 2000). Thompson et al. (2000) reported that dietary modification with flaxseed and its components, such as lignans, could reduce tumor growth in patients with breast cancer in placebo-controlled clinical trials.

Flaxseed also contains significant quantities of both soluble and insoluble dietary fibre. The proportion of soluble to insoluble fibre in flaxseed varies between 20:80 and 40:60 (Morris, 2001). The major insoluble fibre fraction consists of cellulose and lignin, while the soluble fibre fraction or mucilage gum accounts for about 8% of seed mass (Vaisey-Genser & Diane, 2003). Kristensen et al. (2012) introduced flaxseed dietary fibre into different food matrices to determine their effects on blood lipids and fecal excretion of fat and energy by a double blind randomized crossover study. Decreased plasma and low-density lipoprotein cholesterol while increased fat excretion were observed when flaxseed dietary fibre was administered as a drink or in a bread product. This finding indicates that flaxseed dietary fibre could be used as a useful tool for lowering blood cholesterol and potentially plays a role in energy balance. Postprandial lipemia suppression and appetite sensation effects were also reported on flaxseed dietary fibre without any affect on the energy intake (Kristensen et al., 2013). Health Canada approved the labeling of flaxseed products, which indicates that they might lower cholesterol when incorporated in the diet. This labeling is for whole flaxseed products and does not specify gum products.

In addition, flaxseed is a good source of minerals, in particular calcium, magnesium, and phosphorus (Bozan & Temelli, 2008). Flaxseed is also rich in tocopherols, the predominant tocol, γ-tocopherol, is considered to be the most effective against oxidation of low-density lipoprotein LDL (Esterbauer, Schmidt, & Hayn, 1997). The presence of these bioactive flaxseed compounds suggests that flaxseed consumption might offer a wide range of health benefits including anti-inflammatory, Vasodilation, antioxidant, hypocholesterolemic, anticarcinogenic, and attenuation of the postprandial insulin response.

2.3 Flaxseed Gum

Gum, also known as mucilage, is present in the outermost layer (epidermal layer) of flaxseed and makes up approximately 8% of seed mass (Oomah, Kenaschuk, Cui, & Mazza, 1995). It is easily extracted from flaxseed and this extract has potential applications as food hydrocolloid where its thickening and emulsifying properties could prove useful. Aqueous extraction of whole

flaxseed is the most common approach to prepare FG (Ziolkovska, 2012). Researchers have reported different yields and rheological properties depending on extraction conditions and flaxseed genotype (Cui, Kenaschuk, & Mazza, 1996). Cui, Mazza, Oomah, and Biliaderis (1994) optimized FG extraction processes using response surface methodology. Optimum conditions included 85−90 °C water at pH 6.5−7.0 with water to seed ratio of 13:1. FG extracted under optimum conditions exhibited pronounced shear-thinning behaviour and 'weak gel' like properties (FG concentration of 20 g/kg) with low protein content (< 80 g/kg seed mass equivalent to bovine serum albumin, BSA). In general, soluble dietary fibre lowers blood cholesterol/triglyceride lipid levels and/or postprandial blood glucose (Kristensen et al., 2012). FG could be used more as a food hydrocolloid and the application for this purpose is a subject of considerable research (Figure 2.2).

2.3.1 FG Polysaccharides

FG may be separated into two different polysaccharide fractions with very different properties: a neutral fraction and an acidic fraction. The neutral fraction is composed of arabinoxylans with β-D-(1,4)-xylan backbone and is free of uronic acid. The acidic fraction consists mainly of pectic-like polysaccharides containing L-rhamnose, D-galactose, and D-galacturonic acid (Cui, Mazza, & Biliaderis, 1994; Cui, Mazza, Oomah, & Biliaderis, 1994; Qian, Cui, Nikiforuk, & Goff, 2012; Qian, Cui, Wu, & Goff, 2012). Cui, Kenaschuk, and Mazza (1996) investigated the influence of genotype on FG chemical composition. FG extracted from yellow seed flaxseed cultivars contained lower contents of rhamnose (12.8–14.4%) and galacturonic acid (13.8–16.2%), but much higher contents of neutral sugars (e.g. xylose, 39.0–48.7%) than those extracted from the brown seed flaxseed cultivars. However, Qian, Cui, Wu, and Goff (2012) reported that a neutral fraction of FG with molecular weight (MW) of 1,470 kDa contained a small amount (1.8%) of uronic acids. This fraction exhibited pseudo-plastic flow behaviour. The FG acidic fraction is mainly composed of rhamnogalacturonans with a higher MW fraction (1,510 kDa) and a lower MW fraction (341 kDa). This fraction exhibited Newtonian flow behaviour. Similar results were reported by Warrand et al. (2003) with neutral polymer (1.2×10^6 g/mol) made up to 75% of FG. The two acidic fractions account for 3.75% and 21.25% of FG with MWs of 6.5×10^5 g/mol and 1.7×10^4 g/mol, respectively. The structure of the rhamnogalacturonans from flaxseed hulls was partially elucidated by Qian, Cui, Nikiforuk, and Goff (2013) through methylation analysis and 1D/2D NMR spectroscopy (Figure 2.3). A possible structure of the

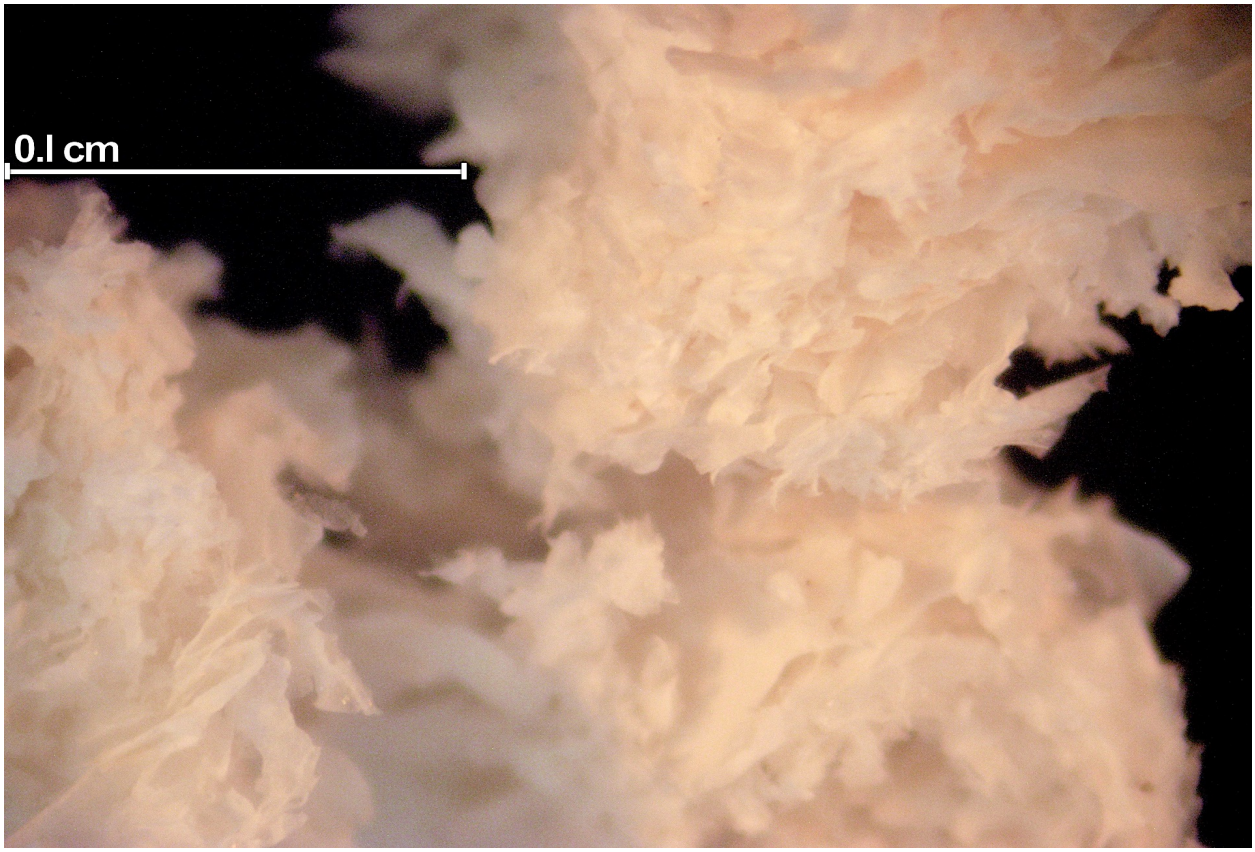

Figure 2.2 FG (*Linum usitatissimum* L., var. CDC Bethune). Images were obtained (1000 magnification) with a Canon Eos 300D digital camera mounted on a Zeiss Stemi SV 11 light microscope. The images were subsequently processed in Photoshop 7.

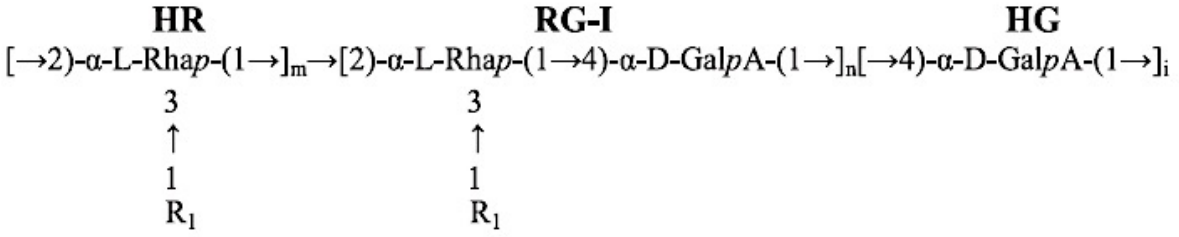

Figure 2.3 Proposed structure of acidic fraction in FG. HR, RG-I, and HG refer to homorhamnan, rhamnogalacturonan-I, and homogalacturonan, respectively. R_1 is mostly a monosaccharide (α/β-D-Gal*p*-(1→, α-L-Fuc*p*-(1→ or β-D-Xyl*p*-(1→). Occasionally R_1 is a longer side chain with more than two residues beginning with →4)-α-Gal*p*A-(1→ or →2)-α-L-Rha*p*-(1→ (Qian, Cui, Nikiforuk, & Goff, 2012).

acidic fraction was proposed as a rhamnogalacturonan-I (RG-I) backbone that features diglycosyl repeating units →2)-α-L-Rha*p*-(1→4)-α-D-Gal*p*A-(1→.

2.3.2 FG Proteins

Food hydrocolloids can be characterized by their functional properties that enable their use in preparing materials that gel, thicken, stabilize, disperse, foam, emulsify, and hold water. When present, protein contributes to hydrocolloid functional properties. Protein has been found in many polysaccharide gums, such as pectin, gum Arabic, microcrystalline-cellulose, galactomannans, and soy soluble polysaccharide (Funami et al., 2007). During FG extraction with water, protein is also extracted. Under optimum conditions proposed by Cui, Mazza, Oomah, and Biliaderis (1994), the gum protein content was lower than 80 g/kg seed mass. Qian, Cui, Wu, and Goff (2012) determined the protein content in FG fractions and no protein was detected in the neutral fraction. Acidic polysaccharide fractions of FG had substantial nitrogen content and if this nitrogen was from protein it could represent up to 8% of the fraction dry mass. Nitrogenous compounds were not covalently linked to FG carbohydrates. After protease hydrolysis of FG a slight surface tension decrease was observed. There was, however, no significant difference between the zero-shear viscosity FG solutions prepared before and after protease hydrolysis (Qian, Cui, Wu, & Goff, 2012). The proteins present in FG have not yet been identified and their contribution to functional properties remains unknown.

2.4 FG Physicochemical Properties

2.4.1 FG Rheological Properties

Rheological properties of polysaccharide gum solutions in water provide important information about structure-function relationship and give guidance toward their potential applications. Like other polysaccharide gums, crude FG exhibited shear-thinning behaviour at concentrations above 1.0% (w/w) over a broad range of shear rates (0.1−1000 s^{-1}) and ‘weak gel’ like properties over the entire frequency range tested. Similar shear-thinning behaviour was also observed for FG with the same concentration after dialysis (Cui, Mazza, & Biliaderis, 1994). The neutral fraction of FG exhibited shear-thinning behaviour at concentrations above 0.5%. Such shear-thinning might be attributed to high arabinoxylan molecular mass. Solutions made with this polymer displayed Newtonian-like properties at concentrations lower than 0.3%.

Newtonian like flow curves were obtained for solutions prepared from FG acidic fractions at all concentrations studied. This finding could be explained by the much smaller molecular size of polysaccharide in FG acidic fraction (Cui, Mazza, & Biliaderis, 1994). FG solutions made from fractions that contained higher arabinoxylan content exhibited similar shear-thinning behaviour as well as 'weak gel' like properties. Solutions made from fractions containing greater amounts of acidic polysaccharides typically behaved like viscous fluids. Rheological properties of FG solutions are related to flaxseed genotype. Solutions prepared from FG extracted from yellow seeded cultivars exhibited stronger rheological properties than solutions prepared from FG from brown seeded cultivars (Cui, Kenaschuk, & Mazza, 1996). Warrand et al. (2005b) reported the presence of large aggregates in FG solution during the filtration procedure when the concentration was made up to 20 g/L. Those association tendencies are thought to be formed by intermolecular associations *via* hydrogen bonding, which lead to pseudo gel behaviour. Using dynamic light scattering Goh, Pinder, Hall, and Hemar (2006) observed a very large molecular species with a hydrodynamic radius in excess of 100 nm in deionized water. The intrinsic viscosity (1030 ± 20 mL/g) generated by this fraction may be due largely to the hydrodynamic volume of the large MW species, which aggregates in water.

The rheological properties that FG imparts on solution can be significantly affected by the drying method employed after extraction. FG solutions prepared from FG that was precipitated with ethanol had greater apparent viscosity than solutions prepared from FG that was dried in other ways. Freeze drying, 105 °C oven drying, 80 °C oven drying, spray drying, and vacuum drying produced FG that imparted less apparent viscosity (Wang et al., 2009b). High-pressure homogenization modified FG solution rheological properties resulting in decreased apparent viscosity, storage, and loss moduli (Wang, Li, Wang, & Xue, 2011). Wu et al. (2010) investigated the influence of both extrusion and enzyme treatment (Pectinex Ultra SP-L, 30000 UPTE mL^{-1} and Pectinex Smash XXL, 26000 PG mL^{-1}) conditions on FG solution rheological properties. They reported that solution consistency index decreased when extrusion temperature was increased and the screw speed was decreased. Decreased apparent viscosity of solutions prepared from the extracted FG was observed during enzymatic treatment. With increased hydrolysis time and enzyme loading the hydrolysis of mucilage polysaccharides was evident. In addition, extrusion can damage compact fibre structure, which improved the accessibility of enzyme.

2.4.2 FG Solution Gelation Properties

A gel is an intermediate between a solid and liquid possessing both elastic (solid) and flow (liquid) characteristics. Aqueous gels are high moisture three-dimensional polymeric networks. Gel viscoelastic properties are described by a storage modulus (G′) and a loss modulus (G″). These moduli describe how a gel can resist flow under pressure and retain a distinct shape (mechanical rigidity) (Banerjee & Bhattacharya, 2012). Based on macroscopic behaviour, gel systems can be divided into two classes: "true gels'' and ''weak gels''. Two main gelation mechanisms are involved in gel formation: cooling or heating. Gels are separated into two categories: ''cold-setting'' and ''heat-setting'' (Doublier, Launay, & Cuvelier, 1992). All gel-forming properties are highly dependent on the intrinsic attributes of the gelling agents.

Functionally, FG resembles gum Arabic more closely than other common gums and it can be used to replace most non-gelling gums for food and non-food applications due to its 'weak gel'-like property and remarkable water-holding capacity (Bhatty, 1993). The formation of FG gel was studied by Chen, Xu, and Wang (2006) and a thermo-reversible cold-setting gel was formed with maximum gel strength at pH 6−9. Increased gel strength was observed when calcium chloride (< 0.3%, w/w) was introduced into the gelation system to form three-dimensional Ca^{2+} cross-linked networks. Higher concentration of calcium chloride had negative effects on FG solution gel strength as it decreased zeta potential. In order to better understand solution gel structure formed by FG, Wang et al. (2011b) performed fractal analysis on FG gels. Analysis showed that both the rheological properties and fractal dimensions could be significantly influenced by solution ionic strength. The highest gel storage modulus was observed at 400 mM.

Gel formation of FG was prepared with other polysaccharides or proteins. Mixed FG-casein gels were prepared in water by Li et al. (2012) with FG concentrations ranging from 0.1 to 0.5% (w/w). The storage (G′), loss (G″) moduli, gelling temperature, and apparent viscosity of the gelled solutions increased with increased FG concentration. Mixed cold-set whey protein isolate-FG gels were produced in water by the addition of $CaCl_2$ or NaCl at fixed ionic strength (150 mM) (Kuhn, Cavallieri, & da Cunha, 2011). However, FG concentration increases reduced gel strength and water holding capacity of the mixed gels. This phenomenon was thought to be associated with phase separation between the biopolymers during the gelation process. When FG was added to native maize starch in water, a gel-like behaviour was found since the storage

modulus (G′) was much larger than the loss modulus (G″) and frequency dependence of both moduli was not significant. The apparent solution viscosity increased with increasing FG concentration with less significant influence by temperature compared to maize starch aqueous gels (Wang et al., 2008).

2.4.3 FG Emulsification Properties

Like most hydrocolloid forming materials, FG can also stabilize oil-in-water emulsion and has been used in preparing emulsion together with whey protein isolate or soybean protein isolate. Qian, Cui, Wu, and Goff (2012) reported the emulsification properties of FG extracted from flaxseed hulls. Hydrolysis of FG protein in solution reduced both surface activity and emulsion stability. During FG processing, the drying method has significant effects on the functional properties FG imparts on solutions. Gels prepared with oven dried (105 °C) FG powders had the best foaming capacity and exhibited the greatest foam stability (Wang et al., 2010b). Khalloufi, Alexander, Goff, and Corredig (2008) described FG emulsification behaviour where FG prepared from the cultivars Emerson and McDuff was included in whey protein coated oil-in-water emulsions. Under neutral pH, emulsion stability was strongly affected by the amount of FG added and depletion flocculation was caused by incompatibility between polysaccharides and whey protein isolates-coated droplets. When pH was lowered to 3.5, the whey protein stabilized oil-in-water emulsion droplet zeta potential decreased from around +30 mV to −10 mV at FG concentrations > 0.2% indicating that the negatively charged FG polysaccharide interacted with the protein adsorbed at the oil-water interface (Khalloufi, Corredig, Goff, & Alexander 2009). Wang, Li, Wang, and Adhikari (2011) determined the effect of adding FG on the stability of soybean protein isolate emulsions. They found that emulsion stability (turbidity) and zeta potential decreased at low gum concentrations (< 0.1%) and increased at higher concentrations (0.1–0.15%).

2.4.4 Potential FG Applications

FG was evaluated as a steric stabilizer for use in salad dressing. It was hypothesized that it would adsorb at the surface of egg yolk emulsion droplets. The most stable salad dressing emulsion was prepared at 0.75% (w/w) FG, 2.5% (w/w) salt at pH 4.0 (Stewart & Mazza, 2000). A desirable synergistic interaction between FG and meat protein was produced with increased

thermal stability of salt-soluble meat protein, as well as increased storage modulus G′, gel strength and decreased synergies of salt-soluble meat protein gels. These favourable interactions of FG with meat protein indicate a potential commercial application for it in meat products (Chen, Xu, & Wang, 2007). Sun, Li, Xu, and Zhou (2011) examined the effects of adding FG to porcine myofibrillar protein (PMP) on water holding capacity and observed a significant decrease in water mobility in PMP due to strengthened electrostatic attraction. This property can be used to modify the yield and texture of related products. When FG was mixed with carrageenan and gellan gum it improved the quality of starch-free emulsion-type sausage. FG contributed to enhanced emulsion stability, sausage product springiness, and sausage redness (Zhou et al., 2010). FG exhibits macromolecular steric repulsion, which is useful in its application as a stabilizer in cloudy carrot juice and a texture ingredient in dairy desserts (Qin, Xu, & Zhang, 2005). Long et al. (2012) immobilized FG to produce gel beads for the adsorption of oil from oily wastewater. Bead performance surpassed that of activated carbon indicating the potential to use them as an environmentally-friendly material for wastewater treatment. Moreover, FG has nutritional value as a dietary fibre, which plays a role in reducing diabetes and coronary heart diseases risk, preventing colon and rectal cancer, and decreasing the incidence of obesity (Thakur, Mitra, Pal, & Rousseau, 2009). In comparison with other commercially available gums, FG imparts lower viscosity. Its low viscosity is favoured in fortification of food dietary fibre, which can improve the sensory attributes without leading to an over-texturization as can occur when a significant concentration of fibre is required to show health benefits (Ibrügger, Kristensen, Mikkelsen, & Astrup, 2012).

2.5 Polysaccharide Gum Structure Modifications

Polysaccharide gums show significant advantages over synthetic materials due to their sustainability, biodegradability, and biosafety. Many natural gums can form three-dimensional interconnected molecular networks known as ‘gels’, which enables their application in different areas based on specific functional properties. However, natural gums do have inherent problems associated with their uses including uncontrolled rates of hydration, pH dependent solubility, thickening, drop in viscosity on storage, and the possibility of microbial contamination (Rana et al., 2011). Chemical modification of gums not only minimizes those drawbacks but also enables their application for specific purposes.

2.5.1 Polysaccharide Gum Crosslinking

Polysaccharides may be cross-linked to alter their swelling properties and to prepare novel hydrogels. The properties of cross-linked gum derivatives depend mainly on cross-linking density (the ratio of moles of cross-linking agent to moles of polymer repeating units). Cross-linking of gums requires availability of active functional groups, including free alcohols, carboxylic acids, polyacrylamide, and phosphate. Ionic bonds are the main interactions of network forming reactions but other interactions, such as hydrogen bonds and hydrophobic interactions also influence properties (Rana et al., 2011).

Many crosslinkers such as multivalent metal ions (Ca^{2+} and Ba^{2+}), trisodium trimetapolyphosphate, acrylamide, glutaraldehyde, and epichlorohydrin have already been used for crosslinking polysaccharides. Bajpai, Saxena, and Sharma (2006) prepared barium ion-cross-linked sodium alginate-carboxymethyl guar gum (CMGG) beads through a gelation process. The biopolymeric beads demonstrated pH-sensitive swelling and were quite stable in the gastrointestinal tract offering the possibility for utilization as drug delivery matrices. Konjac glucomanan (KG) gel was also prepared by crosslinking non-ionic polysaccharides extracted from *Amorphophallus konjac* tubers with organic borate (Gao, Guo, Wu, & Wang, 2008). Hyaluronan and Guar gum based hydrogels were prepared by crosslinking with different amounts trisodium trimetaphosphate (STMP) (Gliko-Kabir, Yagen, Penhasi, & Rubenstein, 2000). Swelling of these cross-linked hydrogels in gastrointestinal fluids was dramatically reduced and highly dependent on the amount of cross-linker used. Other kinds of polysaccharides, including pullulan and hyaluronan, were also cross-linked using the cross-linker trisodium trimetaphosphate (Dulong et al., 2004; Lack et al., 2004). Wen et al. (2009) prepared gels composed of KG, copolymerized with acrylic acid (AA) and cross-linked by *N,N*-methylene-bis-(acrylamide) (MBAAm) then determined the swelling properties. The polymer cross-linking density was inversely related to swelling. Glutaraldehyde aldehyde groups react with polysaccharide hydroxyl groups to form cross-linked acetals. As such, glutaraldehyde has been used extensively for producing polysaccharide polymers. For example, alginate/guar gum hybrid hydrogels were prepared with varied alginate to guar gum mass ratios. Glutaraldehyde crosslinking was achieved but the cross-linking efficiency was low (George & Abraham, 2007). Soppirnath, Kulkarni, and Aminabhavi (2000) reported the preparation of an interpenetrating network microsphere of polyvinyl alcohol and guar gum. The microspheres could be used as

delivery tools for nifedipine, an antihypertensive drug. In addition epichlorohydrin was also reported to cross-link cashew gum after mixing with sodium hydroxide solution. Crosslinking density changed both thermal stability and swelling properties of the product (Silva et al., 2006).

2.5.2 Polysaccharide Gum Esterification

Esterification has been employed as a common method to modify polysaccharide gum physio-chemical properties and improve the utilization of the starting material. Polysaccharide gums may be handled as thermoplastic materials after esterification to facilitate processing. Esterification has additional effects on polysaccharide gum properties as esterified products may have better thermal stability, reduced water absorbency, increased solubility, and reduced crystallinity. Polysaccharide gum ester structures can be modified using esterification protocols such as acetylation (and other carboxylic acid esterification), succinylation, sulfation, and tosylation (Figure 2.4).

Arabinoxylan esters derived from arabinoxylan isolated from corn fibre were prepared by esterification with C2–C4 aliphatic anhydrides using a methanesulfonic acid catalyst (Buchanan et al., 2003). The reaction, which achieved a degree of substitution (DS, average number of substituent groups attached per monomeric unit) of up to 2.1, was conducted over 1 hour at 50 °C. The arabinoxylan esters were soluble in a range of organic solvents with improved thermal-stability (up to almost 200 °C). Pullulan acetate was prepared by reaction of pullulan with acetyl chloride in the presence of pyridine. Increasing the degree of acetylation both improved thermal-stability and reduced biodegration rates (Teramoto & Shibata, 2006). Wheat straw hemicellulose esters were prepared by succinolation in a DMF/LiCl system with pyridine and/or 4-dimethylaminopyridine (DMAP) as the catalyst. Under optimum reaction conditions, 1.5 DS was achieved or 78% of the hemicellulose free hydroxyl groups were succinylated (Fang, Sun, Tomkinson, & Fowler, 2000). Abatangelo, Barbucci, Brun, and Lamponi (1997) reported the synthesis of new heparin-like polysaccharides through sulfation of hyaluronic acid. The sulfated polysaccharides had significantly improved biocompatibility and resistance to enzymatic degradation. Gericke et al. (2012) studied tosylation of cellulose in mixtures of ionic liquids and co-solvents. Due to the beneficial effect of co-solvents, the reaction could be performed at 25 °C without the need for heating (in order to reduce viscosity) or cooling (in order to prevent side reactions). The predominant substitution observed occurred at the primary hydroxyl group of

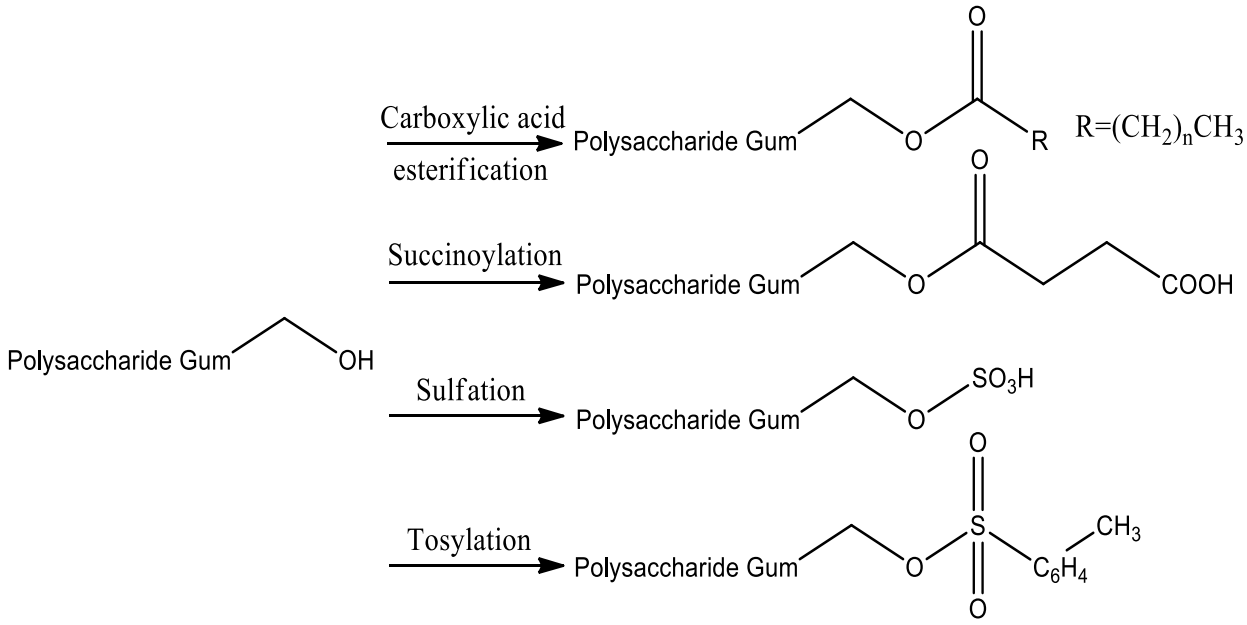

Figure 2.4 Polysaccharide gum ester products.

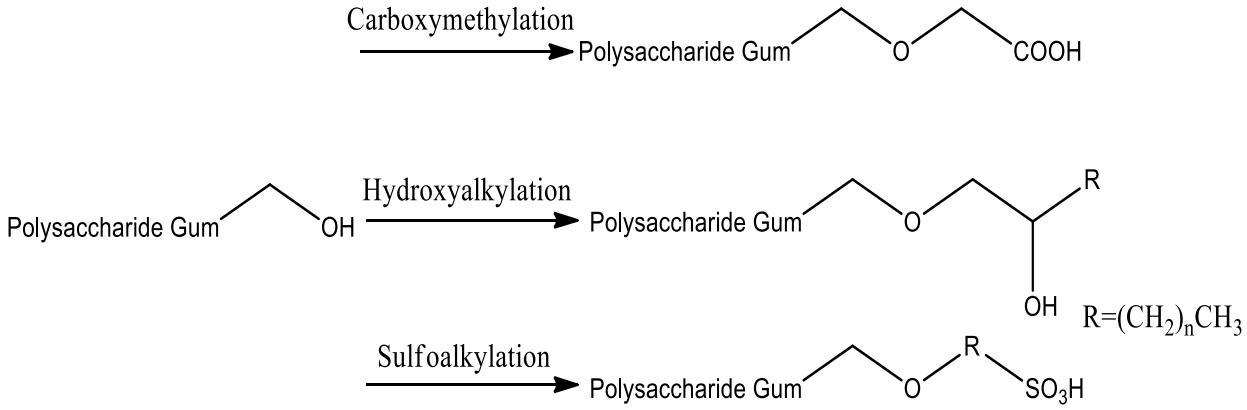

Figure 2.5 Scheme of etherification of polysaccharide gums.

cellulose. Only a moderate degradation of the polymer backbone occurred during the reaction.

2.5.3 Polysaccharide Gum Etherification

Etherification of polysaccharides, including carboxymethylation, hydroxyalkylation, and sulfoalkylation (Figure 2.5), has been wildly investigated by researchers for achieving specific structure modifications and altered physicochemical properties of the reacted materials. Etherification can alter solubility, film-forming ability, and viscosity. In addition, ether products might have stability against microorganisms and resist fermentation. These properties are helpful for polysaccharides utilization in mining, paper, and textile industries.

Guar gum can only be partially dissolved in water and aqueous ethanol solutions. However, after carboxymethylation with monochloroacetic acid in sodium hydroxide solution, guar gum was completely soluble in water and insoluble in ethanol, irrespective of the DS. Regardless of the carboxymethyl content, aqueous solutions of carboxymethylated polysaccharides demonstrated non-Newtonian pseudoplastic behaviour (Gong et al., 2012). All derivatives showed increased stability toward microorganisms (Ragheb, Kamel, Elthalouth, & Nassar, 1994). Khan and Pitochumani (1995) reported hydroxypropylation of guar gum with propylene oxide in 2-propanol under alkaline conditions. These products were intended for use in cosmestics, as a binder and thickening agent in adhesives and as viscosifiers in fracturing fluids. The hydroxypropylation of xylan was investigated by Jain, Sjöstedt, and Glasser (2001) through a reaction in homogeneous aqueous alkaline solution at room temperature with propylene oxide. The DS varied from 0.2 to 1.9 and was found to depend on reaction conditions, especially pH. Hemicellulose obtained from hardwood sulfite and sulfate pulps were sulfopropylated by dimsyl and propanesulfone in dimethyl sulfoxide solution. The reaction conditions led to degradation of hemicellulose polymers (Focher, Marzetti, Naggi, & Torri, 1989).

2.6 Protein and Polysaccharide Gum Interactions

Both proteins and polysaccharides are natural biopolymers that play a key role in food system structuration and stability. These components impart many functional attributes, such as thickening effects, gel-forming capacity, water holding properties, foaming, and stabilization effects on food emulsions. The functional properties of individual biopolymers are highly dependent on their structure and molecular characteristics, including monomer composition,

chain length, monomer bonding, charge, flexibility, and hydrophobicity (de Kruif, Weinbreck, & de Vries, 2004). However, during manufacturing processes, novel or improved functionalities can often be engineered into food systems by biopolymer interactions. The properties of biopolymer complexes reflect not just functional properties of each component they also reflect the complex interaction of multiple components. There has been considerable scientific and industrial interests in understanding interactions between biopolymers in solutions and gels (Schmitt, Sanchez, Thomas, & Hardy, 1998). Complexes formed between biopolymers are to impart desired properties to foods, cosmetics, pharmaceutics, and medicines.

2.6.1 Protein-Polysaccharide Interactions

When biopolymers approach in solutions or gels, either repulsive or attractive interactions determine the functional properties of the biopolymer system (Tolstoguzov, 2003; Turgeon, Beaulieu, Schmitt, & Sanchez, 2003). Electrostatic interactions produce profound effects in biopolymer interactions between charged species. For example, electrostatic attraction is the primary driving forces for complex coacervate formation of gelatin and acacia gum (Tiebackx, 1911). For a protein-anionic polysaccharide biopolymer system, electrostatic interaction can be either attractive or repulsive. Where solution pH is greater than protein pI a repulsive interaction will dominate interactions, as both biopolymers possess negative charges. However, when pK_a of anionic polysaccharide is less than solution pH that is, in turn, less than protein pI, attractive electrostatic interactions will occur (Tolstoguzov, 1997; de Kruif, Weinbreck, & de Vries, 2004). Both attractive interaction of polyions and liberation of counterions along with water molecules contribute to formation of biopolymer complexes (Turgeon, Beaulieu, Schmitt, & Sanchez, 2003). Acid titration is usually employed for titrating pH and in studies of biopolymer complex stability, which is dependent on acid added into the biopolymer system (Dickinson, 1998; McClements, Decker, Park, & Weiss, 2009). Electrostative interactions between biopolymers also rely on charge groups on biopolymer structures and other solution conditions (ionic strength, temperature, destabilization agents, shear, etc.) (Piculell & Lindman, 1992). Typically, biopolymer complexes can be either soluble or insoluble depending on biopolymer net charge. Soluble complexes would be formed due to the solubilization effects of net charges. However, when charges are neutralized, the complexes become insoluble (Tolstoguzov, 1997). Human serum albumin was interacted with dextran, carboxymethyl cellulose, and cellulose sulfate

polysaccharides. Mixtures of human serum albumin-carboxymethyl cellulose and human serum albumin-cellulose sulfate formed complexes at pH 5.2 and 6.5, respectively. However, no complex was observed for mixtures of dextran and human serum albumin, which confirmed the important role electrostatic interactions in complex formation (Noguchi, 1956).

Non-electrostatic interactions also contribute to formation of biopolymer complexes. When biopolymers interact in solution or gels, hydrogen bonding, hydrophobic interactions, covalent bonds, and steric exclusion effects also affect complex formation. Non-electrostatic interactions depend on biopolymer composition and structure (Xia, Dubin, & Kakofuta, 1993). Hydrogen bonding between hydrogen atoms attached to electronegative atoms in gelatin molecules (i.e., nitrogen, oxygen, or sulfur) interact with hydrogen atoms attached to electronegative atoms in pectin (i.e., oxygen of carbonyl or carboxyl group) (Dickinson, 1998; Goh, Sarkar, & Singh, 2009). H-bonds enable formation of molecular structure domains between biopolymers, such as helical and sheet-like regions. In addition, these bonds stabilize aggregate and even biopolymer gel formation (McClements, Decker, Park, & Weiss, 2009). Hydrogen bonding is weakly ionic in nature and hydrogen bond strength decreases as temperature increases (Dickinson, 1998). For gelatin and alginate system where solution pH was greater than gelatin pI, biopolymer complexes were formed by hydrogen bonding rather than electrostatic interactions (Antonov et al., 1996). Hydrophobic interaction originates from structural changes to water molecules surrounding biopolymers that induce the association of non-polar groups of biopolymers in solution (Tolstoguzov, 1997). Hydrophobic interaction is also sensitive to temperature but the strength tends to increase when temperature increases (McClements, Decker, Park, & Weiss, 2009). Moreover, the strength of hydrophobic interaction is also promoted by biopolymer conformational and structural changes. The conformational and structural changes induce solvent rearrangement around the biopolymers. The biopolymers then unfold and exposure more hydrophobic domains enabling the formation of bonds between hydrophobic domains on adjacent molecules (Dickinson, 1998). Thermal pretreatment of a whey protein-xanthan system increased formation of coacervates and increased the stability of biopolymer complexes formed due to increased hydrophobic interaction (Weinbreck, Nieuwenhuijse, Robijn, & de Kruif, 2004a).

2.6.2 Parameters Affect Protein-Polysaccharide Interaction

2.6.2.1 External Parameters

Many parameters, including internal and external parameters, can strongly influence the complex formation between biopolymers in solution. Complex coacervate formation between biopolymers is driven primarily by electrostatic interactions, thus, solution pH of biopolymer blends plays a key role in mediating ionization of amino and carboxylic acid groups (Schmitt, Sanchez, Thomas, & Hardy, 1998, Ye, 2008). For a typical protein-polysaccharide system, complexes would be formed when protein and polysaccharide possess opposite net charge (Dickinson, 1998; Ye, 2008). The strongest complex coacervate is formed due to the maximum electrostatic attractions when there is zero net charge of the biopolymer system, which carry equal and opposite charge (Schmitt, Sanchez, Thomas, & Hardy, 1998). The ionic strength of the biopolymer solution is another important factor that can affect polymer complex formation. Low MW ions present in the biopolymer system would neutralize charge groups on biopolymer structure and hinder the liberation of counter ions associated with the biopolymers. Such ions would decrease electrostatic attraction and decrease the strength of biopolymer complexes formed in their presence (Xia, Dubin, & Kakofuta, 1993). Interestingly, low ionic strength solutions could facilitate biopolymer interactions due to salting-in effects that improve biopolymer solubility (Schmitt & Turgeon, 2011). For a whey protein-carrageenan mixture, the maximum biopolymer interaction occurred at a NaCl concentration of 45 mM. The NaCl here was thought to screen the residual negative charges of carrageenan and promote the exposure of more active sites on protein structure to interact with carrageenan (Weinbreck, Nieuwenhuijse, Robijn, & de Kruif, 2004a).

Biopolymer ratio affects biopolymer interaction due to its influence on biopolymer available sites. Complexes between biopolymers may not form if the biopolymer ratio is “high” with one of the biopolymers is in excess. In this case, charge neutralization cannot be reached and solubility is encouraged (Turgeon & Laneuville, 2009; Schmitt, Aberkane, & Sanchez, 2009). The total biopolymer concentration is also critical for complex coacervate formation. At a high biopolymer concentration, phases may separate due to thermodynamic incompatibility caused by competition of macromolecules for solvent (Tolstoguzov, 1986, 1997). Other physical and mechanical parameters, including temperature, shear rate, shear duration, and pressure, can also affect biopolymer complexes formation and stability (Kelly, Gudo, Mitchell, & Harding, 1994; Tirkkonen, Turakka & Paronen, 1994; Dickinson & Pawlowsky, 1996). Temperature is known to favour several non-electrostatic interactions (Tolstoguzov, 1997; Schmitt, Sanchez,

Desobry-Banon, & Hardy, 1998). Conversely, high temperatures might denature biopolymers resulting in biopolymer conformation and structure changes. Heat treatment of BSA at temperatures higher than 70 ºC enhanced complex coacervate formation with alginate. Heating denatured the BSA and this led to exposure of hydrophobic regions of the protein. The exposed hydrophobic regions favoured the hydrophobic interaction to overcome electrostatic repulsions (Harding et al., 1993). High-pressure treatment can also induce conformation and structure changes in biopolymers (Schmitt, Aberkane, & Sanchez, 2009). Upon high-pressure treatment, a biopolymer system of ovalbumin with both dextran sulfate and ι-carrageenan produced biopolymer complexes under low ionic strength conditions at pH 6.5. Partial denaturation of ovalbumin increased surface hydrophobicity and the number of charged groups were thought responsible for such complex formation (Galazka, Smith, Ledward, & Dickinson, 1999).

2.6.2.2 Internal Parameters

Biopolymer shape, size, conformation, flexibility, and net charge are determined by solution conditions and the structural characteristics (Tolstoguzov, 1997). Biopolymer charge density describes the number of charged moieties for a given area on the biopolymer (Schmitt, Aberkane, & Sanchez, 2009). Biopolymer charge density determines the strength of electrostatic attractive interactions and the structure of biopolymer complexs (Doublier, Garnier, Renard, & Sanchez, 2000; Schmitt, Aberkane, & Sanchez, 2009). Generally, higher charge density such as sulphate side chains favours stronger electrostatic interactions between biopolymers. A more compact complex structure would be formed due to improved local biopolymer dehydration (Schmitt, Aberkane, & Sanchez, 2009; Girard, Turgeon, & Gauthier, 2002a). Higher MW biopolymer could lead to larger occupied volume in solution, producing more available binding sites for other biopolymers under suitable solution conditions (Schmitt, Aberkane, & Sanchez, 2009). Protein and xanthan gum formed larger and more linear electrostatic complexes when higher molecular weight biopolymers were used (Laneuville et al., 2005). Biopolymer contour length, chain flexibility, and protein dimensions were also reported to affect biopolymer interactions. Biopolymers with a more compact conformation have higher charge density and induce stronger charge interactions (Turgeon & Laneuville, 2009). Proteins with more flexible structures (e.g. caseins or gelatin) can bind to polysaccharide more strongly as compared with globular proteins (e.g. BSA or BLG). Thermal denaturation of globular proteins will open up

more interacting groups with improved polysaccharide binding affinity (Doublier, Garnier, Renard, & Sanchez, 2000).

2.6.3 Protein-Polysaccharide Interaction in Food Applications

2.6.3.1 Food Texture Modifier

Biopolymer complexes formed by protein-polysaccharide interaction can serve as food texture modifiers in food products. For homogenized biopolymers in solution, rheological properties are highly dependent on biopolymer concentration, structure features (size, shape, and molecular flexibility), and the extent of biopolymer-solvent interactions. All these properties contribute to the resistance of the biopolymer solution to flow (viscosity): a fundamental rheological parameter (Schmitt & Turgeon, 2011). Biopolymer interactions alter biopolymer rheological properties. Generally, biopolymer interactions that form coacervates with greater particle size exhibit greater viscosity and typical shear-thinning flow behaviour. Shear-thinning flow behaviour is caused by phase separation during the shearing of the polyelectrolyte/micelle system (Liberatore et al., 2009). Characteristics of biopolymer particles, such as volumic fraction, surface charge density, and thickness of the hydration layer, size, shape, and deformability also affect the rheological properties (Schmitt & Turgeon, 2011). When acacia gum was used to form a coacervate with protein, globular particles were formed demonstrating typical viscous flow behaviour. The maximum viscosity was achieved when the net charge neutralization was obtained as observed for whey protein isolate and acacia gum mixtures (Espinosa-Andrews et al., 2010; Weinbreck et al., 2004). Interestingly, gel like properties were observed for biopolymer mixtures of pectins or xanthan gum with proteins but not with acacia gum/protein mixtures. Acacia gum was thought to demonstrate more globular structure while pectins or xanthan gum are more linear (Weinbreck et al., 2004; Wang, Lee, Wang, & Huang, 2010). The influence of protein-polysaccharide interactions is particularly important for dairy products. Carrageenan, an anionic polysaccharide, was used to improve rheological properties of dairy products as well as dairy protein stability. During yogurt production, interactions between dairy proteins and exopolysaccharide produced by lactic acid bacteria occurred and gel like yogurt structure formed as the pH decreased (Syrbe, Bauer, & Klostermeyer, 1998). Protein-polysaccharide complexes can also be used as fat replacers to give a plasticity, smoothness, creaminess, and oily mouthfeel in low-fat food (Schmitt, Sanchez, Desobry-Banon, & Hardy,

1998). Gelatin formed spherical shape particles with acacia gum that melt at 31.5 °C, and impart similar mouthfeel with fat (Bakker et al., 1994). Creamy mouthfeel was observed by Chen et al. (1989) when milk protein complexed with xanthan gum. Minced meat was also produced using protein-polysaccharide complexes such as alginate, pectate, and low-ester pectins complexed with caseins or dried milk, soy, or yeast proteins (Tolstoguzov et al., 1974).

2.6.3.2 Food Emulsifier

Protein-polysaccharide interactions form complexes that combine the intrinsic functional properties of each biopolymer and demonstrate typical hydrophobic/hydrophilic characteristics. Protein-polysaccharide complexes are effective food emulsifiers for stabilizing oil/water interfaces (Schmitt, Sanchez, Desobry-Banon, & Hardy, 1998). Beta-lactoglobulin-pectin complexes can form multi-layer membranes that stabilize oil-in-water emulsion. Improved emulsion stability was observed against salt destabilization effects up to 50 mM as compared with emulsion stabilized by β-lactoglobulin alone (Ganzevles, Cohen Stuart, van Vliet, & de Jongh, 2006). Emulsions using protein-polysaccharide complexes as stabilizer can be produced using following two techniques: (1) mixed emulsion of an oil phase using biopolymer complexes; (2) layer by layer (LBL) technique with a primary emulsion of an oil phase with one biopolymer and, thereafter, interfacial complex formation by a second biopolymer (McClements, 2010). Obviously, the two techniques seem to generate emulsions with different interfacial compositions and structures, but also different colloidal behaviours, such as creaming or flocculation (Jourdain et al., 2009). Emulsions using different approaches exhibit different pH stability (Jourdain et al., 2008). LBL emulsions stabilized by β-lactoglobulin-pectin complexes were produced with the highest emulsion stability with pectin concentration in a range of 0.02−1.0% (w/w). Higher or lower pectin content led to depletion flocculation or bridging flocculation. Either form of flocculation led to decreased emulsion stability (Cho & McClements, 2009). Ionic strength of emulsion systems also affects emulsification properties of protein-polysaccharide complexes. A more stable emulsion stabilized by β-lactoglobulin/citrus pectin system at pH 3–4 was formed at a NaCl concentration of 100 mM as compared to emulsion stabilized by β-lactoglobulin alone (Guzey, Kim, & McClements, 2004). The total biopolymer concentration for producing stable emulsion should also be optimized as this can determine biopolymer coacervate spreading properties at oil droplet surfaces. For whey protein isolate-acacia gum system, stable emulsions can be formed with a total biopolymer concentration of 0.5

to 1.0% (w/w) for a mixing ratio of 2:1 at pH 4.0 for an oil fraction of 5% (Speiciene, Guilmineau, Kulozik, & Leskauskaite, 2007). However, a wide range of total biopolymer concentrations ranging from 3.8 to 11.2 % (w/w) were found to produce stabilized concentrated emulsions of rapeseed oil (40%) (Moschakis, Murray, & Biliaderis, 2010).

2.6.3.3 Food Bioactive Nutrients Encapsulation

Encapsulation serves as one of the main applications of protein-polysaccharide complexes as protection system for sensitive materials. The encapsulation can be achieved through capsule formation (simple, multiple, layer-by-layer, etc.) or emulsion using protein-polysaccharide complexes (Schmitt & Turgeon, 2011). The capsule can entrap a solvent containing sensitive materials with the sensitive materials be distributed in either core or wall of capsules. However, emulsion based encapsulation is more desirable for oil-soluble sensitive products as compared with capsules. Sensitive materials will be encapsulated along with oil phase and mainly distributed in the core of formed particles (McClements, 2009). Upon encapsulation, sensitive materials such as volatile, oils, enzymes, bioactive natural products, and drugs could be protected against harsh processing (heat, redox potential, and shear, etc.) and storage conditions (temperature, light, oxygen, and moisture). Meanwhile, controlled release and targeted delivery of encapsulated sensitive materials to specific gastrointestinal targets under the trigger of mechanical process (chewing), pH variations (acidic conditions in the stomach, neutral in the intestine) or enzymatic action can also be feasible (Schmitt, Sanchez, Desobry-Banon, & Hardy, 1998). Acacia gum-gelatin coacervates were successfully used for encapsulation of lipophilic flavours with the flavours being released by diffusion through coacervate layers during cooking of baked goods with desirable release kinetics (Yeo et al., 2005). Complexes formed between gelatin and gum Arabic have been used for the encapsulation of capsaicin (Xing, Cheng, Yang, & Ma, 2004). BSA has been encapsulated with microspheres of carboxymethyl-chitosan/alginate hardened with $CaCl_2$.

Electrostatic interaction served as the primary interaction for coacervate formation within this mixture, pH was used to trigger the release of encapsulated materials from the biopolymer complex based encapsulation vehicle. This is extremely important for gastrointestinal tract delivery and controlled release of encapsulated nutrients to improve bioavailability. A quick release of BSA was obtained at pH 7.2 (pH of the intestinal fluid) for chitosan-alginate encapsulation system while BSA was hardly released at pH 1.0 (Zhang, Guo, Peng, & Li, 2004).

CHAPTER 3

COMPOSITIONAL ANALYSIS AND FUNCTIONAL PROPERTIES OF GUM FROM SIX CANADIAN FLAXSEED CULTIVARS

3.1 Abstract

Flaxseed gum (FG) was prepared from six Canadian flaxseed (*Linum usitatissimum* L.) cultivars, including CDC Bethune, CDC Sorrel, CDC Arras, CDC Glas, Vimy, and Flanders. FG yield (9.33 ± 0.14 to 14.45 ± 0.49 g/100 g flaxseed), neutral sugar content (367 ± 27 to 592 ± 84 mg/g FG), acidic sugar content (89 ± 25 to 181 ± 17 mg/g FG), protein content (56.6 ± 5.1 to 90.8 ± 4.7 mg/g FG) varied significantly among cultivars. The zeta potential (ζ) of FG solution (−16.4 ± 0.6 to −27.4 ± 1.5 mV) was affected by these changes. FG solutions (1.0%, w/v) rheological properties were cultivar dependent. The highest apparent viscosity (temperature 25 ºC, shear rate 1.0 s^{-1}) was observed in solutions prepared with CDC Glas (2.984 ± 0.204 Pa•s) FG while the lowest viscosity solution was produced by CDC Sorrel (0.048 ± 0.001 Pa•s) gum. FG solutions between 0.5−3.0%, w/v (FG/H_2O) exhibited pseudo-plastic shear-thinning behaviour. Effects of temperature (15−45 ºC), solution pH (3.0−9.0), NaCl concentration (0−200 mM), and sucrose concentration (0−20%, w/v) on FG solution (1.0%, w/v) rheological properties were also examined. Apparent FG solution viscosity increased with FG concentration while increased temperature decreased apparent viscosity and pseudo-plasticity. FG solution rheological properties were not pH sensitive, while NaCl addition decreased apparent viscosity due to charge screening effects. FG interacted with sucrose molecules as added sucrose increased FG solution apparent viscosity. FG solution prepared from Flanders showed the highest emulsion activity index (71.8 ± 2.8 m^2/g) while the lowest was observed for CDC Sorrel (41.3 ± 0.7 m^2/g). However, CDC Arras FG solution had the highest emulsion stability (52.0 ± 1.4%) while CDC Bethune FG solution had the lowest (37.4 ± 1.0%). Both mechanical and steric stabilization effects caused by high viscosity and reduced interfacial properties due to FG protein influenced FG emulsification properties.

Findings here could provide a tool-set to introduce the utilization of FG in food, cosmetic or pharmaceutical industry as a new thickening agent and/or emulsifier. As FG properties are cultivar dependent cultivar specific knowledge of FG solution properties is a requirement for making useful flaxseed products.

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3.2 Introduction

Flax (*Linum usitatissimum* L.) is of Mesopotamic origin and its seed has been used for food and its stems for textile fibre for over 5000 years (Carraro et al., 2012). Flaxseed oil has been used as a drying oil, in personal care products and as a dietary source of omega-3 fatty acid (Singh, Mridula, Rehal, & Barnwal, 2011). Flaxseed oil is rich in polyunsaturated fat (73%) and most cultivars produce an excellent n-6: n-3 fatty acid ratio of approximately 0.3:1 (Cunnane et al., 1993). Flaxseed also serves as a source of both insoluble and soluble dietary fibre with the proportion varying between 80:20 and 60:40 (Morris, 2001; Mazza & Oomah, 1995). Cellulose and lignin constitute the major insoluble fibre fraction, while the soluble fibre is largely FG or mucilage (Mazza & Biliaderis, 1989). FG is found mainly in flaxseed hull outer layers and, thus, it is mostly released from whole or ground seed when soaked in water (Oomah, Kenaschuk, Cui, & Mazza, 1995). Two distinct polysaccharide fractions were identified in FG solutions: a neutral fraction of arabinoxylans (1.2×10^6 g/mol) constituted 75% of FG mass (Cui & Mazza, 1996; Warrand et al., 2003; Qian, Cui, Wu, & Goff, 2012); and an acidic rhamnogalacturonan-I (RG-I) backbone that features diglycosyl repeating units (→2)-α-L-Rha*p*-(1→4)-α-D-Gal*p*A-(1→) (Qian, Cui, Nikiforuk, & Goff, 2012). The acidic fraction can be further separated into two fractions with MWs of 6.5×10^5 g/mol and 1.7×10^4 g/mol which make up 3.75% and 21.25% of FG mass, respectively (Cui & Mazza, 1996; Qian, Cui, Wu, & Goff, 2012). FG is of special research interest due to the marked functional properties of its solutions such as viscosity, emulsification, gellation and foam formation and stability (Chen, Xu, & Wang,

2007; Singh, Mridula, Rehal, & Barnwal, 2011). FG exhibited shear-thinning behaviour at concentrations above 1.0% and 'weak gel' like properties due to the high arabinoxylan molecular mass (Cui, Mazza, & Biliaderis, 1994). Newtonian like flow properties were observed for solutions containing the acidic FG fraction at all concentrations examined (Cui, Mazza, & Biliaderis, 1994). FG can also act as a foaming agent in solutions and stabilize oil-in-water emulsions by increasing system viscosity and decreasing interfacial tension (Khalloufi, Corredig, Goff, & Alexander, 2009). These solution functional properties have led to studies of the use of FG in salad dressing, sausage, carrot juice, and dairy desserts (Stewart & Mazza, 2000; Zhou et al., 2010). Moreover, FG has nutritional value as a dietary fibre that might reduce diabetes and coronary heart diseases risk, prevent colon and rectal cancer, and decrease the incidence of obesity (Cunnane et al., 1993; Qin, Xu, & Zhang, 2005; Thakur, Mitra, Pal, & Rousseau, 2009). Based on the lubrication and moisturizing properties, FG solutions are also suitable for use as laxatives and barium sulfate suspending agents for preparing x-ray contrast suspensions (Izydorczyk, Cui, & Wang, 2005).

FG properties are dependent on extraction conditions, drying processes, shear, and extrusion, as well as flaxseed cultivar used as a source (Cui, Kenaschuk & Mazza, 1996; Wang et al., 2009b; Wang et al., 2010b; Wang, Li, Wang, & Xue, 2011). The influence of flaxseed cultivar on FG content and functional properties has been investigated (Bhatty, 1993; Diederichsen, Raney, & Duguid, 2006; Fedeniuk & Biliaderis, 1994; Oomah, Kenaschuk, Cui, & Mazza, 1995; Wannerberger, Nylander, & Nyman, 1991; Pavlov et al., 2014). FG quality was characterized by a mucilage indicator value (MIV) but this was not correlated with other flaxseed characters, such as seed mass or colour, or even the geographic origin of the accession (Diederichsen, Raney, & Duguid, 2006). However, limited information is available on cultivar dependent FG functional properties and composition. The potential utilization of FG as a commercially viable product requires the selection of cultivars that produce consistent FG.

Canada flaxseed production accounts for nearly 80% of the global trade in flaxseed (Singh, Mridula, Rehal, & Barnwal, 2011). Six Canadian flaxseed cultivars representing the prominent cultivars registered and grown in Canada, including CDC Bethune, CDC Sorrel, CDC Arras, CDC Glas, Vimy, and Flanders, were selected for FG studies reported here. These oilseed flax cultivars were developed by the Crop Development Centre, University of Saskatchewan

(Saskatoon, SK, Canada), and are primarily destined for supplying export markets (Flax Council of Canada). Thus flaxseed cultivars selected here would represent majority of globally traded commercial flaxseed (Saskatchewan Flax Development Commission). Moreover, three cultivars: CDC Bethune, CDC Sorrel, and CDC Glas, were reconstituted to remove CDC Triffid seed, a genetically modified (GM) cultivar that was recalled in 2001 (Triffid Flax-Event FP967) (SeCan Association, Kanata, ON, Canada). FG composition and physicochemical properties of solutions prepared from each cultivar were investigated as were FG yield, protein content, neutral sugar content, acidic sugar content, and zeta potential (ζ). FG solution rheological properties were determined as functions of concentration, temperature, pH, NaCl concentration, and sucrose concentration. FG solution emulsification properties were also examined. Findings from this study provide useful information regarding FG properties as a food additive, cosmetic ingredient or pharmaceutical ingredient. In addition, knowledge of cultivar-dependent FG properties could allow the identification of superior flax cultivars for specific applications and enable flaxseed breeding to select more useful FG.

3.3 Materials and Methods

3.3.1 Materials

Six Canadian flaxseed cultivars (CDC Bethune, CDC Sorrel, CDC Arras, CDC Glas, Vimy, and Flanders) were provided as a generous gift from Dr. Helen Booker and harvested in 2014 from Floral, SK, Canada. Seeds were kept in a desiccator at room temperature (21−23 °C, RT) for subsequent studies. Resorcinol, sodium tetraborate, m-hydroxydiphenyl, D-xylose, and D-galacturonic acid were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Concentrated sulfuric acid (≥ 96%, w/w), Coomassie (Bradford) Protein Assay Kit® containing Pierce Coomassie Assay Reagent®, and albumin standard ampules with BSA solution (2 mg/mL in 0.9% saline and 0.05% sodium azide) were obtained from Fisher Scientific Company (Ottawa, ON, Canada). Anhydrous ethanol (≤ 0.10% water by volume) was purchased from Commercial Alcohols Inc. (Brampton, ON, Canada). Canola oil (Purity 100%, Loblaws Inc., Toronto, ON, Canada) was purchased from a local supermarket (Superstore, Saskatoon, SK, Canada). A Milli-Q deionization reversed osmosis (RO) system (Millipore, Bedford, MA, USA) was used to prepare deionized RO water (resistivity was > 18.2 MΩ•cm at 25 °C). All other reagents were of analytical grade purity and used as received.

3.3.2 FG Extraction

FG used in this work was prepared by an aqueous extraction as suggested by Wang et al. (2009b) with small modifications. Accurately weighed flaxseed (60.0 g) of each selected flaxseed cultivar was rinsed with deionized RO water at room temperature (RT, 22−23 °C) for 1 min to remove surface particulate matter. Thereafter, flaxseed was soaked in deionized RO water with a water to seed mass ratio of 1:10 for 24 h at 60 °C under gentle stirring (300 rpm) with a Teflon coated stirring bar. Subsequent to stirring extracted flaxseed was separated from crude FG solution by filtration through cheesecloth followed by centrifugation of the filtrate at 12,700 × g for 20 min at 4 °C to settle suspended particles. The supernatant was thoroughly mixed with one volume of anhydrous ethanol to precipitate FG. FG precipitate was collected by centrifugation (12,700 × g, 20 min, and 4 °C), frozen and dried in a freeze dryer (LABCONCO, Kansas City, MO, USA). Dried FG was kept in desiccator at RT for subsequent studies. Extraction from each cultivar was performed in triplicate. FG yield (g FG/100 g flaxseed) was calculated and presented as mean ± SD.

3.3.3 FG Solution Preparation

Dried FG was weighed then dispersed in deionized RO water and gently stirred (300 rpm) for 2 h at RT. FG polymer hydration was assured by maintaining the dispersion at 4 °C for 24 h. Air bubbles in the FG dispersion were removed by centrifugation at 2,500 × g for 2 min. The supernatant (FG) was collected and used for subsequent analyses. FG solution (1.0%, w/v) rheological properties were determined after adjusting solution pH from 3.0 to 9.0 using hydrochloric acid solution (0.1 mol/L) and sodium hydroxide solution (0.1 mol/L), respectively. FG solution (1.0%, w/v) ionic strength was adjusted by adding 50−200 mM NaCl. FG solution (1.0%, w/v) rheological properties were studied in the presence of sucrose (0−20%, w/v).

3.3.4 FG Structural Characteristics

3.3.4.1 Neutral Sugar Content

Cultivar FG neutral sugar content was determined by spectrophotometric analysis based on methods previously described by Monsigny, Petit, and Roche (1988) with small modifications. Aliquots (0.4 mL) of FG solution (1.0%, w/v) were mixed with resorcinol solution (0.4 mL, 6.0 mg/mL), then, sulfuric acid was added (2.0 mL, 75% v/v) and samples

were mixed for 30 s in a vortexing mixer. The mixture was maintained at 90 ºC in a water bath for 30 min then cooled to RT. Mixture optical density (OD) was measured at 480 nm with a Genesys 10S UV-vis spectrophotometer (Thermo Scientific, Madison, WI, USA). A blank was prepared following the same procedure with deionized water in place of FG solution. A calibration curve was prepared using D-xylose solution (0.2−10.0 mg/mL). FG neutral sugar content was calculated and expressed as milligrams of D-xylose equivalents per gram of dried FG. All measurements were performed in triplicate and results are presented as mean ± SD.

3.3.4.2 Acidic Sugar Content

FG acidic sugar content was conducted in microtiter plate colourimetric assays (van den Hoogen et al., 1998). FG solution (40.0 µL; 0.1%, w/v) was added to each plate well followed by concentrated sulfuric acid (200 µL, 96%, w/w) with sodium tetraborate (120 mM). Well contents were thoroughly mixed. The plate was maintained at 80 ºC for 60 min then cooled to RT. Absorbance was measured at 490 nm with a Benchmark microplate reader (Bio-Rad, Hercules, CA). Thereafter, 40.0 µL of freshly prepared m-hydroxydiphenyl reagent (100 µL, 100 mg/mL of m-hydroxydiphenyl in dimethyl sulfoxide mixed with 4.90 mL of 80%, w/w, sulfuric acid) was added and plates were maintained at RT for 15 min. Sample absorbance was measured again at 490 nm and the blank absorbance, measured previously, was subtracted. D-galacturonic acid (20.0 to 200.0 µg/mL) was used as a standard and acidic sugar content in FG was expressed as mg/g of D-galacturonic acid/dried FG. All measurements were performed in triplicate and results are presented as mean ± SD.

3.3.4.3 Protein Content

Total FG solution protein content was estimated by dye binding (Coomassie Brilliant Blue G-250®). Samples (5.0 µL, 0.1%, w/v) were diluted with deionized RO water (795 µL) and then mixed with Bio-Rad Protein Assay Dye Reagent (200 µL; Bio-Rad Laboratories, Inc., Mississauga, ON, Canada). Absorbance was monitored in acrylic cuvettes (semi-micro acrylic cuvette, VWR International, Radnor, PA, USA) at 595 nm with a Genesys 10S UV-Vis spectrophotometer (Thermo Scientific, Madison, WI, USA). BSA (95% purity based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, SDS-PAGE) was used to construct a standard calibration curve with a working range between 0 and 100 µg/mL. FG protein content was calculated and is presented as mean ± standard deviation (SD).

3.3.4.4 Zeta Potential

FG solution (0.1%, w/v) zeta potential (ζ, mV) was measured to evaluate overall surface charge. Electrophoretic mobility (UE) was determined by Laser Doppler Velocimetry combined with phase analysis light scattering (Malvern Zeta Nano ZS, Malvern Instruments Ltd., Worcestershire, UK). ζ was calculated based on the Henry equation (Eq. 3.1):

$$U_E = \frac{2\varepsilon \times \zeta \times f(\kappa\alpha)}{3\eta} \quad (3.1)$$

where η and ε are the dispersion viscosity (Pa•s) and FG solution (0.1%, w/v) permittivity, respectively. κ is the Debye length and α is the particle radius, where the f(κα) is equal to 1.5 based on the Smoluchowski approximation (Smoluchowski, 1903). All measurements were performed in triplicate and reported as mean ± SD.

3.3.5 Rheological Properties

FG solution dynamic viscosity was measured under continuous shear over a shear rate range of 0.1–100 s^{-1}. FG solutions made with a range of FG concentrations (0.5−3.0%, w/v), NaCl concentrations (50−200 mM), pH (3.0−9.0), or sucrose concentrations (5.0−20%, w/w) were loaded on the bottom plate of an AR2000ex rheometer (TA Instruments Ltd., Crawley, UK). Rheometer temperature (15−45 ºC) was controlled by a Peltier system (TA Instruments Ltd., Crawley, UK). After loading FG solution samples, the rheometer was equilibrated for 2 min and a solvent trap cover was used to limit evaporation and mitigate interference during analysis. Dynamic viscosity measurements were performed with an acrylic plate (60 mm diameter) and 500 µm gap. Apparent viscosity was obtained by fitting dynamic flow curves of each FG solution to the Power-law model (Eq. 3.2).

$$\eta = k\gamma^{n-1} \quad (3.2)$$

Where k is the consistency coefficient (Pa•s^n), γ is the shear rate (s^{-1}), and *n* is the fluid behaviour index (Rao, 1999). All measurements were performed in duplicate and expressed as the mean value. Data analysis was performed with TA Rheology Advantage Data Analysis software V 5.4.7 (TA Instruments Ltd., Crawley, UK).

3.3.6 Emulsification Properties

FG solutions prepared from each cultivar were mixed with canola oil using a Polytron PT 2100 homogenizer (Kinematica AG, Lucerne, Switzerland) equipped with a 12 mm PT-DA 2112/2EC generating probe at 26,000 rpm to produce oil-in-water emulsions. The emulsion system oil volume fraction (ϕ) and FG concentration were maintained at 0.1 and 0.8% (w/v), respectively. Each emulsion was diluted 121 fold with 0.1% SDS (w/v) and emulsion turbidity (500 nm) was measured immediately after dilution and 60 min later with a Genesys 10S UV-vis spectrophotometer. Emulsion turbidity value (T) was calculated using Eq. 3.3.

$$T = \frac{2.303 \times \mathrm{A} \times \mathrm{V}}{\mathrm{I}} \tag{3.3}$$

where, T is the emulsion turbidity (m^{-1}), A is the emulsion absorbance at 500 nm, V is the dilution factor, and I is the path length (0.01 m).

FG solution emulsion formation activity was determined according to Einhorn-Stoll, Weiss, and Kunzek (2002) using emulsion activity index (EAI, m^2/g) and emulsion stability (ES, %) as indicators. EAI is related to surface area moment mean or Sauter mean diameter, D_{32} (Cameron et al., 1991; Moro et al., 2013). EAI of FG solution was calculated as previously described by Wang et al. (2010b) using Eq. 3.4:

$$\mathrm{EAI} = \frac{2T}{\phi \times \mathrm{c}} \tag{3.4}$$

where, ϕ is the oil volume fraction and c is the emulsifier concentration.

FG solution ES was calculated using Eq. 3.5 (Wang et al., 2010b).

$$\mathrm{ES} = \frac{T_{60}}{T_0} \tag{3.5}$$

where, T_{60} is the diluted emulsions turbidity at 60 min, T_0 is emulsion turbidity immediately after dilution.

All measurements were performed in triplicate and results were expressed as mean ± SD. One-way analysis of variance (ANOVA) was conducted and Duncan's multiple range test was used for mean comparisons. P values of < 0.05 were regarded as significant.

3.4 Results and Discussion

3.4.1 FG Yield

The influence of extraction parameters (Temperature, pH, duration, seed to water mass ratio, microwave, ultrasonication, and stirring etc.) on FG yield has been studied intensively (Cui, Mazza, Oomah, & Biliaderis, 1994; Barbary, Al-Sohaimy, El-Saadani, & Zeitoun, 2009; Ziolkovska, 2012; Fabre, Lacroux, Valentin, & Mouloungui, 2015). Cui, Mazza, Oomah, and Biliaderis (1994) optimized FG extraction conditions using response surface methodology. The highest FG yield, 8.0%, was achieved at 85–90 ºC, 6.5–7.0 pH, and seed to water mass ratio of 1:13. Extraction performed at 100 ºC for 8 h produced a similar FG yield (Barbary, Al-Sohaimy, El-Saadani, and Zeitoun, 2009). Extraction conditions affect FG physicochemical and functional properties (Ziolkovska, 2012). Fabre, Lacroux, Valentin, and Mouloungui (2015) reported 7.0% of seed mass was extracted after 30 min at a temperature of 50 ºC using ultrasound to accelerate mass transfer. However, intrinsic viscosity of this FG was 6.2 dL/g while FG recovered by stirring with a magnetic stir bar was 12.5 dL/g. Harsh extraction conditions including high temperature and ultrasound should be avoided to preserve FG functional properties. However, FG yield was just 3.0−5.2% when extraction was conducted at 25 ºC over 1–8 h (Barbary, Al-Sohaimy, El-Saadani, & Zeitoun, 2009).

FG was extracted from whole seeds of six selected Canadian flaxseed cultivars employing a method previously described by Wang et al. (2009b). Extraction was performed at 60 ºC for 24 h, using a seed to water mass ratio of 1:10, and constant stirring (300 rpm) using a magnetic stir bar. FG was then precipitated with ethanol to prepare enriched FG. Cyanogenic glycosides, present in the FG extract were likely removed by precipitation (Daun & Przybylski, 2000). FG yield from the selected cultivars, expressed as g FG/100 g flaxseed, varied significantly (Table 3.1). The highest FG yields were obtained for Flanders and CDC Glas (14.45 ± 0.49 and 13.62 ± 0.33 g/100 g seed, respectively), whereas, CDC Bethune extraction yield (9.33 ± 0.14 g/100 g seed) was the lowest. Kaewmanee et al. (2014) also observed that FG yield is a function of cultivar. Under mild extraction conditions FG yield ranged from 1.79% to 3.65% of fresh seed mass (i.e. 20 ± 5 ºC extraction temperature, 12 h extraction time and 1:10 seed to water to ratio). FG yields achieved in this study are higher than previously reported. This finding might be ascribed to extraction conditions employed (Barbary, Al-Sohaimy, El-Saadani, & Zeitoun, 2009).

Table 3.1 FG yield, protein, neutral sugar, acid sugar contents, and zeta potentials (ζ) of FG solutions (0.1%, w/v, 25 ºC) for six Canadian cultivars.

Cultivar	Yield (g/100 g seeds)	Protein (mg/g FG)	ζ (mV)	Sugar (mg/g FG)	
				Neutral sugar	Acidic sugar
CDC Glas	13.62 ± 0.33^{b}	64.9 ± 2.3^{c}	-23.3 ± 0.6^{b}	430 ± 39^{b}	98 ± 18^{b}
Vimy	12.75 ± 0.38^{c}	90.8 ± 4.7^{a}	-27.4 ± 1.5^{a}	389 ± 37^{b}	89 ± 25^{b}
Flanders	14.45 ± 0.49^{a}	73.2 ± 13.2^{b}	-25.6 ± 1.7^{a}	578 ± 22^{a}	111 ± 19^{b}
CDC Sorrel	12.71 ± 0.47^{c}	87.3 ± 3.5^{a}	-26.9 ± 1.0^{a}	367 ± 27^{b}	114 ± 23^{b}
CDC Arras	11.90 ± 0.35^{d}	88.3 ± 1.9^{a}	-27.4 ± 0.6^{a}	418 ± 28^{b}	117 ± 26^{b}
CDC Bethune	9.33 ± 0.14^{e}	56.6 ± 5.1^{d}	-16.4 ± 0.6^{c}	592 ± 84^{a}	181 ± 17^{a}

Values are provided as mean ± SD. Different superscripts in the same column indicate significant differences ($p < 0.05$).

Moreover, environmental factors, such as climate, and crop maturity might also affect FG yield (Saag, Sanderson, Moyna, & Ramos, 1975; Diederichsen, Raney, & Duguid, 2006; Pavlov et al., 2014). Extraction yield is an important parameter in determining the economic viability of FG recovery while it is also desirable to determine functional properties.

3.4.2 Neutral and Acidic Sugar Content

Two polysaccharide fractions, a neutral fraction (75%) and an acidic fraction (25%), were reported in FG: (Cui & Mazza, 1996; Warrand et al., 2003; Qian, Cui, Wu, & Goff, 2012). The uronic acid depleted neutral fraction is primarily an arabinoxylan polymer with a β-D-(1,4)-xylan backbone. However, small amounts (1.8 %) of uronic acid were found in the FG neutral fraction polysaccharide (1,470 kDa; Qian, Cui, Wu, & Goff, 2012). FG acidic polysaccharide monomers include D-xylose, L-arabinose, D-glucose, L-galactose, D-galacturonic acid, and L-rhamnose (Cui & Mazza, 1996; Cui, Mazza, & Biliaderis, 1994; Cui, Mazza, Oomah, & Biliaderis, 1994; Qian, Cui, Nikiforuk, & Goff, 2012; Qian, Cui, Wu, & Goff, 2012). FG solutions thin when sheared showing pseudo-plastic rheology with weak gel-like properties. FG solution rheological properties depend on FG sugar monomer composition, which are cultivar specific and determined, in part, by genetics (Cui, Kenaschuk, & Mazza, 1996). For example, FG extracted from yellow seeded flaxseed cultivars contained less rhamnose (12.8–14.4%) and galacturonic acid (13.8–16.2%), but much higher contents of neutral polysaccharides (e.g. xylose, 39.0–48.7%) than those extracted from brown seeded cultivars (Cui, Kenaschuk, & Mazza, 1996).

Neutral and acidic sugar content was determined for FG derived from each flaxseed cultivar. FG neutral sugar content varied from 592 ± 84 to 367 ± 28 mg/g dried FG. FG derived from CDC Bethune and Flanders contained the highest neutral sugar content (592 ± 84 and 578 ± 22 mg/g dried FG, respectively). FG acidic sugar content was also observed to range from 89 ± 25 to 181 ± 17 mg/g dried FG. CDC Bethune had the highest acidic sugar content while Vimy FG had the lowest. Large variations in neutral and acidic sugar content confirm the influence of genotype on FG composition. These variations would impact FG physicochemical and functional properties. Kaewmanee et al. (2014) observed similar genotypic effects on FG sugar composition by observing neutral and acidic sugar content in seven Italian cultivars. In their study, neutral sugars varied from 0.55 (Merlin) to 0.87 (Solal) g/g FG and acidic sugar content varied from 0.58 (Valoal) to 0.83 (Kaolin) g/g FG. This study is also in agreement with previous research

(Izydorczyk, Cui, and Wang, 2005) showing that sugars in neutral and acidic fractions vary substantially with flaxseed cultivar.

3.4.3 Protein Content

Many polysaccharide gums, including pectin, gum Arabic, microcrystalline-cellulose, galactomannans, and soy soluble polysaccharide contain protein and its presence can contribute to rheological and emulsification properties (Funami et al., 2007). The major protein, and only FG protein identified was Conlinin, a 2S flaxseed storage protein (Liu, Shim, Poth, & Reaney, 2016).

Protein content can be up to 80 g/kg of FG powder under some extraction conditions (Cui, Mazza, Oomah, & Biliaderis, 1994). Barbary, Al-Sohaimy, El-Saadani, & Zeitoun (2009) found FG protein content was highly dependent on extraction conditions, especially extraction temperature.

Higher extraction temperatures facilitate protein extraction. Fekri, Khayami, Heidari, and Jamee (2008) reported FG with a protein content of 12.2% (w/w) when extraction was conducted using distilled water (1:20, w/v) with stirring for 3 h at 75 ºC. Wang et al. (2009b) reported that 14.4 ± 0.2% (w/w) FG protein content was attained at a lower extraction temperature of 60 ºC. Flaxseed genotype also partly determines FG protein content. The protein content of FG prepared from seven Italian flaxseed cultivars varied from 0.89 to 1.63% (Kaewmanee et al., 2014). In this study, FG was extracted from six Canadian flaxseed cultivars and protein was determined using the Bradford method with BSA as a standard (Table 3.1). FG from Vimy and CDC Arras had the highest protein (90.77 ± 4.65 and 88.25 ± 1.89 mg/g FG, respectively), while CDC Bethune FG had the lowest (56.60 ± 5.13 mg/g FG). Protein content in the present work was lower than that reported by Kaewmanee et al. (2014) even though a higher extraction temperature of 60 ºC was employed. This could be attributed to different flax cultivars studied and methods employed for protein content measurement. The Bradford method cannot be compared with the Kjeldahl method for protein analysis, therefore, these results cannot be directly compared.

3.4.4 Zeta Potential

In the presence of protein the interfacial tension of two phase mixtures of oil and water with dissolved polysaccharides can be lowered. Protein can anchor polysaccharides onto oil

droplet surfaces (Fedeniuk & Biliaderis, 1994; Bhatty, 1993; Wang et al., 2010b). Lower interfacial tension would help to stabilize emulsions. Protease hydrolysis reduced FG solution surface activity (0.5%, w/v) and emulsion stability regardless of polysaccharide molecular mass, chain flexibility and rheological properties (Qian, Cui, Wu, & Goff, 2012). Thus protein likely plays an important role in these properties. Zeta potential (ζ) is the electro-kinetic potential difference between the dispersion medium and the slip plane (stationary layer of fluid attached to the dispersed particle) of moving particles. ζ is widely used as an indicator of colloid as well as emulsion stability (Acedo-Carrillo et al., 2006). Higher absolute values of ζ induce mutual repulsion between macromolecule electrical double layers and induce greater stability. Conversely, low absolute ζ value favours aggregation and flocculation (Acedo-Carrillo et al., 2006). All FG solution ζ indicate negative macromolecule charge, between −16.4 ± 0.6 to −27.4 ± 0.6 mV (Table 3.1). Solutions prepared with FG from CDC Arras, Vimy, CDC Sorrel, and Flanders had the same charge. The lowest negative potential, −16.4 ± 0.6 mV, was observed in a FG solution prepared from CDC Bethune. Wang et al. (2010b) reported ζ values of FG (0.09%, w/w, in water) prepared by different drying methods were higher than those in the current study, ranging between −27.7 and −39.1 mV. Different extraction methods potentially altered FG sugar and protein content. Also protein conformation, solution electrolytes, and pH might affect the electrical characteristics of solution polysaccharides (Kaewmanee et al., 2014). FG solution ζ and protein content are negatively correlated (R = 0.906). While FG solution ζ was positively correlated with acidic sugar content (R = 0.795). As a general rule, a ζ higher than 30 mV indicates good suspension stability (Sherman, 1970; Wang et al., 2010b). Thus, FG solutions (0.1%, w/v) in this study would likely produce emulsions with limited stability.

3.4.5 Rheological Properties

FG is primarily comprised of hydrophilic high MW arabinoxylans and pectic like acidic polysaccharides (Cui, Mazza, & Biliaderis, 1994). FG molecules would be hydrated when contacted with water and these would form a dissolved polysaccharide network even at low FG concentrations (Cui, Mazza, & Biliaderis, 1994; Cui, Kenaschuk, & Mazza, 1996). Due to high FG solution viscosity, it can potentially be used in food as a thickener, gelling agent, texture modifier, suspending agent, and stabilizer (Chen, Xu, & Wang, 2007; Singh, Mridula, Rehal, & Barnwal, 2011). FG composition plays an important role in determining FG solution rheological

properties (Cui, Kenaschuk, & Mazza, 1996). In real food formulations, FG will encounter other food ingredients including solution molecules and particles (Salehi, Kashaninejad, & Behshad, 2014). Here, FG solution steady shear flow behaviour was studied over a shear rate range of 0.1−100 s^{-1} and a concentration range of 0.5−3.0% (w/v). Effects of temperature (15−45 ºC), solution pH (3.0−9.0), NaCl concentration (0−200 mM), and sucrose concentration (0−20%, w/w) on FG solution rheological properties were investigated. These studies will help to understand the interaction of FG with food systems.

3.4.5.1 Concentration Effects

Steady-state shear flow curves of FG solutions (1.0%, w/v) prepared from each flaxseed cultivar are shown in Figure 3.1A. All solutions exhibited pseudo-plastic or shear-thinning behaviour as FG solution apparent viscosity decreased with increased shear rates. At rest FG polysaccharide molecules are randomly distributed in solution without a regularly ordered solution structure. This arrangement provides the maximum flow resistance. When shear force is applied to the solution, FG polysaccharide chains are deformed and aligned with the flow. The alignment decreases slip resistance and apparent viscosity. When FG polysaccharide chain entanglements are disrupted by shear more rapidly than the rate of forming new entanglements viscosity decreases (Vardhanabhuti & Ikeda, 2006). Warrand et al. (2003) reported FG solution shear-thinning flow behaviour at concentrations of 2.0% (w/v) due to formation of polysaccharide molecular aggregates by intermolecular associations via hydrogen bonds. Similarly, Cui, Mazza, and Biliaderis (1994) observed shear-thinning behaviour in FG solutions above 1.0% (w/v) over a shear rate range of 0.1−1000 s^{-1}. Apparent viscosity of each FG solution at a shear rate of 1.0 s^{-1} is provided as Figure 3.1B. FG solution (1.0%, w/v) prepared from CDC Glas had the highest apparent viscosity of 2.984 ± 0.204 Pa•s while FG solutions prepared from CDC Sorrel and Vimy had the lowest apparent viscosities, 0.048 ± 0.001 and 0.129 ± 0.051 Pa•s, respectively. Typical shear-thinning flow behaviour and high viscosities of FG solutions were attributed largely to high molecular mass neutral polysaccharides (arabinoxylans) (Goh, Pinder, Hall, & Hemar, 2006). However, due to their smaller molecular size Newtonian like flow properties were observed for solutions of FG acidic fractions (Cui, Mazza, & Biliaderis, 1994). Higher apparent viscosity of FG solution prepared from flaxseed cultivar of CDC Glas could be associated with higher neutral sugar content (430 ± 39 mg/g FG, D-xylose equivalent) and low acidic sugar content (98 ± 18 mg/g FG, D-galacturonic acid equivalent). FG solutions of Vimy and CDC Sorrel showed the

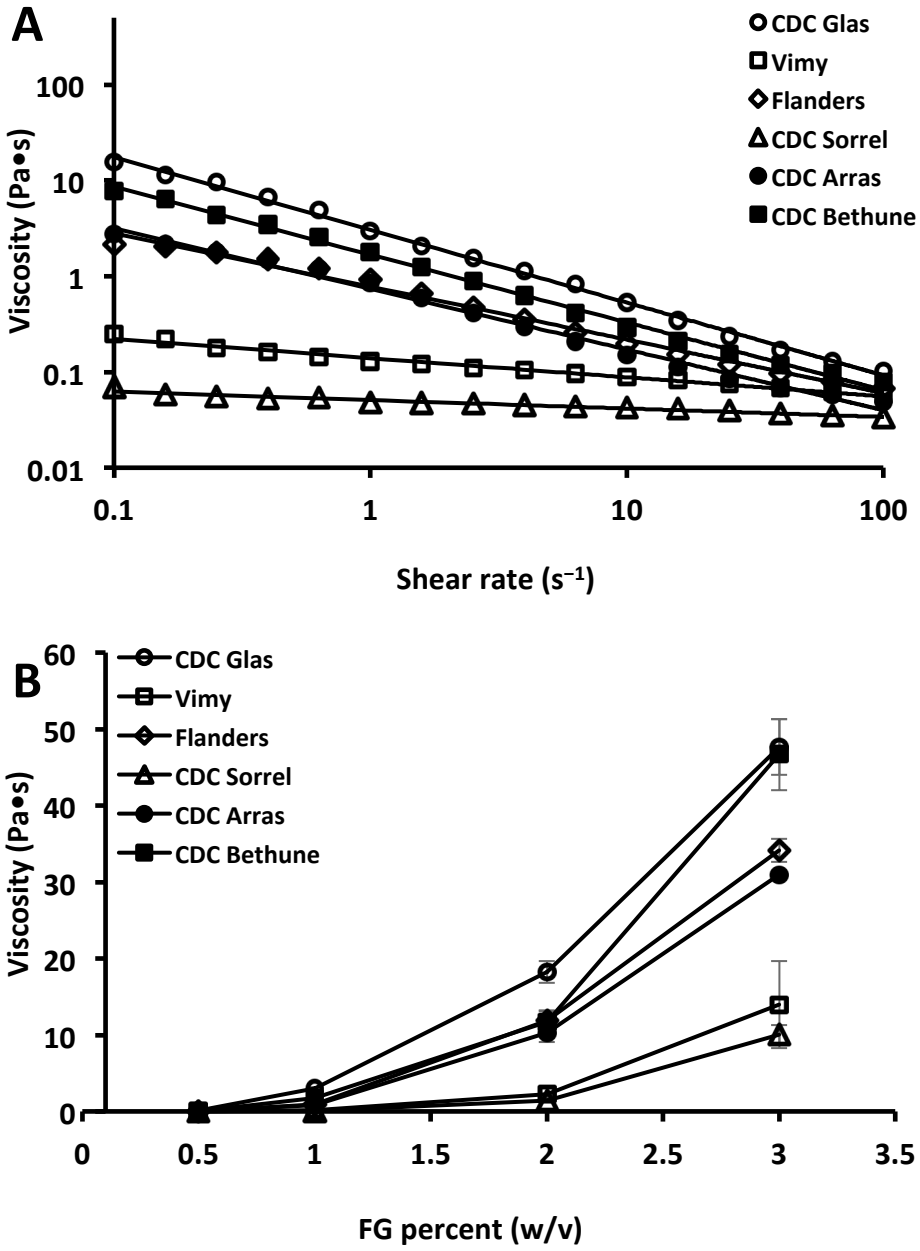

Figure 3.1 Dynamic flow behaviour (A) and apparent viscosity (B) of FG solutions prepared from six Canadian flaxseed cultivars as a function of shear rate (0.1−100 s^{-1}) at fixed concentration (1.0%, w/v) and FG concentrations (0.5−3.0%, w/v) at fixed shear rate (1.0 s^{-1}), respectively.

lowest neutral sugar content of 389 ± 37 and 367 ± 27 mg/g FG (D-xylose equivalent), respectively, resulting in the lowest apparent viscosity.

The k and *n* coefficients were obtained by fitting the Power-law model to flow curves of FG solutions (0.5−3.0%, w/v) (Table 3.2). The least squares fits of flow curves to Eq. (3.2) are illustrated by lines in Figure 3.1A. The coefficient of determination (R^2) was 0.944 or higher for all tested FG solutions (Table 3.2), indicating the appropriateness of the Power-law model to describe FG solution flow properties within the concentration range of 0.5−3.0% (w/v).

The increase in FG concentration raised both pseudo-plasticity and viscosity as indicated by increased k coefficient and decreased *n* coefficient. For FG solutions prepared from CDC Glas, k coefficients increased from 0.103 to 42.831 $Pa•s^n$ as FG concentration increased from 0.5 to 3.0% (w/v). The opposite trend was observed for *n* coefficients that decreased from 0.697 to 0.181 as FG concentration increased from 0.5 to 3.0% (w/v). This indicates predominant pseudo-plastic flow behaviour, which could be attributed to higher solids content in solution, producing an increased restriction of intermolecular motion caused by hydrodynamic forces and interfacial film formation (Maskan & Gogus, 2000). The highest k coefficient (3.053 $Pa•s^n$) was observed with FG solutions prepared from CDC Glas (1.0%, w/v) at 25 ºC while the lowest *n* coefficient was 0.240. FG solutions prepared from CDC Sorrel and Vimy (1.0%, w/v) at 25 ºC produced the lowest k coefficients of 0.051 and 0.140 $Pa•s^n$, respectively, but produced the highest *n* coefficient of 0.912 and 0.800, respectively. Non-Newtonian flow behaviour becomes predominant when *n* coefficients are less than 0.6 (Chhinnan, McWaters, & Rao, 1985). Therefore, the low *n* coefficients represent a departure of flow from Newtonian behaviour. For hydrocolloid solutions, high *n* coefficients are associated with a slimy mouth feel (Szczesniak & Farkas, 1962). Thus, for provision of both high viscosity and good mouth feel, FG solutions prepared from CDC Glas and CDC Bethune would be preferred as lower *n* coefficients were obtained when compared with those from other flaxseed cultivars (Marcotte, Taherian, & Ramaswamy, 2001a).

3.4.5.2 Temperature Effects

When dissolved hydrocolloid molecules arrange themselves to form a stable network structures as a result of intra- and inter-molecular interactions. Motion of hydrocolloid molecules are restricted due to these interactions, during shear such interactions induce viscosity (Hassan & Hobani, 1998).

Table 3.2 Power-law coefficients of fits for FG solution rheological measurements as a function of concentration.

Treatment		Model parameter		
Flaxseed cultivar	Concentration (%, w/v)	*n*	k (Pa•s^n)	R^2
CDC Glas	0.5	0.697	0.103	0.983
	1.0	0.240	3.053	0.997
	2.0	0.190	16.472	0.997
	3.0	0.181	42.831	0.997
Vimy	0.5	0.940	0.022	0.968
	1.0	0.800	0.140	0.985
	2.0	0.499	2.306	0.997
	3.0	0.390	11.982	0.993
Flanders	0.5	0.828	0.039	0.964
	1.0	0.444	0.781	0.991
	2.0	0.275	9.168	0.983
	3.0	0.226	29.586	0.994
CDC Sorrel	0.5	0.981	0.014	0.886
	1.0	0.912	0.051	0.953
	2.0	0.484	1.378	0.987
	3.0	0.317	9.129	0.990
CDC Arras	0.5	0.815	0.034	0.963
	1.0	0.367	0.736	0.993
	2.0	0.216	8.083	0.989
	3.0	0.212	24.221	0.988
CDC Bethune	0.5	0.806	0.048	0.944
	1.0	0.292	1.678	0.996
	2.0	0.260	10.753	0.996
	3.0	0.195	38.792	0.987

Increasing the hydrocolloid solution temperature changes interaction strength, rearranges hydrocolloid network structure, and reduces solution viscosity (Garcia-Ochoa & Casas, 1992). Temperature effects on FG solution viscosity are shown in Figure 3.2A. For all FG solutions, apparent viscosity decreased as temperature increased from 15 to 45 ºC. At a shear rate 1.0 s^{-1}, apparent CDC Glas FG solution (1.0%, w/v) viscosity decreased from 6.601 ± 0.341 to 0.330 ± 0.019 Pa•s as temperature increased from 15 to 45 ºC. However, FG solutions (1.0%, w/v) prepared from CDC Sorrel and Vimy did not show similar temperature sensitivity as apparent viscosity decreased from 0.091 ± 0.002 to 0.028 ± 0.009 Pa•s and from 0.363 ± 0.021 to 0.061 ± 0.013 Pa•s, respectively as the temperature increased from 15 to 45 ºC.

FG solutions flow curves were fitted to the Power-law model with all R^2 values higher than 0.953, indicating a good fit. As temperature increased from 15 to 45 ºC, *n* coefficient of CDC Glas FG solution (1.0%, w/v) increased from 0.188 to 0.530, while k coefficient decreased from 5.655 to 0.363 Pa•s^n, indicating a decrease of pseudo-plastic flow of these FG solutions (Figures 3.2B & C). However, a FG solution (1.0%, w/v) prepared from CDC Sorrel demonstrated Newtonian like flow property as *n* coefficient was close to 1.0, within the temperature range tested (Figures 3.2B & C). With increasing solution temperature hydrocolloid gum prepared from *Gleditsia amorphoides* seeds demonstrated decreased apparent viscosity (lower k coefficient) and pseudo-plasticity (higher *n* coefficient) (Perduca et al., 2013). Galactomannan solution apparent viscosity decreased by 50% when temperature was raised from 20 to 80 ºC (Wielinga & Maehall, 2000). Mucilage obtained from *Opuntia ficus indica* was also reported to undergo temperature dependent (5−70 ºC) thinning that was independent of mucilage concentration in a range of 3−10% (w/v) (Medina-Torres, Brito-De La Fuente, Torrestiana-Sanchez, & Katthain, 2000). Similar effects of temperature on rheological properties were also reported with xanthan gum (Marcotte, Taherian, & Ramaswamy, 2001a), carrageenan (Marcotte, Taherian, & Ramaswamy, 2001b), and mucilage extracted from *Alyssum homolocarpum* seed (Koocheki et al., 2009).

3.4.5.3 Salt Effects

Acidic sugar content in FG prepared from six Canadian flaxseed cultivars varied from 89 ± 25 (Vimy) to 181 ± 17 (CDC Bethune) mg/g FG (D-galacturonic acid equivalent) (Table 3.1). Acidic carboxyl groups in FG polysaccharide chains induce intra- and inter-molecular repulsive forces that result in a more expanded polysaccharide molecular configuration and arrangements in

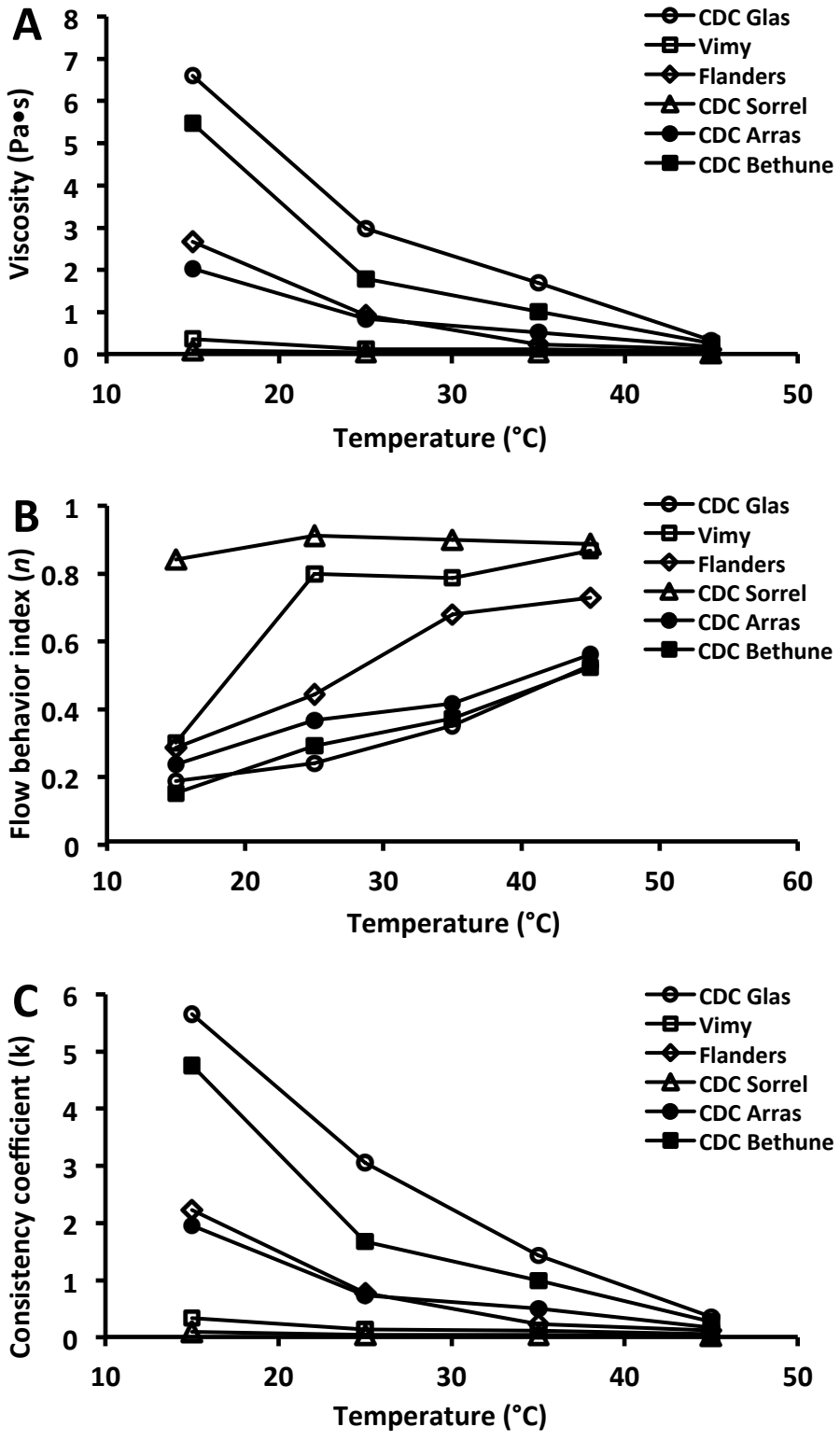

Figure 3.2 Apparent viscosity (A, shear rate: 1.0 s^{-1}), Power-law coefficients of *n* (B), and k (C) value of FG solutions (1.0%, w/v) as a function of temperature (15−45 ºC).

solution that, in turn, lead to higher FG solution viscosity (Lin & Lai, 2009). Ionic strength can be increased by addition of counter ions, which, in turn, screen FG anionic group charges. Charge screening reduces charge repulsion and molecule expansion and, thereby, decreases viscosity (Simas-Tosin et al., 2010). The effects of NaCl concentration on FG solution viscosity were investigated.

FG solution viscosity was affected by NaCl concentration as it behaves as a polyelectrolyte (Figure 3.3A). With a NaCl concentration of 50 mM, CDC Glas FG solution had an apparent viscosity of 3.315 ± 0.438 Pa•s at a shear rate of 1.0 s^{-1}, which was greater than the apparent viscosity of the same FG in distilled water 2.984 ± 0.204. However, in 200 mM NaCl FG solution apparent viscosity was just 1.214 ± 0.042 Pa•s (Figure 3.3A). Other solutions prepared with FG from other cultivars had lower apparent viscosity with increased NaCl concentration up to 200 mM. The shear rates versus dynamic shear flow data were well fitted to the Power law model. Flanders FG solution *n* coefficients were 0.444 and 0.787 for FG solutions prepared in distilled water and 200 mM NaCl, respectively, while k coefficients were 0.781 and 0.126 $Pa•s^n$ for the same FG solutions (Figures 3.3B & C). However, the *n* coefficients of FG solutions prepared from CDC Sorrel FG were not significantly changed by NaCl content indicating a predominantly Newtonian like flow behaviour at all NaCl concentrations tested. Results are in agreement with findings previously reported by Mazza and Biliaderis (1989). Lower viscosity of FG solutions with increased NaCl concentration was ascribed to reduce electrostatic repulsion of anionic carboxyl groups on FG polysaccharide chains (Medina-Torres, Brito-De La Fuente, Torrestiana-Sanchez, & Katthain, 2000). Similar salt concentration effects on hydrocolloid solution viscosity were also reported with cress seed (*Lepidium sativum*) gum (Behrouzian, Razavi, & Karazhiyan, 2013), *Opuntia ficus indica* mucilage gum (Medina-Torres, Brito-De La Fuente, Torrestiana-Sanchez, & Katthain, 2000), *Alyssum homolocarpum* seed mucilage (Koocheki et al., 2009), Balangu seed (*Lallemantia royleana*) gum (Salehi, Kashaninejad, & Behshad, 2014) due to suppressed repulsive forces between adjacent hydrocolloid chains, resulting in compaction of hydrocolloid polymer chains and a significant decrease in viscosity.

3.4.5.4 pH Effects

Effects of solution pH and cultivar on steady state shear viscosity of FG solution (1.0%, w/v) were determined. For FG solutions prepared from all flaxseed cultivars except CDC Glas apparent viscosity increased with pH from 3.0 to 7.0. Apparent FG solution viscosity at pH to 9.0

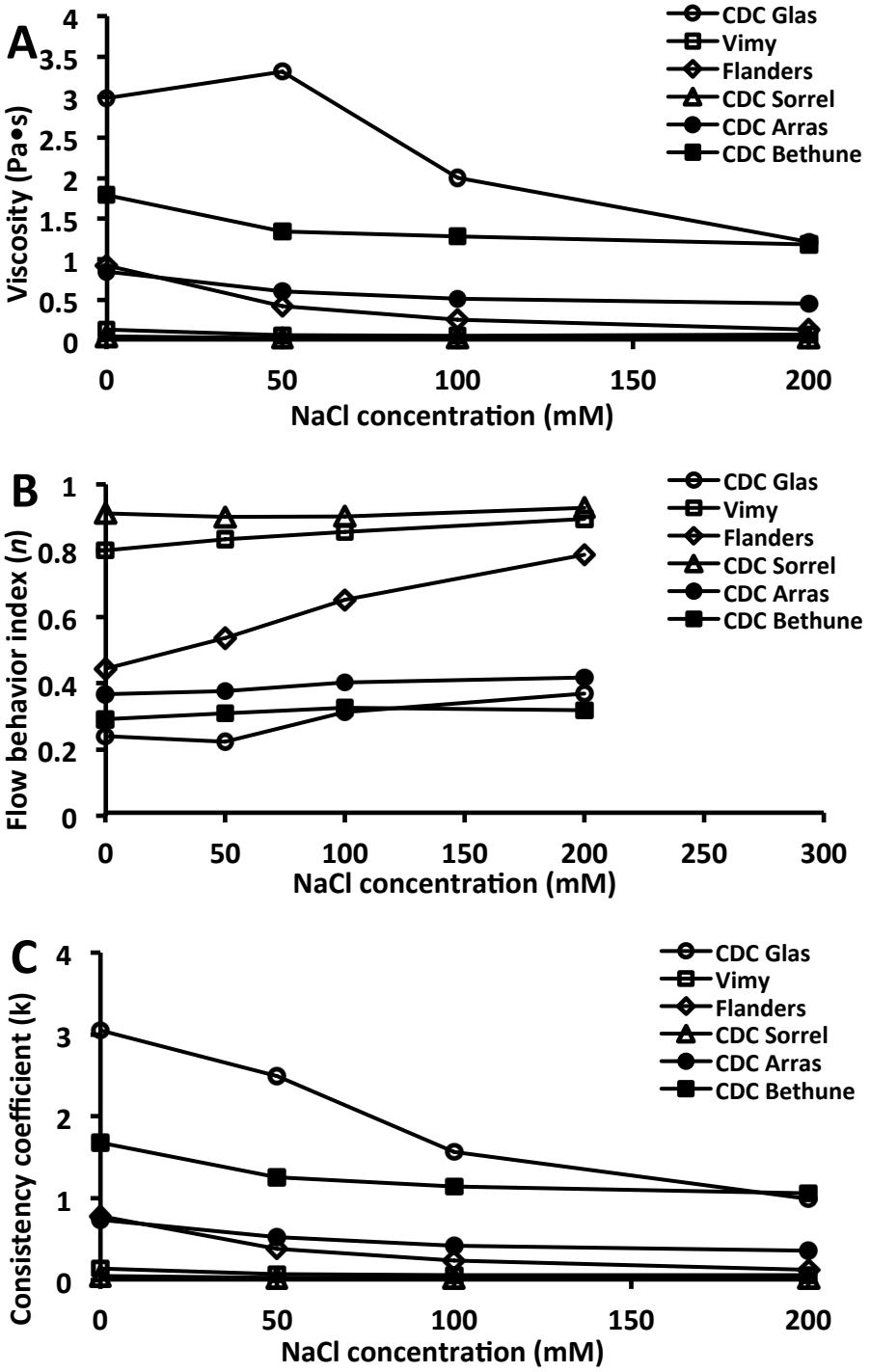

Figure 3.3 Apparent viscosity (A, shear rate: 1.0 s^{-1}), Power-law coefficients of *n* (B), and k (C) value of FG solutions (1.0%, w/v) as a function of NaCl concentration (0−200 mM).

was similar to the apparent viscosity at pH 7.0. FG molecular structure is thought to exist in a random coil at low pH (3.0) but become a rigid rod-like conformation at pH 7.0 and above. The more expansive rod-like conformation induces more flow resistance and increases apparent viscosity (Goh, Pinder, Hall, & Hemar, 2006; Medina-Torres, Brito-De La Fuente, Torrestiana-Sanchez, & Katthain, 2000). FG carboxyl group ionization increases as pH increases from 3.0 to 7.0, which might increase apparent viscosity due to enhanced repulsive inter- and intra-molecular interactions (Medina-Torres, Brito-De La Fuente, Torrestiana-Sanchez, & Katthain, 2000). Feng, Gu, and Jin (2007) observed a maximum k coefficient when hydrocolloid chains reached rod-like conformations. The maximum apparent viscosity of CDC Glas FG solution (3.826 ± 0.910 Pa•s) was observed at pH 5.0 (Figure 3.4A). At this pH, FG carboxyl group ionization would be at a maximum. Accordingly, FG solution pseudo-plasticity and k coefficients increased as solution pH increased from 3.0 to 7.0 for solutions from all cultivars (Figures 3.4B & C). Electrostatic repulsion within and between FG molecules due to ionization of anionic carboxyl groups was thought to be responsible for increased apparent viscosity as a more extended form of FG polysaccharide chains were induced (Onweluzo, Obanu, & Onuoha, 1994).

3.4.5.5 Sucrose Effects

Dissolved sucrose changes hydrocolloid gum solution rheological properties in several ways. Sucrose can increase aqueous phase viscosity and can interact directly with the hydrocolloid gum molecules (Yanes, Duran, & Costell, 2002). Under certain conditions sucrose may compete with hydrocolloids for bound water molecules and reduce hydrocolloid hydration, this competition could reduce solution viscosity (Amini & Razavi, 2012). Sucrose effects on FG solution rheological properties were determined on all FG solutions (Figure 3.5). The highest apparent viscosity for a 1.0% (w/v) FG solution occurred with CDC Glas preparations. CDC Glas FG solution apparent viscosity increased from 2.984 ± 0.204 to 7.788 ± 1.472 Pa•s when 20% (w/v) sucrose was present. CDC Sorrel FG solution induced the lowest apparent viscosity. CDC Sorrel FG solution apparent viscosity was greater in the presence of 20% (w/v) sucrose (0.109 ± 0.007 Pa•s) than without (0.048 ± 0.001).

All samples were well fitted by the Power-law model. FG solution *n* coefficients for all flaxseed cultivars increased while k coefficients decreased with increased sucrose concentration up to 20% (w/v) (Table 3.3). Nishinari, Watase, Williams, & Phillips (1990) reported an increase of κ-carrageenan solution viscosity with increased sucrose concentration. Sucrose was assumed

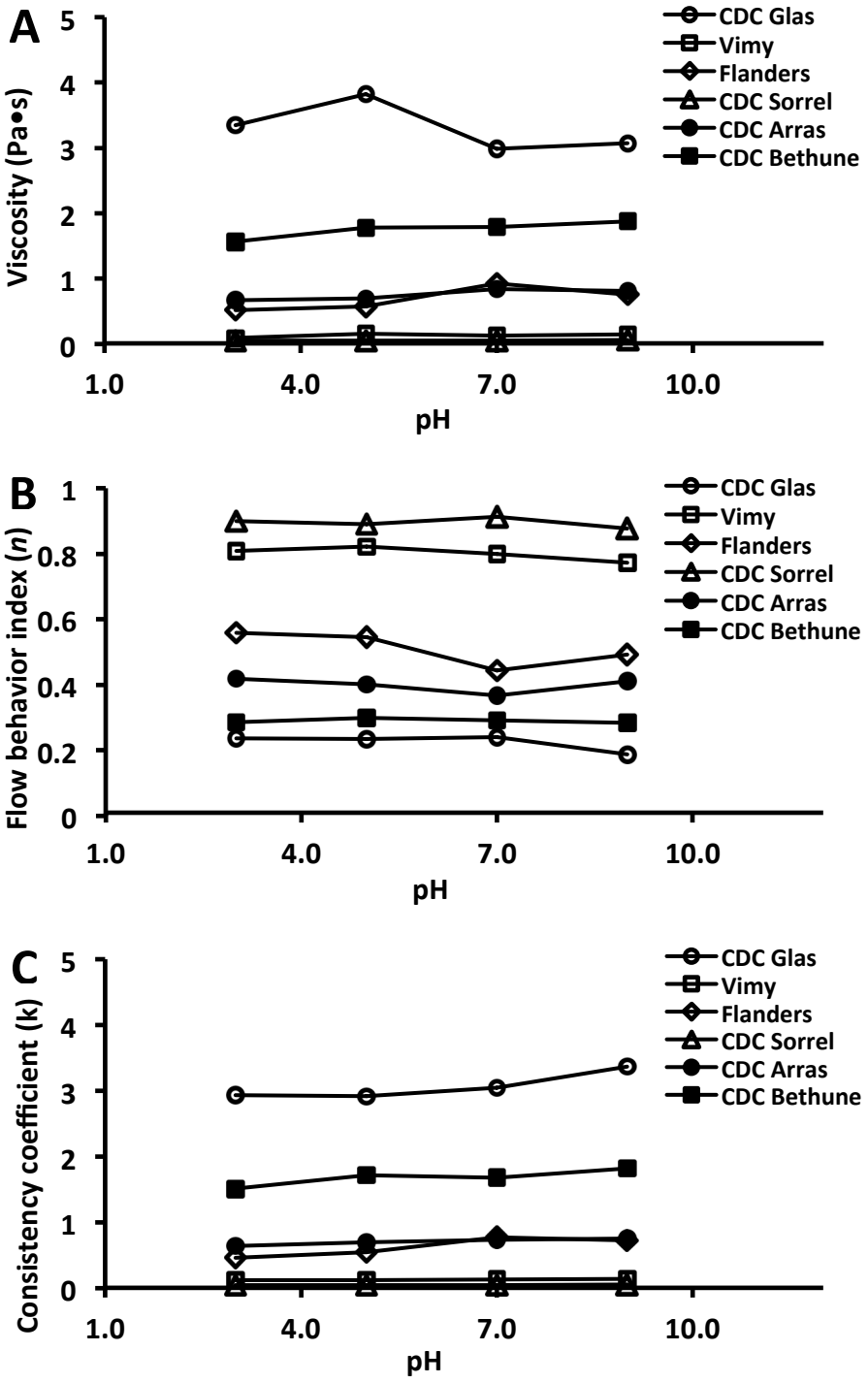

Figure 3.4 Apparent viscosity (A, shear rate: 1.0 s^{-1}), Power-law coefficients of *n* (B), and k (C) value of FG solution (1.0%, w/v) as a function of solution pH (3.0−9.0).

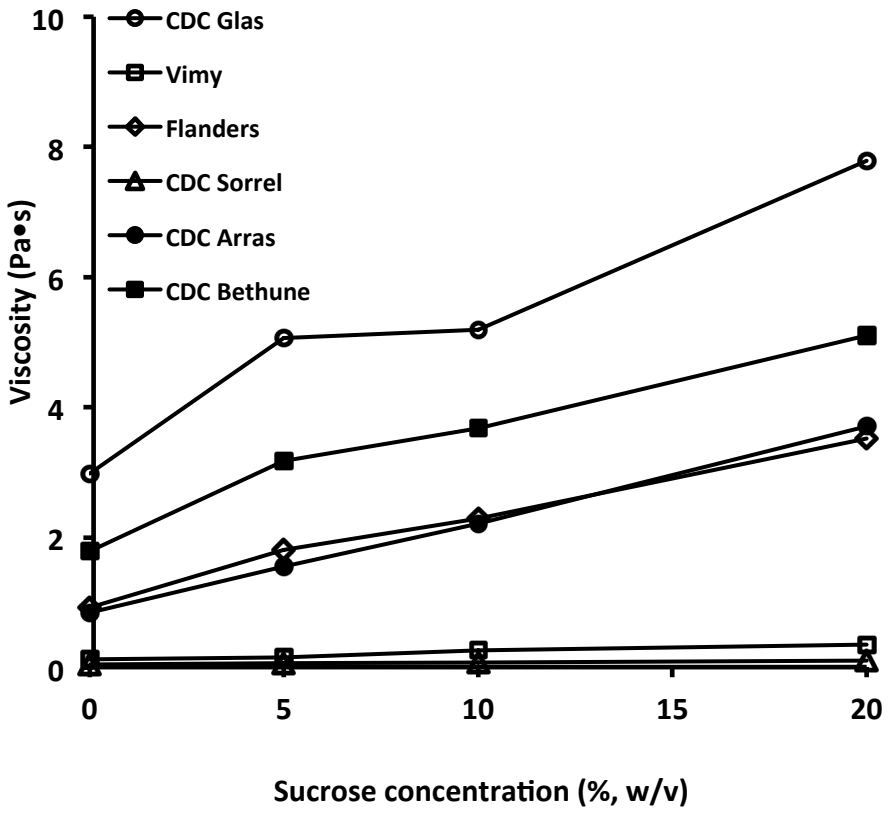

Figure 3.5 Cultivar differences in FG solutions (1.0%, w/v) apparent viscosity as a function of sucrose concentration (0−20%, w/v) at fixed shear rate of 1.0 s^{-1}.

Table 3.3 FG solution Power-law coefficients of sucrose (0−20%, w/v) FG mixtures.

Treatment		Model parameter		
Flaxseed cultivar	Sucrose concentration (%, w/v)	*n*	k (Pa•s^n)	R^2
CDC Glas	0	0.240	3.053	0.997
	5	0.187	4.248	0.993
	10	0.175	4.740	0.996
	20	0.167	7.262	0.995
Vimy	0	0.800	0.140	0.985
	5	0.789	0.161	0.999
	10	0.747	0.259	0.998
	20	0.703	0.344	0.998
Flanders	0	0.444	0.781	0.991
	5	0.300	1.582	0.994
	10	0.277	2.110	0.995
	20	0.265	3.284	0.997
CDC Sorrel	0	0.912	0.051	0.953
	5	0.845	0.076	0.988
	10	0.882	0.075	0.992
	20	0.856	0.114	0.986
CDC Arras	0	0.367	0.736	0.993
	5	0.273	1.404	0.995
	10	0.227	1.988	0.994
	20	0.216	3.415	0.997
CDC Bethune	0	0.292	1.678	0.996
	5	0.208	2.696	0.995
	10	0.202	3.289	0.996
	20	0.186	4.845	0.997

to form hydrogen bonds with k-carrageenan molecules in solution that linked k-carrageenan polysaccharide chains and stabilized polysaccharide polymer structures. Similar findings were also reported with guar gum, *Alyssum homolocarpum* seed gum, and mixtures of waxy maize starch and xanthan gum (Chenlo, Moreira, & Silva, 2011; Koocheki et al., 2009; Wang et al., 2009a), due to specific interactions between hydrocolloid polymers and sucrose. No obvious impact was observed on Balangu seed (*Lallemantia royleana*) gum (BSG) solution, as *n* coefficient was not changed with addition of sucrose. The k coefficient of BSG solution increased with sucrose concentration up to 4% (w/w) (Salehi, Kashaninejad, & Behshad, 2014). Conversely, apparent viscosity was lower when pectin solution was mixed with glucose and maltose (0−30%) (Kar & Arslan, 1999).

3.4.6 Emulsification Properties

FG, like most hydrocolloids, can stabilize oil-in-water emulsions and has been used in preparing emulsions with whey or soybean protein isolates (Khalloufi, Alexander, Goff, & Corredig, 2008; Wang, Li, Wang, & Adhikari, 2011). FG solutes are predominantly hydrophilic polysaccharides, while FG proteins increase both hydrophobicity and emulsification properties due to their surface activity at the oil-water interfaces (Yadav, Igartuburu, Yan, & Nothnagel, 2007). Gum Arabic, an emulsifier widely used in food processing, contains about 2% protein that is rich in hydrophobic hydroxyprolyl, prolyl and seryl residues (Osman et al., 1993; Osman et al., 1995). Accordingly, the hydrophobic polypeptide chains of gum Arabic anchor the polysaccharide onto oil droplet surfaces and can stabilize 20% (w/w) orange oil-in-water emulsions (Randall, Phillips, & Williams, 1988). FG solution EAIs were measured for FG prepared from each of six Canadian flaxseed cultivars (Table 3.4). FG solution EAIs ranged from 59.6 ± 1.0 to 71.8 ± 2.8 m^2/g with the highest EAI observed for FG solution prepared from Flanders, while the lowest EAIs occurred with CDC Sorrel and CDC Bethune FG (41.3 ± 0.7 and 44.6 ± 0.3 m^2/g, respectively). CDC Bethune FG solutions also had the lowest (56.6 ± 5.1 mg/g FG powder) among the cultivars investigated. Qian, Cui, Wu, and Goff (2012) reported similar results where surface tension of FG solution increased from 55.0 ± 0.5 dyn/cm to 62.0 ± 0.5 dyn/cm after complete removal of protein. They noted decreased emulsion stability of FG solution and revealed the significant contribution of proteins to emulsification properties.

Table 3.4 Emulsion activity index (EAI, m^2/g) and emulsion stability (ES, %) of FG solutions (1.0%, w/v).

Cultivar	EAI (m^2/g)	ES (%)
CDC Glas	49.10 ± 0.39^{c}	42.63 ± 1.50^{c}
Vimy	53.56 ± 1.18^{b}	38.38 ± 0.77^{d}
Flanders	59.63 ± 0.95^{a}	47.69 ± 0.59^{b}
CDC Sorrel	41.30 ± 0.71^{e}	42.94 ± 0.53^{c}
CDC Arras	54.79 ± 0.39^{b}	52.03 ± 1.39^{a}
CDC Bethune	44.58 ± 0.32^{d}	37.39 ± 0.95^{d}

Columns labeled with the same letters are not significantly different ($p < 0.05$).

In this study, FG solutions prepared from the cultivars CDC Glas and Flanders had protein contents of 64.9 ± 2.3 and 73.2 ± 13.2 mg/g, respectively. However, those FG solutions showed high EAI values. The higher emulsification activity of FG solutions prepared from CDC Glas and Flanders could be ascribed to elevated hydrophilic polysaccharide content (Nakamura et al., 2004a). Improved rheological properties of hydrophilic polysaccharides induced steric and mechanical stabilization effects, which slow or prevent emulsion droplet aggregation by forming thick charged layers (Randall, Phillips, & Williams, 1988). ES of FG solutions extracted from whole seeds of six Canadian flax cultivars was also investigated in this study and followed a similar pattern as seen in EAI. The ES ranged from 52.0 ± 1.4% to 37.4 ± 1.0% with the highest ES be obtained for CDC Arras FG preparations, while the lowest ES be observed for solutions prepared with CDC Bethune FG (Table 3.4). FG solution from CDC Arras showed the highest ES with the highest absolute ζ (−27.4 ± 0.6 mV), while the lowest absolute ζ was observed in FG solution from cultivar of CDC Bethune (−16.4 ± 0.6 mV), which correlated with the lowest ES of FG solutions tested. Surprisingly FG solution prepared from Vimy exhibited relatively lower ES (38.4 ± 0.8%) in spite of its higher absolute ζ (−27.4 ± 1.6 mV). Low FG solution viscosity may have contribution to the lower ES (Liu, Shim, Poth, & Reaney, 2016).

3.5 Conclusions

Physicochemical and functional properties of FG solutions prepared from FG extracts of six Canadian flaxseed cultivars (CDC Bethune, CDC Sorrel, CDC Arras, CDC Glas, Vimy, and Flanders) were investigated to understand effects of genotype on FG properties. Significant variation on FG yield, neutral sugar content, acidic sugar content, protein content, as well as ζ were observed among the selected flaxseed cultivars. All FG solutions demonstrated typical shear-thinning behaviour and apparent viscosity was cultivar dependent and positively correlated with neutral sugar content but negatively with acidic sugar and protein content. Cultivar dependent FG solution rheological properties were also observed with changes in solution temperature (15−45 ºC), solution pH (3.0−9.0), NaCl concentration (0−200 mM), and sucrose concentration (0−20%, w/v). FG solution apparent viscosity decreased with higher solution temperature, but not sensitive to solution pH. NaCl (> 50 mM) can screen FG anionic carboxyl group charges, reducing solution viscosity. FG solution rheological properties were enhanced by sucrose indicating interaction with FG molecules, most likely through hydrogen bonding. FG

solution emulsification properties EAI and ES were also affected by flaxseed cultivars. Protein fractions in FG could anchor the polysaccharide chains onto the surface of oil droplet to stabilize oil-in-water emulsions. While steric and mechanical stabilization effects induced by hydrophilic polysaccharide chains could prevent emulsion droplet aggregation by forming thick charged layers. Results here will help to design useful FG products with consistent rheological and emulsification properties for targeted utilization as food thickener or emulsifier. These can be achieved by selection of flaxseed genotype and controlling of FG constituents. CDC Glas FG and CDC Bethune FG are more suitable for using as food thickener. Solutions of both have higher viscosity than solutions from FG of other cultivars. However, FG yield from CDC Bethune was low which restricts the utilization of CDC Bethune as a economic cultivar source for producing FG due to the expensive gum extraction process. CDC Glas and Flanders have the highest FG yields, thus, FG could be produced from seeds and meal of those two cultivars and used as food thickener. Solutions of Vimy FG and CDC Sorrel FG demonstrated low viscosity, in turn, have greater potential as dietary fibres to show health benefits. Greater amount of Vimy FG and CDC Sorrel FG can be induced into food products than FG from other cultivars without over-texturization to affect food mouth feel. Flanders FG solutions showed higher EAI and ES than FG solutions from other cultivars, which could be applied as food emulsifiers. However, FG is not an ideal food emulsifier, while emulsification properties could be improved by blending CDC Glas FG and Flanders FG together or using Flanders FG in protein based food products. Both strategies could optimize FG constituents (protein and polysaccharide), which give advantages to show better performance as both protein and polysaccharides in FG contribute to FG solution emulsification properties.

3.6 Connection to Next Study

Protein was found in FG samples prepared from all of the selected Canadian flaxseed cultivars but the concentration of protein varied substantially. FG solutions also could stabilize emulsions as indicated by EAI and ES. Furthermore, FG solution emulsification properties were positively correlated with FG protein content. Knowledge of the nature of this protein and its contribution to FG emulsion properties is crucially important for developing FG products. Thus, the next study determined the identity of FG protein present in FG prepared from CDC Bethune, the most grown flaxseed cultivar in Canada at this time. The properties of this protein will help to understand its contributions to FG emulsification properties and to screen flaxseed cultivars for use as food emulsifiers.

CHAPTER 4

CONLININ IN FLAXSEED (*LINUM USITATISSIMUM* L.) GUM AND ITS CONTRIBUTION TO EMULSIFICATION PROPERTIES

4.1 Abstract

Flaxseed gum (FG) is a mixture of natural polysaccharide and protein derived from flaxseed (*Linum usitatissimum* L.) that has potential for thickening foods, stabilizing emulsions, and gelling solutions. The composition and identity of protein in FG have never been reported. In this study, gum prepared from whole flaxseed was deglycosylated by treating with trifluoromethanesulfonic acid (TFMS). The resultant proteins were separated by 2D-gel electrophoresis. The major protein spots with estimated molecular weights (MW) of 10–11 kDa and 11–12 kDa were excised, digested with trypsin, and analyzed using matrix-assisted laser ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Peptide MS of tryptic digestion fragments was compared to MS of peptides from gene models available through National Center for Biotechnology Information (NCBI) database. Fragments consistent with the seed storage protein conlinin, the low-molecular-mass 2S storage flaxseed protein, were identified as the major spot constituents. Emulsification properties of FG were determined before and after protease hydrolysis with emulsion activity index (EAI) and emulsion stability (ES) as indicators. Both EAI and ES decreased from 98.7 ± 5.4 to 59.9 ± 3.2 m^2/g and from 66.4 ± 1.1 to 42.1 ± 2.0%, respectively, after protease treatment. Conlinin is the major protein associated with FG and it plays a fundamental role in determining FG emulsification properties.

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4.2 Introduction

Flax (*Linum usitatissimum* L.), one of the most ancient crops, has been cultivated for over 5000 years (Cardoso Carraro et al., 2012; Oomah & Mazza, 1998). The value of flaxseed lies in its functional components including polyunsaturated fatty acids (α-linolenic acid), proteins, lignans (secoisolariciresinol diglucoside), orbitides, and soluble polysaccharides (Oomah & Mazza, 1998; Shim et al., 2014). Water-soluble flaxseed polysaccharides, commonly referred to as FG, are of special interest due to their functional properties when included in aqueous solutions. Solutions of FG have considerable viscosity and readily form stable emulsions, gels and foams (Chen, Xu, & Wang, 2006; Singh, Mridula, Rehal, & Barnwal, 2011). Accordingly, FG has been proposed for inclusion in products such as salad dressing, sausage, carrot juice, and dairy desserts (Stewart & Mazza, 2000; Zhou et al., 2010). Moreover, FG has nutritional value as a source of dietary fibre, potentially playing a role in reducing diabetes and coronary heart diseases risk, preventing colon and rectal cancer, and decreasing the incidence of obesity (Cunnane et al., 1993; Thakur, Mitra, Pal, & Rousseau, 2009).

FG mainly occurs in the outermost layer of flaxseed hulls and constitutes approximately 8% of seed dry mass (Oomah, Kenaschuk, Cui, & Mazza, 1995). FG is readily extracted from flaxseed by immersing the seed in water (Cui, Mazza, & Biliaderis, 1994). The monomer composition and structure of FG has been widely investigated, with D-xylose, L-arabinose, D-glucose, L-galactose, D-galacturonic acid, and L-rhamnose being major constituents of the polysaccharide polymers (Cui, Mazza, & Biliaderis, 1994; Cui, Mazza, Oomah, & Biliaderis, 1994; Qian, Cui, Nikiforuk, & Goff, 2012; Qian, Cui, Wu, & Goff, 2012). Two distinct polysaccharide fractions were identified in FG. One polysaccharide fraction is neutral comprising arabinoxylans with β-D-(1,4)-xylan backbones and a molecular weight (MW) of 1,200 kDa. It is largely free of uronic acid. These arabinoxylans constitute 75% of the FG fraction mass (Cui, Mazza, & Biliaderis, 1994). In addition, FG possesses an acidic fraction, which can be separated into two sub-fractions with MWs of 650 and 17 kDa. These acidic gums contribute between 3.8 and 21.3% of FG mass, respectively (Warrand et al., 2005a). However, Qian, Cui, Wu, and Goff (2012) reported that a fraction of FG with a MW of 1,470 kDa that was thought to be neutral contained a small amount (1.8%) of uronic acid. Structures of polysaccharides from the acidic FG fraction of flaxseed hulls were partially elucidated through

methylation analysis and 1D/2D nuclear magnetic resonance (NMR) spectroscopy (Qian, Cui, Nikiforuk, & Goff, 2012). A possible structure of the acidic fraction was proposed as a rhamnogalacturonan-I (RG-I) backbone that features a diglycosyl repeating unit of [→2)-α-L-Rha*p*-(1→4)-α-D-Gal*p*A-(1→]. Protein has also been observed in extracted FG (Qian, Cui, Nikiforuk, & Goff, 2012) where protein content varies from 4 to 20% of gum dry mass dependent upon flaxseed cultivar and extraction conditions (Cui, Mazza, Oomah, & Biliaderis, 1994). Nevertheless, the composition and identity of protein components in FG have not been reported.

Like most hydrocolloids, FG can stabilize oil-in-water emulsions, and proteins in FG are thought to play an important role in its emulsification properties (Bhatty, 1993; Wang et al., 2010). Removal of protein using protease reduces FG solution surface activity and decreases FG emulsion stability regardless of polysaccharide molecular mass, chain flexibility and rheological properties (Qian, Cui, Wu, & Goff, 2012). Similar emulsification properties have also been reported for other natural food hydrocolloids, including sugar beet pectin (Funami et al., 2007), gum Arabic (Yadav, Igartuburu, Yan, & Nothnagel, 2007), corn fibre gum (Yadav, Nunez, & Hicks, 2011), and soy soluble polysaccharide (Nakamura et al., 2004a, 2004b; Nakamura, Yoshida, Maeda, & Corredig, 2006). The protein components in gum Arabic (about 2% protein by mass), are rich in hydrophobic hydroxyprolyl, prolyl and seryl residues. These components are, in part, responsible for stable emulsions produced in gum Arabic solutions (Yadav, Igartuburu, Yan, & Nothnagel, 2007). The protein fraction is proposed to adsorb onto oil-water interfaces during emulsion formation, and the highly branched polysaccharide structure stabilizes emulsions through steric and mechanical effects (Funami et al., 2007; Yadav, Nunez, & Hicks, 2011). The above explanation is also congruent with reported emulsification properties of soluble soy derived polysaccharides, with protein covalently bound to a high molecular weight fraction of the carbohydrate backbone (Nakamura et al., 2004a, 2004b; Nakamura, Yoshida, Maeda, & Corredig, 2006).

In the present study, proteins in FG from whole flaxseeds were isolated and identified. The proteins were extracted from FG by deglycosylation using trifluoromethanesulfonic acid (TFMS), to totally remove carbohydrates, and then separated by 2D-gel electrophoresis. The protein gel spots were identified by matrix-assisted laser ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and confirmed by comparison with a flaxseed genome

database. Using protease treatment to remove FG proteins, the contribution of proteins to emulsification properties of FG solutions was evaluated and compared to gum Arabic solution. Findings from this study will help to further understand the effects of protein on FG solution emulsification properties.

4.3 Materials and Methods

4.3.1 Materials

TFMS (≥ 99%), gum Arabic from acacia tree (branched polysaccharide), sodium dodecyl sulfate (SDS, ≥ 99%), urea (for electrophoresis, ≤ 0.005% Ammonia), thiourea (≥ 99%), CHAPS hydrate (≥ 98% by TLC), glycerol (≥ 99% by GC), mineral oil (0.84 g/mL at 25 °C), pyridine (≥ 99%), α-cyano-4-hydoxycinnamic acid (Suitable for MALDI-TOF-MS), and trifluoroacetic acid (TFA, ≥ 99%) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Iodoacetamide (IAA), dithiothreitol (DTT), polyacrylamide, 0.5 M Tris-HCl buffer solution (pH 6.8), 1.5 M Tris-HCl buffer solution (pH 8.8), bromophenol blue, SDS running buffer (10 × premixed electrophoresis buffer, contains 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3 following dilution to 1 × with water), ammonium persulfate (APS), 2-mercaptoethanol (14.2 M, ≥ 98%), Coomassie Brilliant Blue® dye, Bio-Lyte ampholyte (pH 3−10), and *N,N,N′,N′*-tetramethylethylenediamine (TEMED) were purchased from Bio-Rad (Richmond, CA, USA). Anhydrous toluene, methanol, acetic acid, PageRuler Prestained Protein Ladder (MW marker with contrasting coloured reference bands at 10−170 kDa), Pierce® Trypsin protease (MS grade, lyophilized), and acetonitrile (HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA), as well as the Coomassie (Bradford) Protein Assay Kit® containing Pierce Coomassie Assay Reagent® and albumin standard ampules with bovine serum albumin (BSA) at a concentration of 2 mg/mL in a solution of 0.9% saline and 0.05% sodium azide. Protease (Subtilisin A from *Bacillus licheniformis*) was obtained from Megazyme International Ireland Ltd. (Bray, Co. Wicklow, Ireland) and stored in 50% glycerol at 4 °C. Canola oil (Purity 100%, Loblaws Inc., Toronto, ON, Canada) was purchased from a local supermarket (Superstore, Saskatoon, SK, Canada). A Milli-Q® deionization reversed osmosis (RO) system (Millipore, Bedford, MA, USA) was used to prepare deionized RO water (resistivity was > 18.2 MΩ•cm at 25 °C). All other reagents were of analytical grade and used as received.

4.3.2 Preparation of FG

FG was prepared from whole flaxseed according to procedures previously described by Wang et al. (2009b) with small modifications. Whole flaxseed (1.0 kg, var. CDC Bethune, Floral, SK, Canada) was weighed accurately and washed with deionized water for 1 min at room temperature (RT, 22–23 °C) to remove surface dust. Thereafter, FG was extracted by soaking flaxseed in deionized water at 60 °C for 24 h with water to seed mass ratio of 10:1 (w/w) and gentle stirring (300 rpm with a Teflon-coated stirring bar). Following this, seed was removed by filtration through cheesecloth to produce FG extracts. Insoluble particles were removed by centrifugation at 12,700 g for 20 min at 4 °C. FG was precipitated with ethanol using a volume ratio of 1:1 (extract: ethanol) at RT. Precipitated FG was collected by centrifugation at 12,700 g for 20 min at 4 °C, lyophilized (LABCONCO®, Kansas City, MO, USA), and kept in a desiccator at RT for subsequent analyses.

Neutral sugar content in FG was 592 ± 84 mg (D-xylose equivalent)/g FG, measured by spectrophotometric analysis at 480 nm (Genesys 10S UV-vis spectrophotometer, Thermo Scientific, Madison, WI, USA) based on methods previously described by Monsigny, Petit, and Roche (1988). FG acidic sugar content was 181 ± 17 mg (D-galacturonic acid equivalent)/g FG, determined by colourimetric assay at 490 nm using Benchmark microplate reader (Bio-Rad, Hercules, CA) (van den Hoogen et al., 1998). All measurements were performed in triplicate.

4.3.3 Total Protein Content

Total protein content in FG was measured using a Bradford protein assay kit (Pierce, Rockford, IL, USA). FG solution (0.1%, w/v) was prepared in deionized RO water with magnetic stirring (300 rpm) at RT for 24 h to maximize dissolution. Aliquots of FG solutions (5.0 µL) were mixed with 200 µL of Bio-Rad Protein Assay Dye Reagent (Bio-Rad Laboratories, Inc., Mississauga, ON, Canada) and 795 µL of deionized RO water. After binding of FG proteins with Coomassie Brilliant Blue G-250® in Bio-rad® Protein Assay Dye Reagent, the solutions were placed in acrylic cuvettes (semi-micro acrylic cuvette, VWR International, Radnor, PA, USA) and absorbance was monitored at 595 nm with a Genesys 10S UV-Vis spectrophotometer. A standard calibration curve of BSA (95% purity based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, SDS-PAGE) with a working range between 0 and 100

μg/mL was constructed for estimation of protein content in FG samples. All measurements were performed in triplicate and results were presented as mean ± SD.

4.3.4 Deglycosylation of FG

FG was deglycosylated to totally remove carbohydrate while leaving proteins intact using the method previously described by Yadav, Nunez, and Hicks (2011). FG samples were dehydrated in a vacuum oven (Isotemp™ Model 281A, Fisher Scientific, Pittsburgh, PA, USA) at 50 ºC until a constant weight was reached. Aliquots of dried FG (20 mg) were accurately weighed into screw-capped glass vials (15×45 mm) with Teflon-lined caps. Before the start of deglycosylation reactions, vials were kept in a dry ice/ethanol bath to maintain a temperature of –72 ºC. The deglycosylation reagent (0.5 mL of TFMS/anhydrous toluene mixture 90/10, v/v) was pre-cooled in the –72 ºC bath for 3 min and gradually added to FG. The vials were gently shaken for 5 min to ensure total dissolution of FG then placed in the bath. During the reaction, vapor was released every hour by opening the vials. After four hours the reaction was neutralized by addition of a pre-chilled (–72 °C) mixture of pyridine/methanol/water (1.0 mL, 3/1/1, v/v/v). Subsequently, sample vials were kept in crushed ice at 0 ºC for 15 min. The reaction mixture was adjusted to pH 6–7 with 2.0% (w/v) ammonium bicarbonate solution and then dialyzed against distilled water for 72 h using Spectra/Por® molecular porous membrane tubing (Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) with a 2,000 Da MW cut-off retaining the FG protein fraction. The protein fraction was freeze-dried (LABCONCO, Kansas City, MO, USA) and stored at –20 ºC until analyzed.

4.3.5 2D-Gel Electrophoresis of FG Protein

2D-gel electrophoresis was performed to separate FG proteins collected after deglycosylation by both isoelectric point (pI) and MW. Dried protein fractions (1.0 mg) were solubilized in isoelectric focusing (IEF) sample buffer (200 μL) consisting of 8 M urea, 2 M thiourea, 2% (w/v) CHAPS, 0.002% (w/v) bromophenol blue, 50 mM DTT, and 0.5% (v/v) ampholyte pH 3–10. Protein (100 μg) was applied to the linear immobilized pH gradient (IPG) 7 cm strips with pH ranges 3–10 (Bio-Rad, Mississauga, ON, Canada) for 16 h at 4 ºC. IEF was performed by applying a voltage of 250 V for 1 h, ramping to 3,500 V over 2 h, and holding at 3,500 V until a total of 75 kVh was reached. The strips were then equilibrated for 15 min in

equilibration buffer I (2.5 mL) containing 6 M urea, 30% glycerol, 2% SDS, 50 mM Tris-HCl pH 8.8, 0.01% (w/v) bromophenol blue, and the reducing agent DTT (10 mM). Alkylation was conducted for 15 min by addition of equilibration buffer II (2.5 mL) consisting of 2% (w/v) IAA following removal of DTT in equilibration buffer I. After equilibration, strips were subjected to SDS-PAGE chromatography using a Mini-protean® II system (Bio-Rad, Richmond, CA, USA) sealed with stacking gel (5%). The polyacrylamide gels for electrophoresis were poured as follows: 15% resolving gel: 3.55 mL of sterile H_2O, 3.75 mL of 40% acrylamide, 2.5 mL of 1.5 M Tris-HCl buffer solution (pH 8.8), 100 µL of 10% (w/v) SDS, 100 µL of 10% (w/v) APS, and 8.0 µL of TEMED; 5% stacking gel: 3.01 mL of sterile H_2O, 0.64 mL of 40% acrylamide, 1.25 mL of 0.5 M Tris-HCl buffer solution (pH 6.8), 50 µL of 10% (w/v) SDS, 50 µL of 10% (w/v) APS, and 8.0 µL of TEMED, respectively. After loading protein samples, electrophoresis was conducted with Bio-Rad SDS running buffer (Richmond, CA, USA) at a constant voltage of 90 V for 1.5 h. Thereafter, polyacrylamide gels were removed from the apparatus and stained for 2 h with Coomassie brilliant blue dye (Bio-Rad, Richmond, CA, USA). Gels were destained with an aqueous solution containing 20% (v/v) methanol and 10% (v/v) acetic acid. The MW of protein spots were estimated based on their migration relative to known proteins present in the standard PageRuler Prestained Protein Ladder (Fisher Scientific, Ottawa, ON, Canada) with corresponding MWs ranging from 10 to 170 kDa.

4.3.6 In-gel Digestion of Protein Gel Spots

In-gel digestion of Coomassie-stained protein gel spots obtained via 2D-gel electrophoresis was performed on a MassPrep II Proteomics Workstation (Micromass, Manchester, UK) following procedures described by Sheoran, Olson, Ross, and Sawhney (2005). The selected protein gel spots were dissected by a stainless steel surgical blade and transferred to 96 well polypropylene v-bottom microtiter plates (Corning Costar, Corning, New York, NY, USA). Protein-containing gel pieces were destained twice with 100 µL of ammonium bicarbonate/acetonitrile mixture (1/1, v/v) for 10 min. Afterwards, protein cysteine residues in gel pieces were reduced and alkylated by sequential addition of 50 µL of 10.0 mM DTT (30 min, 37 ºC) and 55.0 mM IAA (20 min, 37 ºC) in 0.1 M ammonium bicarbonate solution. Gel pieces were then washed and dehydrated with acetonitrile and cleaved with 25.0 µL porcine trypsin solution (6 ng/µL) (sequencing grade, Promega, Madison, WI, USA) in 50.0 mM ammonium

bicarbonate for 5 h at 37 ºC. Peptides were recovered from gel pieces by addition of 30 µL of 0.1% (v/v) TFA in 3% (v/v) acetonitrile for 30 min, followed by two extractions with 24.0 µL of 0.1% (v/v) TFA in 50% (v/v) acetonitrile for 30 min, respectively. The tryptic peptide extracts were combined and dried under vacuum using a centrifugal vacuum evaporator (Thermo-Savant SpeedVac, BioSurplus, San Diego, CA, USA) prior to MS analysis.

4.3.7 Mass Spectrometry and Protein Identification

Peptides arising from tryptic digestion were reconstituted in filtered (0.45 µm, ChromSpec, Brockville, ON, Canada) 0.2% (v/v) formic acid, 3% (v/v) acetonitrile aqueous solution. Aliquots (0.5 µL) of reconstituted tryptic peptides were mixed with 0.5 µL matrix solution of α-cyano-4-hydoxycinnamic acid (5 mg/mL) in aqueous acetonitrile solution (50%, v/v) containing 0.1% (v/v) TFA. Matrix containing peptide samples (1 µL) were spotted onto a stainless steel plate for MALDI-TOF-MS. The MALDI-TOF-MS was operated in positive reflectron mode. The MS survey scan was performed with a scan range from *m/z* 700 to 3,200, averaging 1,000 acquired spectra, and then processed with Protein Lynx Global Server 2.4 (Waters, Milford, MA, USA). Subsequently, MS fragment data was used to search the National Center for Biotechnology Information (NCBI) database. The MASCOT (Matrix Science, London, UK) database search engine (http://www.matrixscience.com) was employed for this search. Carboxyamidomethylation of cysteine was set as a fixed modification and oxidation of methionine was used as a variable modification during the database searches. A maximum of 1 missed cleavage site during trypsin digestion of protein gel spots was allowed with a mass tolerance for precursor peptide ions set as ± 50 ppm and ± 0.4 Da for fragment ions. Protein identification was considered unambiguous if at least 20% of the whole protein sequence was covered by the matched peptides with a minimum of four matched peptides.

4.3.8 Protease Hydrolysis of Proteins in FG

Protease hydrolysis was conducted as previously described by Qian, Cui, Wu, and Goff (2012) with small modifications to remove FG proteins. FG solution (1%, w/v) prepared in 80 mM phosphate buffer (pH 7.5) was mixed with protease stock solution (Megazyme, 350 tyrosine U/mL, 0.2 mL/g FG) under constant stirring (Teflon-coated magnetic stirring bar, 300 rpm) at 60 ºC for 60 min. The temperature of the mixture was then raised to 80 ºC to inactivate protease

then cooled to RT. The protease treated FG solution was dialyzed against distilled water at 4 ºC for 72 h with a MW cut-off of 3.5 kDa. The resultant FG solution was recovered and lyophilized (FreeZone Console Freeze Dry System with Stoppering Tray Dryer, LABCONCO, Kansas City, MO, USA) for subsequent analyses.

4.3.9 Emulsification Properties

Emulsions were prepared by mixing canola oil with FG solution before and after protease treatment using a Polytron PT 2100 homogenizer (Kinematica AG, Lucerne, Switzerland) with a 12 mm PT-DA 2112/2EC generating probe at 26,000 rpm for 3 min. The final concentrations of emulsifiers in oil-in-water emulsion systems were adjusted to 0.4–0.8% (w/v) and the oil volume fraction (ϕ) was constant at 0.1. Emulsions were then diluted 121 fold with 0.1% SDS (w/v). The absorbance of diluted emulsions was measured immediately and 60 min later with a Genesys 10S UV-vis spectrophotometer. Absorbance of SDS solution (0.1%, w/v) was recorded as the blank. Gum Arabic solutions (0.4–0.8%, w/v) were prepared for comparison with FG.

Emulsion formation activity was determined according to Einhorn-Stoll, Weiss, and Kunzek (2002). The turbidity values (T) of emulsions were calculated using Eq. 4.1.

$$T = \frac{2.303 \times \mathrm{A} \times \mathrm{V}}{\mathrm{I}} \qquad (4.1)$$

where, T is the emulsion turbidity (m^{-1}), A is the emulsion absorbance at 500 nm, V is the dilution factor, and I is the path length (0.01 m). Emulsion activity index (EAI) is a widely used indicator of emulsion formation (Moro Baez, Ballerini, Busti, & Delorenzi, 2013). EAI is related to the surface area moment mean or Sauter mean diameter, D_{32} (Cameron et al., 1991). EAI was calculated as previously described by Wang et al. (2010b) using Eq. 4.2:

$$\mathrm{EAI} = \frac{2T}{\phi \times \mathrm{c}} \qquad (4.2)$$

where, ϕ is the oil volume fraction and c is the emulsifier concentration.

The emulsion stability (ES, %) of FG solution before and after protease treatment was expressed as the ratio of turbidity measured at 60 min and immediately after dilution of emulsions (Eq. 4.3) (Wang et al., 2010b).

$$\mathrm{ES} = \frac{T_{60}}{T_0} \qquad (4.3)$$

where, T_{60} is the turbidity of diluted emulsions at 60 min, T_0 is the turbidity of emulsions immediately after dilution.

All measurements were performed in triplicate and results were expressed as mean ± SD. One-way analysis of variance (ANOVA) was conducted and Duncan's multiple range test was used for mean comparisons. P values of < 0.05 were regarded as significant.

4.4 Results and Discussion

4.4.1 Protein in FG

Due to the functional properties of FG solution, including rheological, emulsification, gelation, and foaming properties, FG is of special research interest (Chen, Xu, & Wang, 2006; Singh, Mridula, Rehal, & Barnwal, 2011). FG might also be used as a source of soluble dietary fibre, which may afford health benefits (Cunnane et al., 1993; Thakur, Mitra, Pal, & Rousseau, 2009). The use of FG as food ingredient in salad dressing, sausage, carrot juice, and dairy desserts has been proposed (Chen, Xu, & Wang, 2006). The chemical composition and structure of FG has been widely investigated (Cui, Mazza, & Biliaderis, 1994; Cui, Mazza, Oomah, & Biliaderis, 1994; Qian, Cui, Nikiforuk, & Goff, 2012; Qian, Cui, Wu, & Goff, 2012). FG was characterized as an anionic hetero-polysaccharide mixture composed of neutral (75% of FG mass) and acidic fractions (25% of FG mass). The neutral fraction is primarily arabinoxylans with β-D-(1,4)-xylan backbones, while the structure of the pectic-like acidic fraction was proposed as an RG-I backbone that features a diglycosyl repeating unit of [→2)-α-L-Rha*p*-(1→4)-α-D-Gal*p*A-(1→] (Qian, Cui, Nikiforuk, & Goff, 2012).

The protein content in dried FG prepared under the optimum extraction conditions (a temperature of 85−90 °C, a pH of 6.5−7.0, and a seed to water mass ratio of 1:13) was previously determined to be 80 g/kg (Cui, Mazza, Oomah, & Biliaderis, 1994). The protein content measured in extracted FG is known to vary depending on the assay employed and extraction conditions. FG extraction efficiency is especially dependent upon temperature (Cui, Mazza, Oomah, & Biliaderis, 1994). Previously, Qian, Cui, Wu, and Goff (2012) prepared FG from flaxseed hulls and separated the polysaccharides into different fractions. Nitrogen was not detected in neutral fractions, while substantial nitrogen content, which could represent up to 8% protein, was present in an FG acidic fraction. It was proposed that proteins are not covalently linked to FG polysaccharides (Qian, Cui, Wu, & Goff, 2012). However, there is no literature

report describing the nature of the protein or proteins in FG extracts and contribution of FG proteins to solution functional properties. In this study, FG was extracted from whole flaxseeds according to previously described methods with small modifications (Wang et al., 2009b). FG protein content was determined to be 55.7 ± 0.7 mg (BSA equivalent)/g FG (Bradford), which is reasonable as protein content in FG was reported to be 40−200 mg/g FG (Oomah, Kenaschuk, Cui, & Mazza, 1995). It should be noted that the Bradford assay cannot determine absolute protein content in FG and cannot be compared directly with protein content determined by measuring nitrogen (Bradford, 1976). However, the assay was performed to confirm the action of the protease during hydrolysis.

4.4.2 Deglycosylation and 2D-Gel Electrophoresis of FG

It was thought that a portion of proteins in FG might be glycosylated and difficult to analyze using conventional SDS-PAGE (Ray et al., 2013). Thus, TFMS was used as a deglycosylation agent to obtain intact carbohydrate-free FG proteins. Due to the difference in stability of glycosidic versus peptide bonds in the presence of TFMS, TFMS can remove glycans from glycoproteins to release intact polypeptides (Desai, Allen, & Neuberger, 1983; Lind, Bacic, Clarke, & Anderson, 1994; Takeichi, Takeuchi, Kaneko, & Kawasaki, 1998). In comparison with enzymatic deglycosylating procedures, TFMS-mediated deglycosylation enables a simple method to remove carbohydrates from glycoproteins regardless of protein linkage, carbohydrates and composition of protein and carbohydrate in glycoproteins (Edge, 2003). Yadav, Nunez, and Hicks (2011) successfully employed TFMS for deglycosylation of corn fibre gum and identified alpha-zein Z1 as a major storage protein associated with corn fibre gum. In this study, TFMS-mediated deglycosylation of FG was performed under anhydrous conditions to afford solvolytic cleavage of glycosidic bonds without peptide bond hydrolysis. The resulting FG protein fraction was collected by dialysis against distilled water.

Dialyzed proteins were separated by 2D-gel electrophoresis with a protein loading content of 100 mg (Figure 4.1). Two protein spots (1 and 2) were observed on the gel with MWs of 11−12 kDa (Spot 1) and 10−11 kDa (Spot 2). Two major storage proteins have been identified from flaxseed including a salt-soluble 11−12S globulin which accounts for 70−85% of total flaxseed protein content, and a water-soluble 1.6−2S albumin (Dev, Quensel, & Hansen, 1986; Madhusudhan & Singh, 1983; Sammour, 1999). The MW of globulins was approximately 250

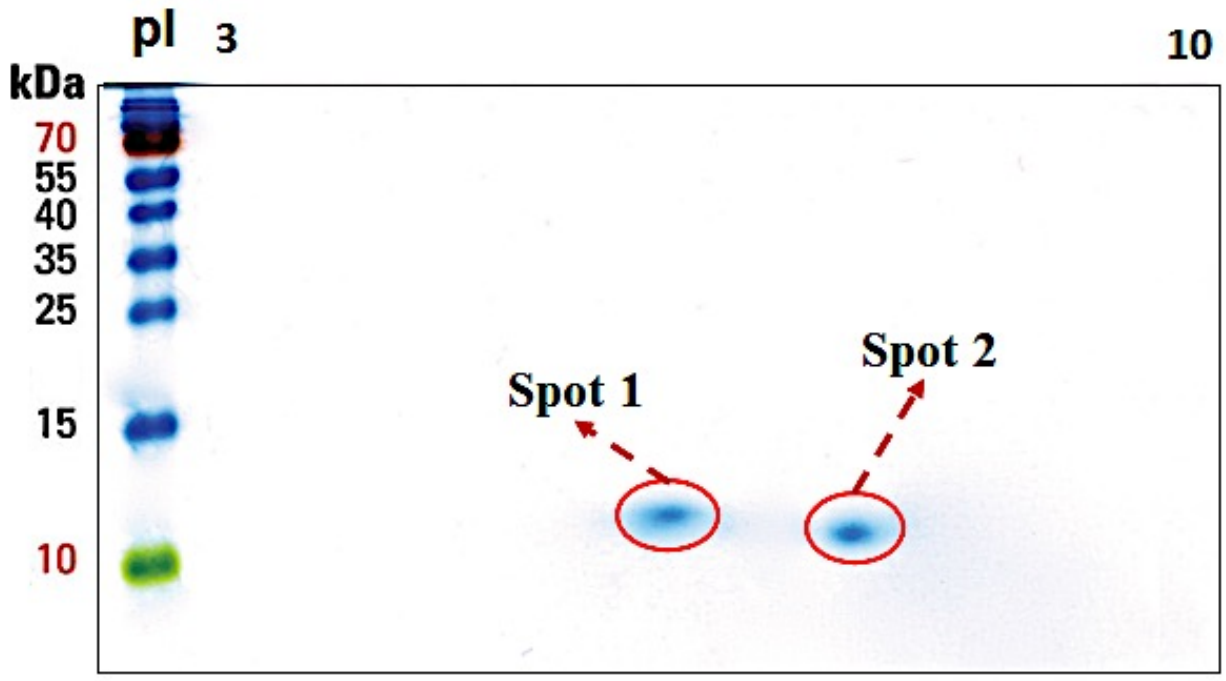

Figure 4.1 2D-gel electrophoresis of proteins collected after FG deglycosylation.

kDa. The water-soluble albumins demonstrated a MW of 25 kDa with a low MW subunit of 11 kDa (Madhusudhan & Singh, 1983). Sammour, Elshourbagy, Aboshady, and Abasary (1994) identified protein bands with MWs between 9 and 17 kDa in SDS-PAGE profiles of reduced protein fractions of flaxseed. Based on the analysis above, the protein gel spots obtained by deglycosylation of FG contain the water-soluble flaxseed albumin.

4.4.3 Trypsin Digestion of the Protein Gel Spots and Mass Spectrometric Analysis

The two well-defined protein gel spots (1 and 2) from Figure 4.1 were selected for protein identification by MALDI-TOF-MS. The protein gel spots were carefully excised, cleaned, and digested with porcine trypsin, which selectively cleaves polypeptide chains after lysine (K) or arginine (R) (Funami et al., 2007). Tryptic peptides were subjected to MALDI-TOF-MS analysis. The MS spectra data were queried against the NCBI protein database, limited to flax, using Mascot® as a search engine. A significant match to tryptic fragments was reported where matches had a confidence level ≥ 95% and a protein threshold score value of ≥ 72 as determined by Mascot®. After digestion of protein gel spots 1 and 2 with trypsin, MALDI-TOF-MS spectra revealed the presence of peptide masses shown in Figures 4.2 and 4.3, respectively. For protein gel spot 1, matched peptides corresponded to a flax hypothetical protein (NCBI accession number CAC94010) with a protein score of 394 and coverage of 41% of the sequence (Table 4.1).

Protein gel spot 2 (Table 4.2) matched a protein (NCBI accession number CAC94011) with a protein score of 395 and sequence coverage of 36% (Truksa, MacKenzie, & Qiu, 2003). The corresponding full database sequences for the hypothetical proteins identified in protein gel spots 1 and 2 (Figure 4.1) are presented in Tables 4.3 and 4.4, respectively. Accordingly, the hypothetical proteins from FG after deglycosylation were identified as containing conlinin. Interestingly, both the MALDI-TOF mass spectra of protein gel spots 1 and 2 after trypsin digestion demonstrated several common peptide masses. It appeared that both spots contained traces of both conlinin species. Conlinin, a low MW storage protein of flaxseed, was first prepared from flaxseed meal after dioxane extraction and purified subsequently with glycol (Vassel & Nesbitt, 1945). In previous work, the protein was characterized and found to have a sedimentation coefficient of 1.6 and a MW of 15−18 kDa after purification through CM-Sephadex C-50 chromatography (Madhusudhan & Singh, 1985).

However, the predicted MWs of the proteins identified in the current study were approximately

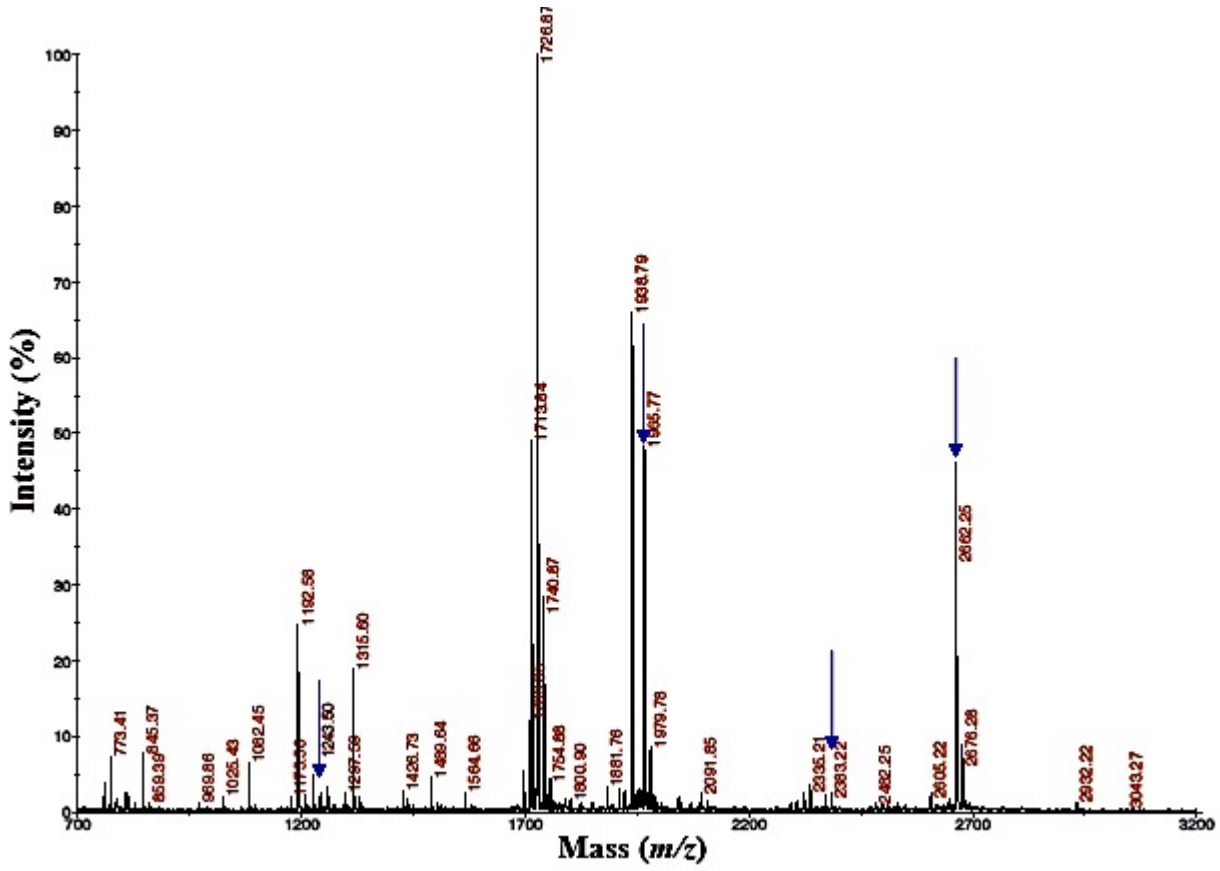

Figure 4.2 MALDI-TOF MS spectrum of tryptic peptides in protein spot 1 in 2D-gel electrophoresis.

Table 4.1 MASCOT-matched peptides for hypothetical flaxseed protein in protein spot 1[a] from 2D-gel electrophoresis.

Calculated mass (*m/z*)[b]	Observed mass (*m/z*)[b]	Position[c]	Sequence
2662.20	2662.25	39–62	GGGQGGQGQQQSCEQQIQQQDFLR
1243.52	1243.50	63–71	SCQQFMWEK
1965.75	1965.77	86–102	GGGEQSQYFDSCCDDLK
2383.18	2383.22	116–135	AIGQMRQEIQQQGQQQEVQR

[a]NCBI accession number, gi:20502190; protein score, 394; sequence coverage, 41%.

[b]The calculated and observed masses are the protonated form, $[M+H]^+$, of the peptides.

[c]The sequence position of the starting and ending amino acids are based on the database sequences presented in Table 4.3.

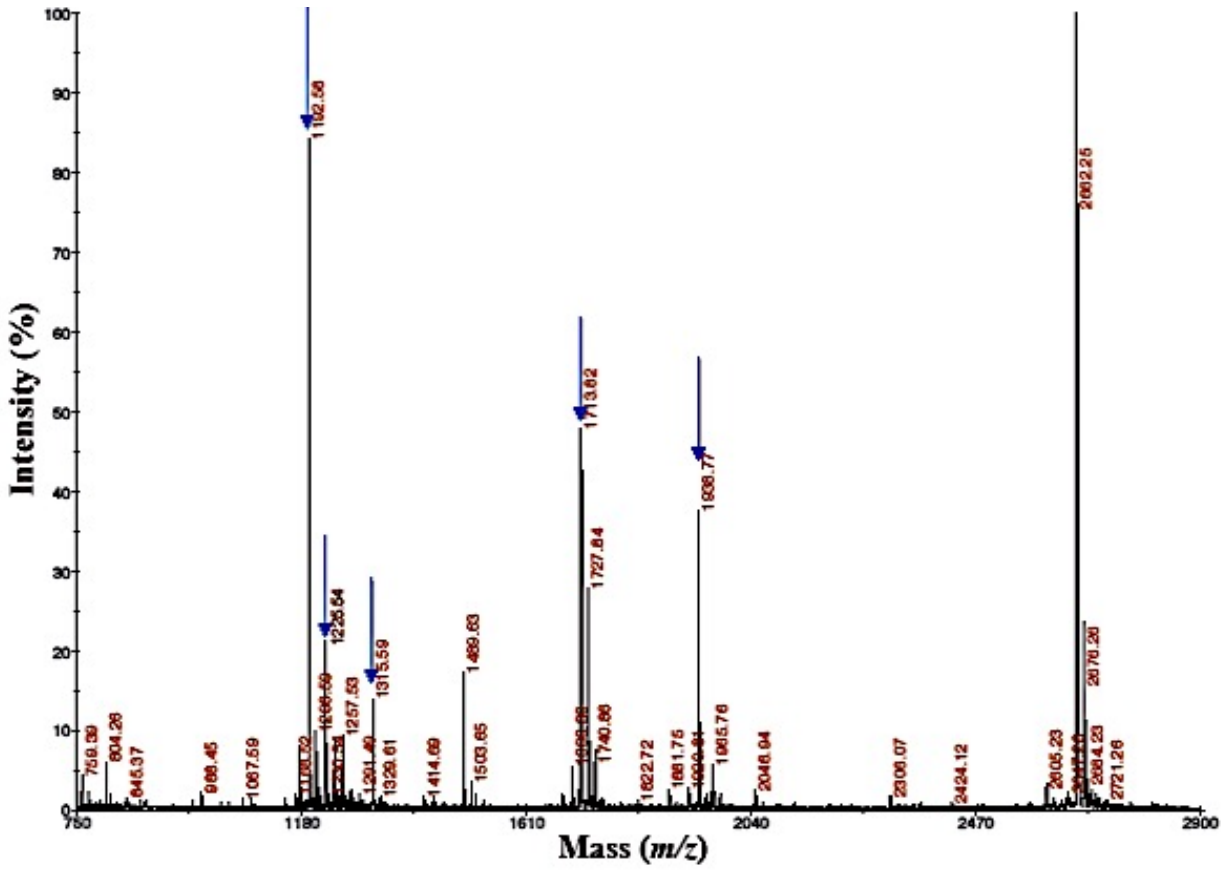

Figure 4.3 MALDI-TOF MS spectrum of tryptic peptides in protein spot 2 in 2D-gel electrophoresis.

Table 4.2 MASCOT-matched peptides for hypothetical flaxseed protein in protein spot 2[a] from 2D-gel electrophoresis.

Calculated mass (*m/z*)[b]	Observed mass (*m/z*)[b]	Position[c]	Sequence
1192.59	1192.58	53–61	QIQEQDYLR
1225.56	1225.54	62–70	SCQQFLWEK
1315.60	1315.59	145–156	DLPGQCGTQPSR
1713.81	1713.82	121–134	QDIQQQGQQQEVER
1938.76	1938.77	85–101	GGGQQSQHFDSCCDDLK

[a]NCBI accession number, gi:20502192; protein score, 395; sequence coverage, 36%.

[b]The calculated and observed masses are the protonated form, $[M+H]^+$, of the peptides.

[c]The sequence position of the starting and ending amino acids are based on the database sequences presented in Table 4.4.

19 kDa (Tables 4.3 & 4.4), which was greater than the MWs characterized by 2D-gel electrophoresis based on MW markers (approximately 11 kDa). The discrepancy may arise from the use of different flaxseed cultivars (Cui, Kenaschuk, & Mazza, 1996). Truksa, MacKenzie, and Qiu (2003) identified two cDNAs, *conlinin1* (*cnl1*) and c*onlinin2* (*cnl2*), which encode flaxseed conlinin. In previous work, the expression of conlinin genes has been reported to vary with flaxseed maturity, and post-translational modifications, such as the removal of a signal peptide, may also have occurred during the biosynthesis of conlinin, which could be responsible for inconsistencies of conlinin MW. This hypothesis was tested in silico using SignalP 4.1 online software to predict likely signal peptides. Based on the software output both predicted conlinin molecules include sequences that are consistent with signal peptide sequences of MAKLMSLAAVATAFLFLIVVDA as indicated in Tables 4.3 and 4.4 (Petersen, Brunak, von Heijne, & Nielsen, 2011). The signal peptide sequence would be cleaved after the synthesis of conlinin molecules resulting in a processed peptide of substantially reduced mass. As shown in Figure 4.1, pIs of protein gel spots 1 and 2 could be estimated as 6.5 and 7.5, respectively, based on 2D-gel electrophoresis. The estimated pI of protein gel spot 2 was consistent with the predicted pI of the conlinin database sequence (Table 4.4) after cleavage of the signal peptide (Theoretical pI: 7.48, ExPASy ProtParam, SIB Swiss Institute of Bioinformatics).

However, the predicted pI of protein gel spot 1 (7.48) without the signal peptide sequence was significantly different from the estimated pI by 2D-gel electrophoresis. The difference in pI could also be a result of additional post-translational modifications occurring after conlinin translation, such as phosphorylation of serine, threonine, tyrosine, histidine and/or arginine or lysine residues. For example, phosphorylation of amino acid residues would increase protein acidity, and alter protein pI but leave MW largely unchanged.

Gene expression is specifically activated in embryos and the inner seed coat layer, and so deposition of conlinin in the flaxseed coat would be consistent with the presence of conlinin in FG extracts. Conlinin was reported to account for 42% of flaxseed protein with 93 and 99% of the conlinin dissolved in water and 0.05 M NaCl solution, respectively (Youle & Huang, 1981). Conlinin was noted to be a single polypeptide chain with 26−30% α-helix and 51% β-sheet (Madhusudhan & Singh, 1985).

Table 4.3 Total peptide sequence[a] of the NCBI-matched protein for protein spot 1 from 2D-gel electrophoresis.

Position	Sequence				
1–50	MAKLMSLAAV	ATAFLFLIVV	DASVRTTVII	DEETNQGRGG	GQGGQGQQQS
51–100	CEQQIQQQDF	LRSCQQFMWE	KVQRGGRSHY	YNQGRGGGEQ	SQYFDSCCDD
101–150	LKQLSTGCTC	RGLERAIGQM	RQEIQQQGQQ	QEVQRWIQQA	KQIAKDLPGQ
151–169	CRTQPSQCQF	QGQQQSAWF			

[a]NCBI accession number, gi:20502190, 19,507 Da. Peptide sequence in red is signal peptide. Shaded areas in the primary sequence request peptides identified by MS of tryptic fragments.

Table 4.4 Total peptide sequence[a] of the NCBI-matched protein for protein spot 2 from 2D-gel electrophoresis.

Position	Sequence				
1–50	MAKLMSLAAV	ATAFLFLIVV	DASVRTTVII	DEDTNQGRGG	QGGQGQQQQC
51–100	EKQIQEQDYL	RSCQQFLWEK	VQKGGRSYYY	NQGRGGGQQS	QHFDSCCDDL
101–150	KQLRSECTCR	GLERAIGQMR	QDIQQQGQQQ	EVERWVQQAK	QVARDLPGQC
151–168	GTQPSRCQLQ	GQQQSAWF			

[a]NCBI accession number, gi:20502192, 19,456 Da. Peptide sequence in red is signal peptide. Shaded areas in the primary sequence request peptides identified by MS of tryptic fragments.

4.4.4 Emulsification Properties of FG Solution

The contributions of FG proteins to emulsification properties of FG solutions were evaluated before and after protease treatment alongside gum Arabic solution at the same concentration (Figure 4.4). For gum Arabic solutions, EAI was significantly decreased from 102.8 ± 3.7 to 71.8 ± 2.8 m^2/g when concentration decreased from 0.8 to 0.4% (w/v). Similarly for FG solutions, EAI significantly decreased from 98.7 ± 5.4 to 60.3 ± 1.6 m^2/g with decreasing concentration, from 0.8 to 0.4% (w/v). The EAI of FG solution (98.7 ± 5.4 m^2/g, 0.8%, w/v) was not significantly different to the gum Arabic solution (102.8 ± 3.7 m^2/g, 0.8%, w/v), however EAI decreased significantly after protease treatment (62.31 ± 0.84 m^2/g, 0.8%, w/v). Qian, Cui, Wu, and Goff (2012) reported similar results where the surface tension of FG solution increased from 55.0 ± 0.5 dyn/cm to 62.0 ± 0.5 dyn/cm after complete removal of protein. They noted decreased ES of FG solution revealed the significant contribution of proteins to emulsification properties. FG solution EAI after protease hydrolysis did not vary significantly over the concentration range from 0.8 to 0.4% (w/v). FG solutes are predominantly hydrophilic polysaccharides, while the proteins present in FG are known to increase the hydrophobicity and emulsification properties due to the substantial surface activity at oil-water interfaces (Yadav, Igartuburu, Yan, & Nothnagel, 2007). Similar properties have been observed for gum Arabic, an emulsifier widely used in food processing. Gum Arabic contains about 2% of a protein that is rich in hydroxyprolyl, prolyl and seryl residues as well as a highly branched arabinogalactan polysaccharide (Osman et al., 1993, 1995). Accordingly, the hydrophobic polypeptide chains of gum Arabic anchor the polysaccharide onto oil droplet surfaces and can stabilize 20% (w/w) orange oil-in-water emulsions (Randall, Phillips, & Williams, 1988). The ES of FG solutions before and after the protease treatment was also examined and compared with gum Arabic solutions. As shown in Figure 4.5, the ES of FG solution (66.4 ± 1.1%) was lower, but not significantly different from gum Arabic solution (73.0 ± 2.5%). After protease treatment of FG solution, the ES was significantly decreased to 42.1 ± 2.0%. Viscous hydrophilic polysaccharide chains of FG have been reported to prevent aggregation of emulsion droplets by steric and mechanical stabilization effects due to the formation a charged layer around the oil droplets (Nakamura et al., 2004b).

Protein in sugar beet pectin lowers interfacial tension between water and oil phases when it is adsorbed onto droplet surfaces and acts like an anchor. Proteins enhance both emulsion activity

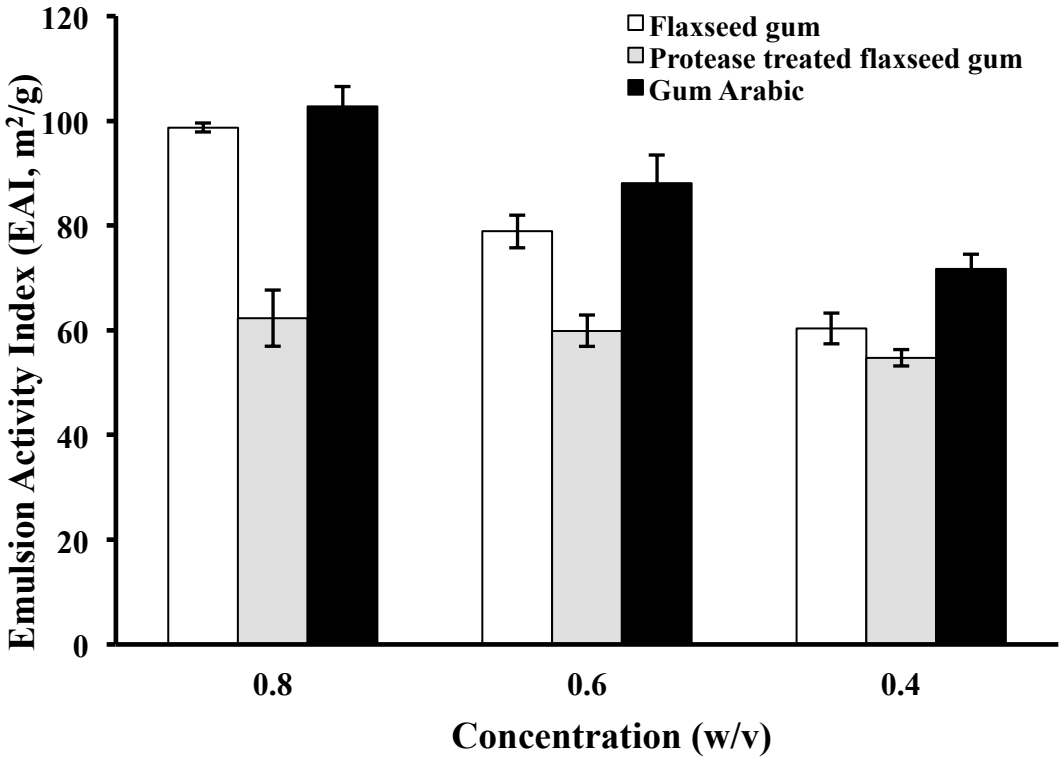

Figure 4.4 EAI of the control of gum Arabic and FG before and after protease treatment.

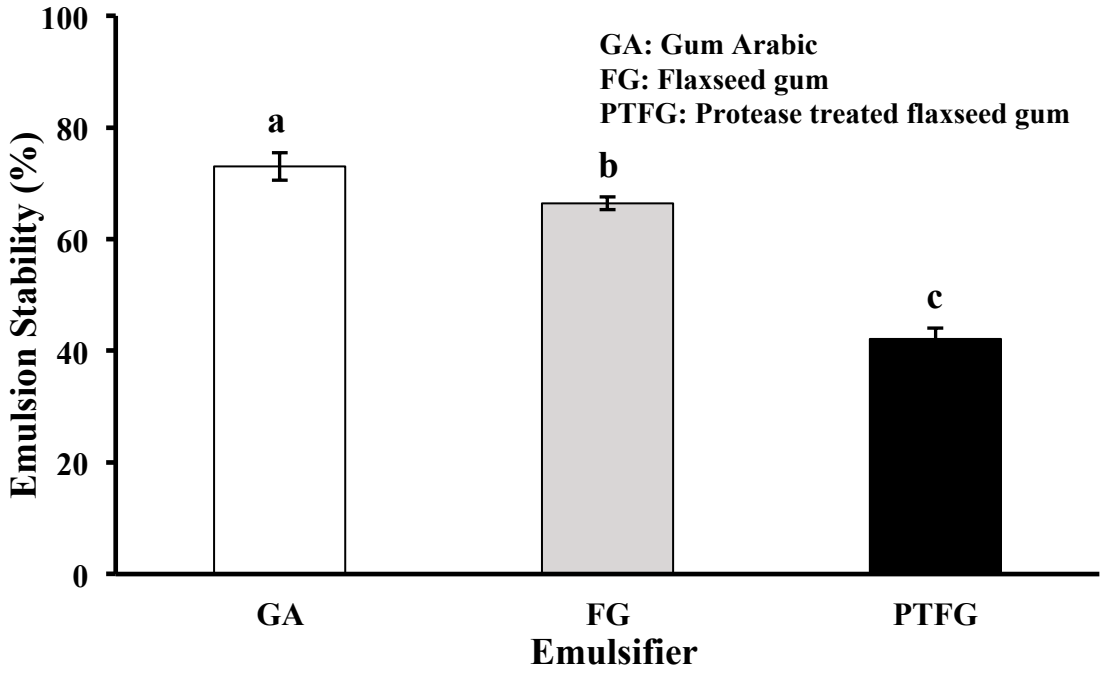

Figure 4.5 ES of the control of gum Arabic and FG before and after protease treatment. Values of each bar with different labels were significantly different ($p < 0.05$).

and stability (Funami et al., 2007). Soybean water-soluble polysaccharide (SSPS) preparations stabilize beverage emulsions as well as acidic milk beverages because of their high water solubility, pH stability, low bulk viscosity, emulsification properties, and ability to form strong interfacial films (Buffo, Reineccius, & Oehlert, 2001).

A high molecular weight fraction (HMF, 310 kDa) of SSPS exhibited better emulsification properties than whole SSPS. This phenomenon was ascribed to the protein fraction in SSPS/HMF (2.2%). The protein had a molecular mass of 50 kDa and was rich in both proline (23.1%) and glutamic acid (15.2%) (Nakamura et al., 2004b). The contribution of proteins to gum Arabic emulsification properties was confirmed by the reduced emulsion activity after protease treatment that removed proteins from gum Arabic (Randall, Phillips, & Williams, 1988). However, emulsion-forming properties of gum Arabic solution were improved by pasteurization and demineralization. This was ascribed to the denaturation and unfolding of proteins, promoting effective adsorption at the oil-water interface and enhancing double electrical layer intensity (Randall, Phillips, & Williams, 1988).

4.5 Conclusions

Proteins extracted from FG were enriched by deglycosylation with TMFS and separated via 2D-gel electrophoresis. Two protein gel spots with estimated MW of 10−11 kDa and 11−12 kDa were observed. These spots were selected, excised, digested with trypsin, and analyzed using MALDI-TOF-MS. Peptide MS data of protein fragments produced from the selected gel spots were queried against primary sequences from the NCBI database, and both proteins were identified as conlinin, a 2S storage protein of flaxseed. The contributions of proteins to FG solution emulsification properties were also examined. FG solutions showed comparable EAI and ES to solutions prepared with gum Arabic at the same concentration. Both EAI and ES were significantly decreased after protease treatment due to depletion of FG proteins. FG readily forms coacervates with BSA for example (Liu, Shim, Wang, & Reaney, 2015). FG is a naturally occurring coacervate between negatively charged polysaccharide chains and positively charged albumins. This coacervate would scatter light and be responsible for the dull colour of FG. We are conducting a detailed study of this phenomenon. Findings from this study will help to understand the mechanisms underlying FG emulsification properties and to screen flaxseed cultivars to find those with improved emulsification properties for use as food emulsifiers.

4.6 Connection to Next Study

The previous manuscript clearly shows that conlinin was the major protein present in FG from CDC Bethune and that this protein greatly affected FG solution properties. It is likely that FG used in food systems will, potentially, be mixed with other proteins. The next study was designed to examine interactions between FG and food protein in solution. While the mechanisms by which such interactions occur are known in other systems, a systematic examination of the interaction of FG with a food protein in coacevate formation has never been reported. Knowledge of interaction between FG and food protein will be used to predict the utility of FG to act as a food texture modifier, food emulsifier, and carrier of food bioactive nutrients.

CHAPTER 5
INTERMOLECULAR INTERACTION AND COMPLEX COACERVATION BETWEEN BOVINE SERUM ALBUMIN AND GUM FROM WHOLE FLAXSSED

5.1 Abstract

The formation of bovine serum albumin (BSA) coacervates with gum, extracted from whole flaxseed, was investigated by turbidimetric analysis, zeta potentiometry, and dynamic light scattering as a function of pH (6.0−1.4), biopolymer mass ratio (R = 1:15 to 15:1, w/w), salt concentration (NaCl, 0−100 mM), and urea concentration (0−150 mM). Critical pH dependent phase transitions (pH_c, $pH_{\phi 1}$, and $pH_{\phi 2}$) associated with formation of soluble and insoluble complexes of a BSA: FG mixture (R = 1:1) with a total biopolymer concentration (C_T) of 0.05% (w/w) were observed at pH 5.4, pH 4.8, and pH 2.0, respectively. The maximum interaction, indicated by the highest optical density (OD_{600}), was found at pH 3.4 (pH_{max}). In the absence of destabilization agents, the maximum coacervate formation at pH_{max} (OD_{600} = 0.818 ± 0.005) occurred at R = 2:1. As R increased from 1:15 to 15:1 the critical phase transition pH also increased ($pH_{\phi 1}$ from 4.2 to 5.2, and $pH_{\phi 2}$ from 1.8 to 2.8). The shift of pH_{max} from 2.80 to 4.80 was consistent with the isoelectric point of BSA-FG mixtures found by electrophoretic mobility measurements, while the pH_c was independent of R. NaCl significantly suppressed biopolymer interactions and decreased the pH_c, $pH_{\phi 1}$, and pH_{max}, while the $pH_{\phi 2}$ was increased. An overall shift of turbidity curve towards more acidic pH was observed in the presence of urea with less suppression of maximum OD_{600} than NaCl. Particle size distribution of BSA-FG (R = 1:1, C_T = 0.05%, w/w) obtained by dynamic light scattering at different pHs provided further insight into the association and disassociation processes during complex coacervation. Findings from this study will help to understand the nature of interactions between FG and food proteins and design new food materials for functional food and bio-material applications.

Liu, J., Shim, Y. Y., Wang, Y., & Reaney, M. J. T. (2015). Intermolecular interaction and complex coacervation between bovine serum albumin and gum from whole flaxseed (*Linum usitatissimum* L.). *Food Hydrocolloids, 49*, 95−103. Copyright ©2015, Reproduced with the permission of Elsevier.

5.2 Introduction

Coacervation of protein and polysaccharide, the most widely used hydrocolloids in food industry, is a fundamental physicochemical phenomenon that is of great importance in determining the structure and physical properties of formulated foods (Schmitt, Sanchez, Desobry-Banon, & Hardy, 1998; Strauss & Gibson, 2003). The phenomenon has attracted considerable research interest as coacervates affect the performance and utility of food, pharmaceutical, and cosmetic products (Turgeon, Schmitt, & Sanchez, 2007). Coacervation phenomena affect processes such as encapsulation, protein separation and recovery, enzyme immobilization, gelation, emulsification, and foam stabilization (Navratil & Sturdik, 2000; Roy & Gupta, 2003; Thimma & Tammishetti, 2003; Zhao et al., 2014). Controlled coacervation can be used in the production of fat replacers, meat analogues, coatings, edible films, and texturized food (Wang, Gao, & Dubin, 1996; Yang, Chen, & Chang, 1998; Azzam et al., 2002; Tolstoguzov, 2002; Burova et al., 2007). Coacervate complexes typically form when favourable intermolecular electrostatic, hydrogen bonding, and hydrophobic interactions are established during mixing of oppositely charged biopolymers in aqueous media (Lamprecht, Schafer, & Lehr, 2001; Espinosa-Andrews, Baez-Gonzalez, Cruz-Sosa, & Vernon-Carter, 2007). Coacervates can be either soluble or insoluble. Complex coacervate properties are sensitive to chemical, physical, and structural characteristics of the polymers mixture forming the coacervate including the biopolymer charges, molecular weight (MW), flexibility, and conformation (de Kruif, Weinbreck, & de Vries, 2004). Coacervate properties are also affected by the ratio and concentration of biopolymers used in producing the coacervate (Schmitt, Sanchez, Desobry-Banon, & Hardy, 1998). These biopolymer characteristics and the ratio and concentration of biopolymers, in turn, contribute to the entropy of biopolymer mixing and affect the intensity of electrostatic interactions. Solution and processing variables during coacervates preparation including pH, ionic strength, applied shear (mixing), pressure, and temperature also determine coacervates properties (Schmitt, Sanchez,

Desobry-Banon, & Hardy, 1998; de Kruif & Tuinier, 2001).

Since the first systematic study of complex coacervate formation between fish gelatin and gum Arabic (GA) (Tiebackx, 1911), additional studies were conducted of complex coacervates formed between proteins and anionic polysaccharides, such as GA, pectin, alginate, and carboxymethyl cellulose (de Kruif, Weinbreck, & de Vries, 2004). Flaxseed (*Linum usitatissimum* L.) gum contains an anionic polysaccharide that occurs mainly in the outermost layer of the flaxseed hull. It is comprised of both neutral (75%) and acidic fractions (25%) (Cui & Mazza, 1996; Warrand et al., 2003; Qian, Cui, Wu, & Goff, 2012). The neutral, uronic acid free, fraction is primarily arabinoxylans with β-D-(1,4)-xylan backbones. However, Qian, Cui, Wu, and Goff (2012) reported that a fraction of FG with MW of 1,470 kDa that was thought to be neutral contained a small amount (1.8%) of uronic acid. The acidic fraction consists mainly of pectic-like polysaccharides containing L-rhamnose, D-galactose, and D-galacturonic acid (13.8–16.2%) with a higher MW fraction (1,510 kDa) and a lower MW fraction (341 kDa) (Cui & Mazza, 1996; Qian, Cui, Wu, & Goff, 2012). FG has been used as food ingredient based on its marked water-holding capacity, good swelling properties, and rheological properties in aqueous solution (Chen, Xu, & Wang, 2006). It has been suggested that FG can be used to provide similar functionality to other non-gelling gums (Chen, Xu, & Wang, 2007; Singh, Mridula, Rehal, & Barnwal, 2011). FG increases the thermal stability of salt-soluble meat protein and water holding capacity of porcine myofibrillar protein (Sun, Li, Xu, & Zhou, 2011).

The rheological properties (apparent viscosity and viscoelasticity) and gelling temperature of FG-casein gels (casein concentration: 15−23%, w/w) increased with increases in FG concentration (0.1−0.5%, w/w) (Li, Li, Wang, Wu, & Adhikari, 2012). When FG was mixed with whey protein isolate, gel strength increased with FG concentration. Thermodynamic incompatibility caused excessive phase separation with FG concentrations higher than 0.5% (w/w), resulting in decreased gel strength (Zhang, Li, Wang, & Adhikari, 2013). Similar results were observed with cold-set whey protein isolate-FG gels induced by the addition of $CaCl_2$ or NaCl at fixed ionic strength (150 mM). Increases in FG concentration decreased both gel strength and water-holding capacity and induced phase separation between whey protein isolate and FG mixtures (Kuhn, Cavallieri, & da Cunha, 2011). However, mechanisms underlying interactions between FG and proteins in aqueous environments have not been systematically studied. Bovine serum albumin (BSA) from cow whey is an ingredient in many food products and its properties

are well characterized (Jayaprakasha & Brueckner, 1999). BSA is a globular protein with 583 amino acid residues, a MW of 66.5 kDa and an isoelectric point of 4.7 (Zhao, Li, Carvajal, & Harris, 2009). In this study, BSA was employed as a protein biopolymer to characterize interactions between protein and FG molecules in aqueous solution. The influence of pH, biopolymer mixing ratio, and destabilizing agents (NaCl and urea) on the formation of soluble and insoluble complexes was investigated through turbidimetric analysis and zeta potential. Changes of particle size in solution were studied and phase diagrams were established. Findings from this study will help to advance the understanding of mechanisms underlying associative phase behaviour between BSA and FG for expanding the utilization of FG in food products.

5.3 Materials and Methods

5.3.1 Materials

Flaxseed (var. CDC Bethune), provided as a generous gift from Dr. G. Rowland, was harvested in 2011 from Floral, SK, Canada. BSA (lyophilized powder, MW ~66 kDa; protein content > 96%) was purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Sodium azide (NaN_3, ≥ 99.99% trace metals basis) and sodium hydroxide (NaOH, ≥ 97%) were also obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Anhydrous ethanol (≤ 0.10% water by volume) was purchased from Commercial Alcohols Inc. (Brampton, ON, Canada) and hydrochloric acid (37% by mass) was obtained from Fisher Scientific Company (Ottawa, ON, Canada). A Milli-Q deionization reversed osmosis (RO) system (Millipore, Bedford, MA, USA) was used to prepare deionized RO water (resistivity was 18.2 MΩ•cm at 25 °C). All other reagents were of analytical grade purity and used as received.

5.3.2 Extraction of Gum from Whole Flaxseed

Accurately weighed whole flaxseed (1,000.0 g) was washed with deionized water for 1 min at room temperature to remove surface dust. Thereafter, the extraction was performed according to procedures previously described by Wang et al. (2009b) with small modifications. The flaxseed was soaked in deionized water with a water to seed mass ratio of 10:1 for 24 h at 60 °C under gentle magnetic stirring (300 rpm). After extraction, flaxseed was separated from gum extracts by filtration through cheesecloth. The insoluble particles in the extracts were removed through centrifugation (12,700 g at 4 °C for 20 min). The supernatant was mixed with

anhydrous ethanol at a volume ratio of 1:1 to precipitate FG. The precipitated gum was collected by centrifugation (12,700 g at 4 °C for 20 min) and lyophilized (LABCONCO, Kansas City, MO, USA). The dried FG was kept in desiccator at room temperature for subsequent analyses.

5.3.3 Preparation of Biopolymer Mixtures

Stock solutions of FG and BSA were prepared by dissolving accurately weighed amounts of each powder in deionized RO water with a concentration of 0.05% (w/w), respectively. The stock solutions were kept at room temperature (22–23 °C) for 2 h with constant stirring at 300 rpm and then incubated at 4 °C for 24 h. In order to examine the systematic effects of biopolymer mass ratio (R = BSA: FG, w/w) and pH on the interactions between FG and BSA, the R was varied from 15:1 to 1:15 with a constant total biopolymer concentration (C_T) of 0.05% (w/w). The pH of the biopolymer mixtures (6.0–1.4) were achieved *via* the addition of an internal acidifier of glucono-δ-lactone (0.005%, w/w) to slowly lower the pH to 4.4, followed by the dropwise addition of HCl solutions (0.05 M, 0.5 M, 1.0 M, and 2.0 M). To mitigate dilution during neutralization, a gradient of HCl concentrations were used based on the pH of the biopolymer mixtures (0.05 M > pH 3.6, 0.5 M > pH 2.8, 1.0 M > pH 2.0, and 2.0 M > pH 1.4) (Weinbreck, de Vries, Schrooyen, & de Kruif, 2003). The effects of destabilizing agents (NaCl and urea) on solution turbidity, as a measure of complex coacervate formation between FG and BSA, during acid titration were investigated. The concentration of NaCl and urea was varied from 0–100 mM, and 0–150 mM, respectively while R of 2:1 (w/w) was maintained. Homogenous BSA and FG solutions with corresponding concentration (0.003–0.047%, w/w) were used as controls. All measurements were performed in triplicate.

5.3.4 Turbidity Measurement

Changes of the optical density of the biopolymer mixtures during turbidimetric titration with acid were measured at 600 nm (OD_{600}), using a Genesys 10S UV-vis spectrophotometer (Thermo Scientific, Madison, WI, USA) with Semi-Micro Acrylic (PMMA) plastic cuvettes (VWR International, LLC, Radnor, Pennsylvania, USA). Turbidity (τ, cm^{-1}) was defined as:

$$\tau = -\left(\frac{1}{L}\right)\ln\left(\frac{I}{I_0}\right) \qquad (5.1)$$

Where L is the light path length of 1 cm, I is the intensity of radiation on the detector when the sample is present, and I_0 is the intensity of radiation on the detector when a blank is placed in the spectrometer. A structure formation transition point of pH_c indicated the formation of soluble "primary" complexes with a slight increase in turbidity during acid titration. The $pH_{\phi 1}$ and $pH_{\phi 2}$ were determined graphically as the intersection between two curve tangents as described by Weinbreck, Nieuwenhuijse, Robijn, and de Kruif (2003). pH_{max} was determined as the maximum OD_{600}. All measurements were performed in triplicate at room temperature and reported as mean ± SD.

5.3.5 Zeta Potential Measurement

To evaluate the overall surface charge, zeta potential (ζ, mV), of BSA, FG, and their complexes was determined by measuring electrophoretic mobility (U_E) using Laser Doppler Velocimetry combined with phase analysis light scattering (Malvern Zeta Nano ZS, Malvern Instruments Ltd., Worcestershire, UK). Biopolymer mixtures (10.0 mL) with varied R as well as the corresponding homogeneous BSA and FG (0.05%, w/w) were titrated with a MPT-2 autotitrator (Malvern Instruments Ltd., Westborough, MA, USA) using sodium hydroxide (0.5 M), HCl (0.5 M and 0.1 M) as titrants. ζ was measured at 0.4 pH unit increments. ζ was calculated based on the Henry equation:

$$U_E = \frac{2\varepsilon \times \zeta \times f(\kappa\alpha)}{3\eta} \qquad (5.2)$$

where η and ε is the dispersion viscosity (Pa·s) and permittivity of the biopolymer mixture with different biopolymer ratios, respectively. κ is the Debye length and α is the particle radius, where the f(κα) is equal to 1.5 based on the Smoluchowski approximation (Smoluchowski, 1903). All measurements were performed in triplicate and reported as mean ± SD.

5.3.6 Particle Size Measurement

The hydrodynamic diameter (d_H, nm) of BSA-FG complexes (R = 2:1, C_T = 0.05%, w/w) and the corresponding homogeneous BSA and FG solutions were determined by measuring their diffusion coefficient (D, m^2/s) as a function of pH. Samples solutions were subjected to dynamic light scattering (DLS) by a He-Ne laser with a wavelength of 633 nm at various pH levels

(Malvern Zeta Nano ZS, Malvern Instruments Ltd., Worcestershire, UK). Data analysis was performed with CONTIN program according to the Stokes–Einstein equation:

$$d_H(\times 10^9) = \frac{kT}{3\pi\eta D} \qquad (5.3)$$

where k is the Boltzmann's constant and T is the absolute temperature (K).

5.4 Results and Discussion

5.4.1 Turbidity Measurement

5.4.1.1 Effects of pH and R on Complex Formation

The charge density of weak polyelectrolyte biopolymers that participate in coacervate formation between proteins and polysaccharides is governed largely by pH (Schmitt, Sanchez, Desobry-Banon, & Hardy, 1998). For protein-polysaccharide systems, complex coacervate formation typically occurs between the isoelectric point (IEP) of the protein and the pK_a of the polysaccharides reactive groups. Although the mechanisms of the complex coacervate formation is not fully elucidated, it is widely believed that the following sequential processes are involved (Figure 5.1): (1) formation of soluble "primary" complexes (pH_c) between biopolymers with a slight increase in turbidity during acid titration; (2) initiation of the nucleation and growth of primary soluble complexes to form quasineutralised insoluble complexes ($pH_{\phi 1}$) with a rapid increase in turbidity. By further titration, the turbidity will reach a maximum point (pH_{max}) where electrical equivalence is achieved between the biopolymers; and (3) the insoluble complexes begin to disassociate after pH_{max} due to the protonation of anionic groups (e.g. carboxyl groups) on polysaccharide structures and the complexes will be largely disassociated ($pH_{\phi 2}$) when the biopolymers carry a similar net charge (de Kruif & Tuinier, 2001; Turgeon, Beaulieu, Schmitt, & Sanchez, 2003).

The effect of pH (6.0–1.4) on complex coacervate formation between BSA and FG at R = 1:1 (C_T = 0.05%, w/w) in the absence of NaCl and urea was investigated (Figure 5.2). Homogenous biopolymer solutions of BSA and FG under the same condition were also studied as controls. Four phases (I, II, III, and IV) were indicated by turbidity changes during the acid titration of BSA-FG mixture (Figure 5.2). As can be observed, a slight increase in turbidity was found at pH 5.4 (pH_c) which signified the formation of soluble complexes between BSA and FG

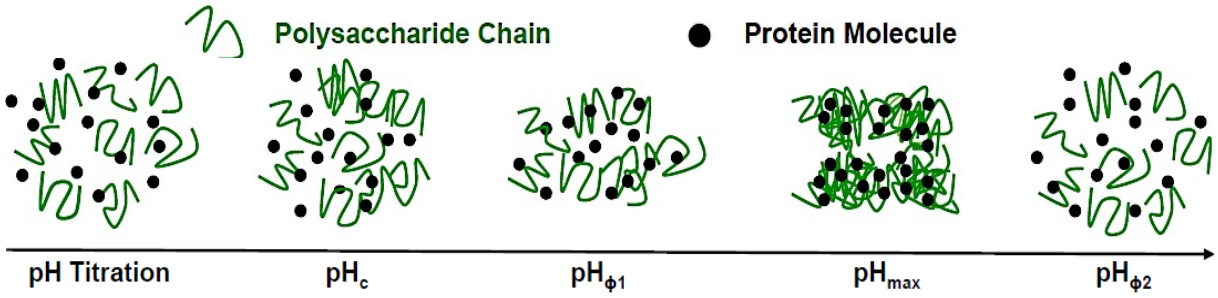

Figure 5.1 Sequential processes involve in complex coacervates formation between FG and BSA.

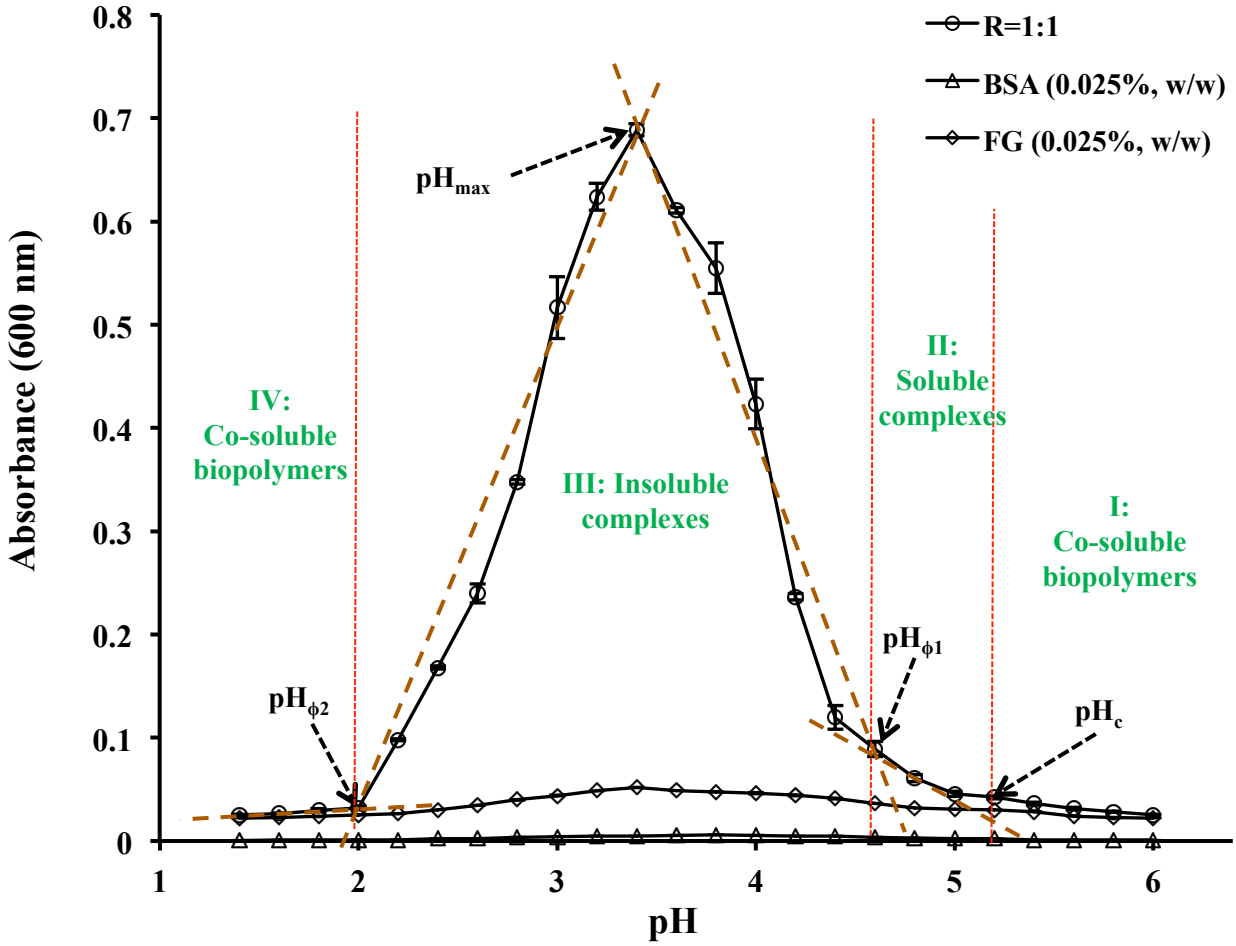

Figure 5.2 Turbidity curve of BSA-FG mixture with R = 1:1 (C_T = 0.05%, w/w) and homogenous BSA and FG solutions at 0.025% (w/w). Critical pH transition points (pH_c, $pH_{\phi1}$, and $pH_{\phi2}$) were boundaries of separate regions (I, II, III, and IV) corresponding to structure forming events during acid titration and pH_{max} referred to the pH with the maximum OD_{600} during acid titration.

as discussed by Weinbreck, de Vries, Schrooyen, and de Kruif (2003). Interestingly, the pH_c was above the IEP_{BSA} (4.96) and, theoretically, both the BSA and FG were negatively charged and repelling each other with repulsive coulombic forces that should prevent complexation. The formation of soluble complexes at pH 5.4 (pH_c) was probably due to the presence of positive patches on the BSA that interact with the carboxyl groups on the FG molecules (Park, Muhoberac, Dubin, & Xia, 1992). Similar pH_c was observed at pH 5.5 with an increase in scattering intensity but no corresponding increase in absorbance at 600 nm when BSA was mixed with GA (Vinayahan, Williams, & Phillips, 2010). This finding is in agreement with Khalloufi, Corredig, Goff, and Alexander (2009) who reported that the static and dynamic behaviour of whey protein isolate stabilized emulsions remained unchanged with addition of low concentrations (< 1.0%) of FG at neutral pH. At acidic pH the polysaccharide chains of FG adsorbed onto the emulsion droplet surfaces due to the electrostatic interactions between polysaccharides (negative) and proteins adhering to droplet surfaces (positive).

With further acidification to pH 4.8 ($pH_{\phi 1}$) < IEP_{BSA} = 4.96, BSA and FG molecules carried opposite charges and began to attract each other. The BSA-FG mixture underwent a transition from transparent-to-cloudy that was measurable as increased of OD_{600}. The turbidity corresponded to formation of insoluble BSA/FG complexes. The OD_{600} rose to 0.689 ± 0.006 at pH 3.4 (pH_{max}) indicating the maximum formation of insoluble complexes between BSA and FG. Beyond pH_{max}, insoluble complexes began to disassociate due to the progressive protonation of the FG carboxyl groups. Total disassociation of these complexes occurs at pH 2.0 ($pH_{\phi 2}$) where both biopolymers carry the same net charge. However, over the pH range of 4.8 ($pH_{\phi 1}$) to 2.0 ($pH_{\phi 2}$), turbidity measurements of homogenous BSA and FG solution showed much lower scattering intensities caused by protein aggregation and conformational changes of polysaccharides during acid titration (Weinbreck, de Vries, Schrooyen, & de Kruif, 2003).

R is critical to the charge balance between proteins and polysaccharides, which subsequently affects the intensity of complex formation (Ye, 2008). The effect of R (1:15 to 15:1), on complex coacervate formation between BSA and FG was investigated at constant total biopolymer concentration (C_T = 0.05% w/w) without NaCl and urea (Figure 5.3). Interactions between BSA and FG were almost negligible with no significant change of turbidity during acid titration when BSA concentration was low (R ≤ 1:8). Similar results were also found during coacervate formation between pea protein isolate and GA (Liu, Low, & Nickerson, 2009).

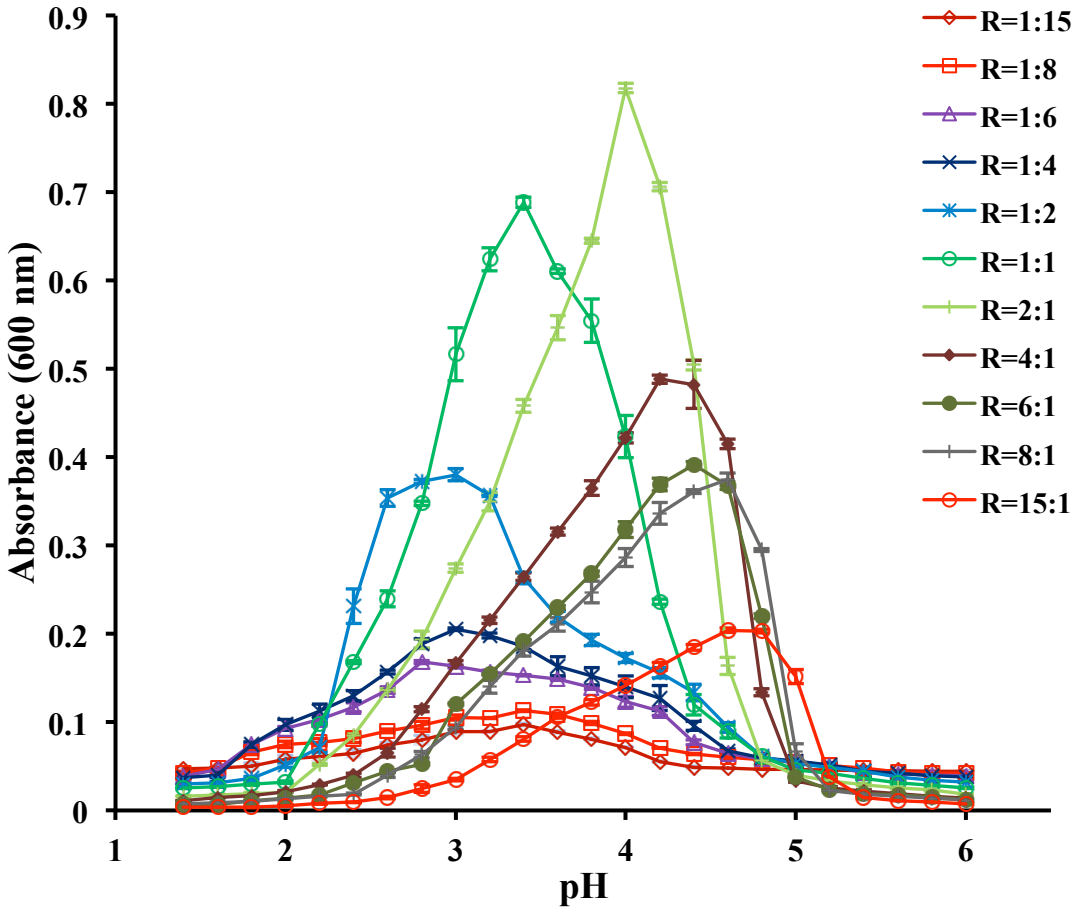

Figure 5.3 Turbidity curve of BSA-FG mixture as a function of biopolymer ratio R and pH with C_T of 0.05% (w/w).

These authors hypothesized that the polysaccharide in the biopolymer mixture, which is less effective at scattering light than protein, may prevent or reduce inter-polymeric and intra-polymeric interactions and aggregation when present at higher concentrations (Schmitt, Sanchez, Thomas, & Hardy, 1999). During the acid titration process within the pH range ($pH_{\phi 1}$ to $pH_{\phi 2}$) turbidity significantly increased with increasing BSA portion (R ≥ 1:6). The highest OD_{600} was observed where the concentration of protein exceeded that of the polysaccharide (R = 2:1) at pH_{max} 4.0. The strong OD indicated the greatest BSA-FG complex formation.

$pH_{\phi 1}$ and $pH_{\phi 2}$ tended to occur at higher pH with increasing R (Figure 5.2). Under these conditions more BSA molecules would be available in the biopolymer mixture to achieve electro-neutrality of the insoluble complex. Therefore BSA has a smaller positive charge at higher pH and higher R (Yang, Anvari, Pan, & Chung, 2012). A similar trend was evident for pH_{max} as a function of R, which exhibited a shift closer to the isoelectric point of BSA as R increased. In addition, changes in pH_{max} with R were consistent with changes in zeta potential when net zero surface charges of complexes were achieved. At that critical point, formed complexes demonstrated the highest stability, insolubility, as well as concentration due to charge neutralization (Klemmer et al., 2012). However, pH_c was not significantly changed as a function of R. Vinayahan, Williams, and Phillips (2010) reported a similar phenomenon for BSA-GA systems, indicating the formation of soluble complexes between single polysaccharides and a given amount of protein (~10 BSA molecules per GA molecule).

5.4.1.2 Effects of Destabilizing Agents on Complex Formation

Low levels of salts enhance the solubility of biopolymers by increasing molecular coiling (Schmitt, Sanchez, Thomas, & Hardy, 1999). Conversely, greater concentrations of salt inhibit complex formation by disrupting electrostatic attractive forces between biopolymers in solution (Girard, Turgeon, & Gauthier, 2002). The influence of salt (NaCl) on BSA-FG (R = 2:1, C_T = 0.05%, w/w) complex formation was studied by turbidimetric analysis. Both pH_c (from 5.4 to 4.8) and $pH_{\phi 1}$ (from 5.0 to 4.4) were lower in the presence of NaCl (100 mM) than in the absence of NaCl (Figure 5.4). In NaCl solutions Na^+ and Cl^- ions compete with the negative charge binding sites of polysaccharide molecules and positive charge binding sites of protein molecules. In these solutions the charge of dissolved ions mask the biopolymer charge and reduced interaction between BSA and FG. Similarly, NaCl reduced FG gel strength by decreasing intramolecular charge repulsions and the number of junction zones between FG polysaccharide

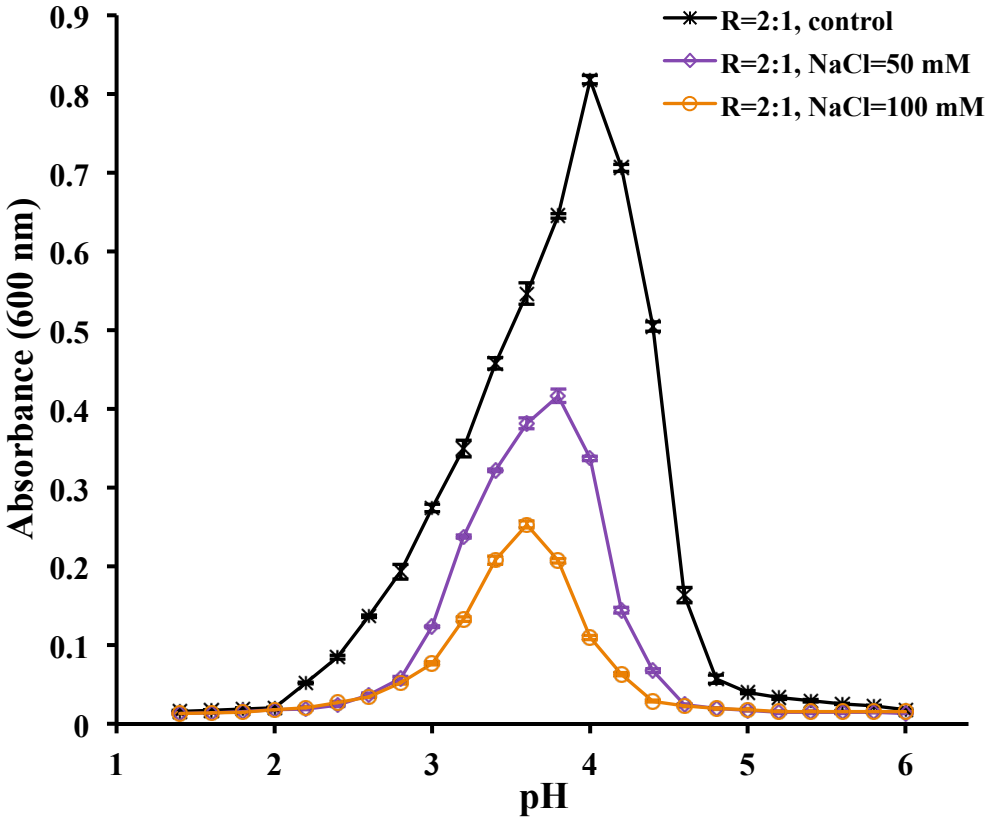

Figure 5.4 Turbidity curves of BSA-FG mixture as a function pH in the presence and absence of NaCl (R = 2:1, C_T = 0.05%, w/w).

chains (Chen, Xu, & Wang, 2006). Decreasing the solution pH ensured associative interactions between biopolymers enabling complex formation in the presence of salts, where the density of positive charges was greater on the protein (Girard, Turgeon, & Gauthier, 2002). Meanwhile, the OD_{600} at both pH_c and $pH_{\phi 1}$ was lower in NaCl indicating decreased number and stoichiometry of the soluble complexes. The pH_{max} as a function of the concentration of NaCl followed a similar trend as the OD_{600} at pH_{max} was significantly decreased due to the disruption of the electrostatic attractive forces between the biopolymers in solution. However, $pH_{\phi 2}$ increased from 2.0 to 2.4 when the NaCl concentration increased to 100 mM. Under these conditions the range between $pH_{\phi 1}$ and $pH_{\phi 2}$ decreased. Weinbreck, de Vries, Schrooyen, and de Kruif (2003) observed similar trends for whey protein isolate-GA mixtures with the decreasing of pH_c, $pH_{\phi 1}$, and turbidity in the presence of NaCl. However, Singh et al. (2007) reported that the pH_c was independent of NaCl concentrations below 200 mM and $pH_{\phi 1}$ remained stable until a critical salt concentration of 50 mM was achieved for gelatin-agar mixtures. For β-lactoglobulin-pectin mixtures $pH_{\phi 1}$ shifted to higher values when salt concentrations were lower than 100 mM. NaCl solutions with concentrations below 800 mM did not suppress turbidity. This phenomenon was ascribed to the aggregation of β-lactoglobulin in solution during acid titration (Wang, Lee, Wang, & Huang, 2007).

Complex coacervate formation between BSA and FG (R = 2:1, C_T = 0.05%, w/w) was also studied by turbidimetric analysis in the presence of urea as a standard denaturant. In general, urea can change the structure of the aqueous phase around biopolymer molecules and increase the solubility of hydrophobic groups, resulting in disrupted hydrogen bonds and hydrophobic interactions (Liu, Low, & Nickerson, 2009). During acid titration in the presence of urea the turbidity profile of the biopolymer mixture followed a pattern similar to the control (Figure 5.5). At a urea concentration of 150 mM, the turbidity curve peak occurred at a more acidic pH than for urea free controls. The critical pH values of $pH_{\phi 1}$, pH_{max}, and $pH_{\phi 2}$ for the urea treatment were 5.0, 4.0, and 2.2 while the controls were 4.8, 3.8, and 1.8. However, in agreement with the results for β-lactoglobulin-high methylated pectin system (Girard, Turgeon, & Gauthier, 2002), no effect on the pH_c value was observed in the presence of urea below 150 mM. Meanwhile, OD_{600} at pH_{max} (0.664 ± 0.009) was lower than controls (0.818 ± 0.005) when the concentration of urea was 150 mM, which indicated lower BSA: FG complex formation, most likely due to breakage of hydrogen bonds. Lii, Liaw, Lai, and Tomasik (2002) reported that the solubility of

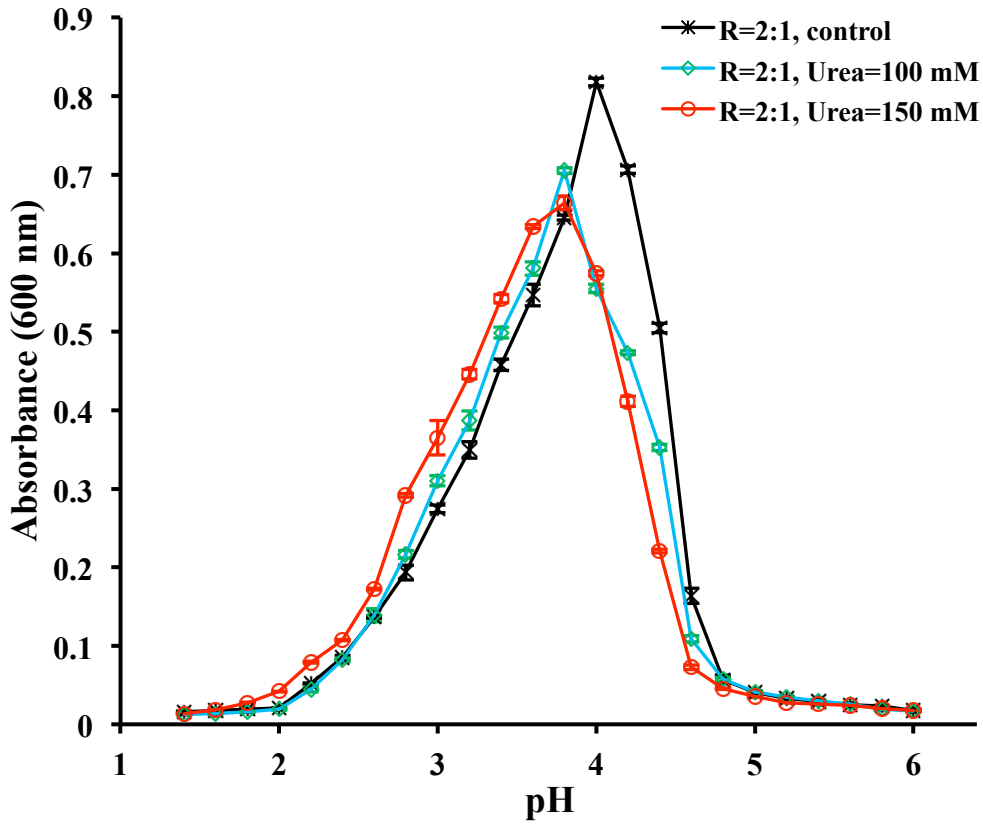

Figure 5.5 Turbidity curves of BSA-FG mixture as a function pH in the presence and absence of urea (R = 2:1, C_T = 0.05%, w/w).

xanthan gum-gelatin complexes were greater in the presence of urea at concentrations of up to 7.0 M. Both hydrogen bonding and electrostatic interactions were thought to be responsible for the complex formation. Similar results were also reported for lentil protein isolates-GA system with a pH_c, $pH_{\phi 1}$, and pH_{max} of 4.23, 3.77, and 3.60 that shifted to 3.98, 3.63, and 3.40 in the presence of 100 mM urea (Aryee and Nickerson, 2012). In principle, electrostatic interactions that lead to complex coacervate formation between protein and anionic polysaccharide through the decrease in electrostatic free energy of the biopolymer system are affected by many parameters (Tiebackx, 1911). However, much less is known about other nonspecific low energy interactions involved in complex coacervate formation such as hydrogen bonding. The pH_c of β-lactoglobulin-high methylated pectin system (5.0) was 0.5 pH units lower (4.5) in the presence of 110 mM urea. This pH shift indicates a major role of hydrogen bonding in complex coacervate formation while stronger electrostatic attractive interactions were needed to induce complex formation (Girard, Turgeon, & Gauthier, 2002). Results here provide evidence that the electrostatic attractive forces between oppositely charged biopolymers are the primarily driving force for the initial stage of complex coacervates formation. Thereafter, hydrogen bonds could serve to stabilize complexes formed between BSA and FG.

5.4.2 Zeta Potential

Complex coacervates between BSA and FG are, in large part, stabilized by electrostatic interaction between oppositely charged biopolymers. The influence of biopolymer surface electrical properties provides important information regarding complex formation and stability. Zeta potential (ζ), the electro-kinetic potential difference between the dispersion medium and the slip plane (stationary layer of fluid attached to the dispersed particle) of moving particles was measured for homogenous BSA and FG solutions and BSA-FG mixtures with varying R (1:15 to 15:1, w/w) and pH. ζ of homogenous BSA solution increased from –17.5 ± 1.8 to 32.8 ± 0.7 during titration from pH from 5.96 to 1.65 due to the protonation of BSA amine ($–NH_2$) and carboxyl groups ($–COO^-$; Figure 5.6). In agreement with literature, the IEP of BSA (0.05%, w/w), where ζ was zero, was pH 4.96 (Vinayahan, Williams, & Phillips, 2010). ζ of homogenous FG solution (0.05%, w/w) increased from –42.3 ± 1.0 to 0.03 ± 1.15 (pH from 5.98 to 1.74) with the IEP of 1.73 due to decreased ionization of carboxyl residues on the FG molecular structure with the decrease in pH (Cui & Mazza, 1996; Warrand et al., 2003). Above the IEP of FG and

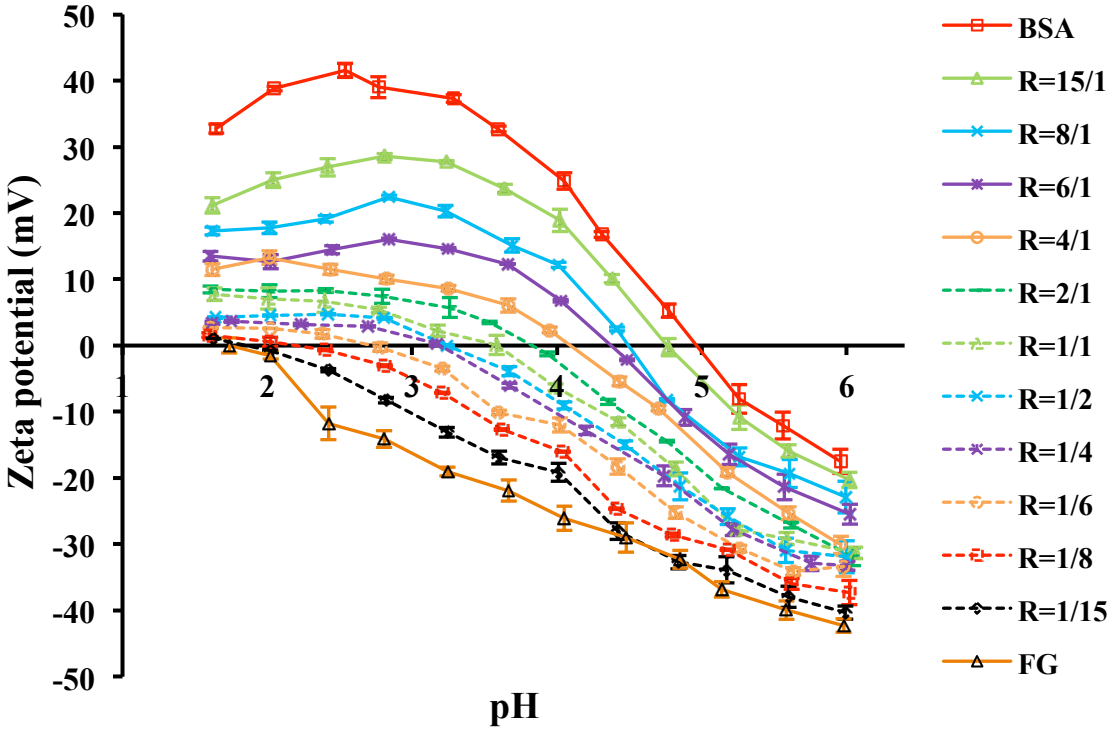

Figure 5.6 Zeta potential (mV) of BSA, FG, and mixtures thereof (C_T = 0.05%, w/w) with different biopolymer ratio (R = BSA: FG, w/w).

below the IEP of BSA, where the FG molecules were negatively charged while the BSA molecules were positively charged, complex coacervate formation occurred between BSA-FG biopolymers due to electrostatic interactions.

ζ of BSA-FG biopolymer mixture as represented by net ζ of BSA–FG complexes and non-interacting individual biopolymers in solution, increased with increasing R in the pH range tested. A similar trend was observed for BSA-FG biopolymer mixture IEP with IEP increasing from 1.86 to 4.76 as R increased from 1:15 to 15:1 due to the relatively higher ζ of BSA than FG under the same conditions (Figure 5.6). The values of IEP of BSA-FG mixtures (Figure 5.7) approximately coincided with pH_{max} determined by turbidimetric analysis at each given R (Figure 5.2). This observation indicated that complex coacervates formed between BSA and FG were stabilized by electrostatic interactions and the most intensive interaction occurred at the point where the electrical charge of the biopolymer mixtures were neutralized (Yang, Anvari, Pan, & Chung, 2012).

5.4.3 Particle Size

Particle size distribution of BSA and FG during complex coacervate formation was monitored by DLS to provide further insight into interactions between these biopolymers. The scattering intensity profiles of homogenous BSA and FG solutions (0.05%, w/w), as well as their 1:1 mixture (C_T = 0.05%, w/w) were determined between pH 2.0 and 5.6 (Figure 5.7). At pH 5.6 (Figure 5.7A), two peaks, indicating particles with diameters of 3.7 and 65 nm, were observed in homogenous BSA solution. The larger peaks indicate the presence of protein aggregates or other impurities in the BSA solution. However, the content of larger particles is negligible (data not shown) and the observed BSA particle size was consistent with reported literature values of 3–5 nm (Valstar, Almgren, Brown, & Vasilescu, 2000; Hiroshi, Kikuchi, Ogawa, & Kokufuta, 2007; Zhao, Li, Carvajal, & Harris, 2009). In FG solution two peaks with maxima at 820 and 83 nm were observed at pH 5.6 (Figure 5.7B). These comprised 88 and 12% of particles by volume (data not shown), respectively, which was consistent to the different polysaccharide fractions in FG: neutral fraction (75%) with MW of 1,470 kDa and an acidic fraction (25%) (Cui & Mazza, 1996; Qian, Cui, Wu, & Goff, 2012). When homogenous BSA and FG solutions were mixed together, R = 1:1 (C_T = 0.05%, w/w) and pH 5.6 > pH_c = 5.4 (Figure 5.7C), three separate peaks were observed with maxima indicating particle diameters of 657, 81, and 8.0 nm, respectively.

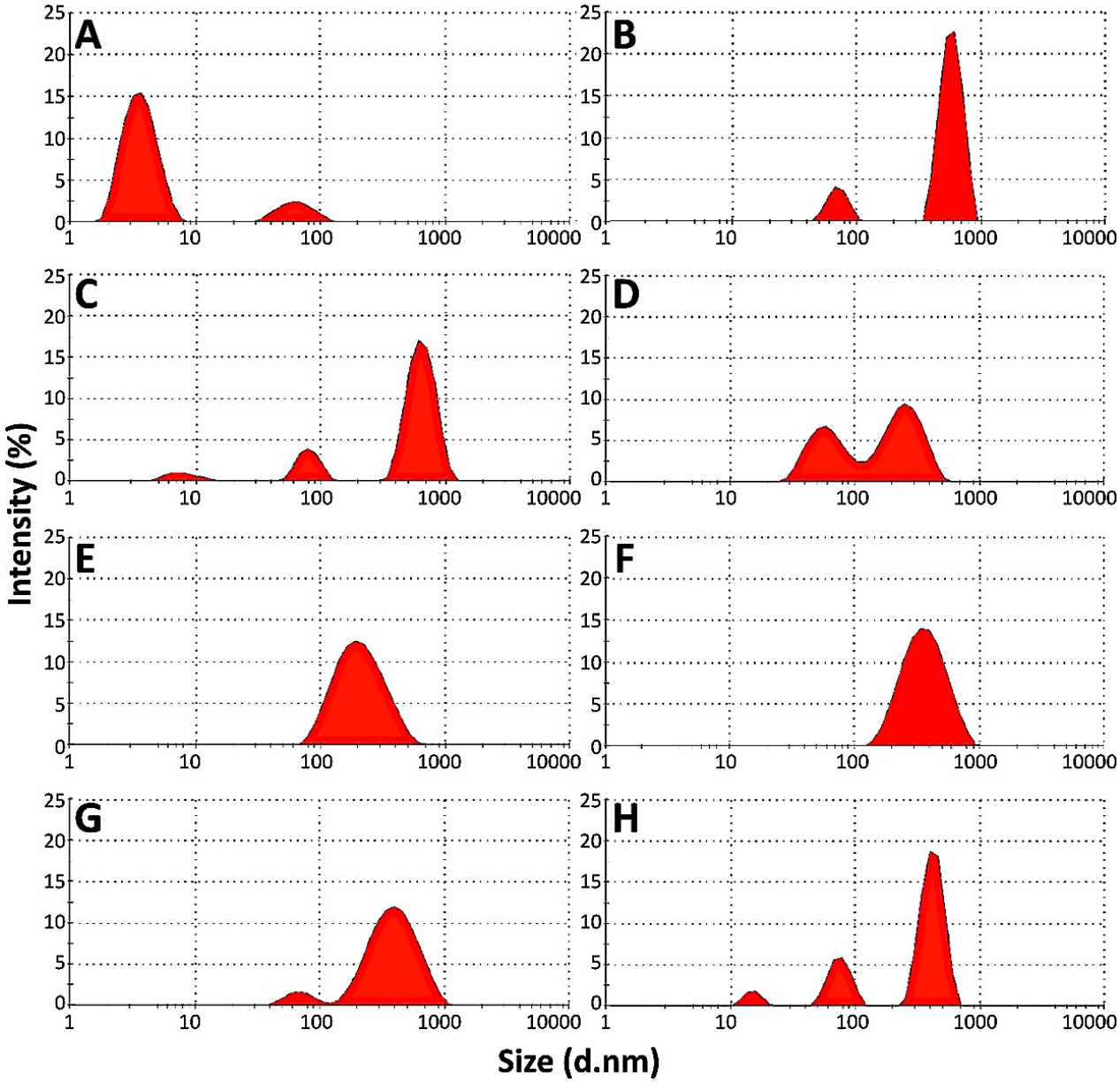

Figure 5.7 Apparent particle sizes for BSA, FG, and mixtures thereof (C_T = 0.05%, w/w) at varying pH during the complex coacervation. A: BSA solution (C_T = 0.05%, w/w) at pH 5.6; B: FG solution (C_T = 0.05%, w/w) at pH 5.6; C–H: BSA-FG mixture (R = 1:1, C_T = 0.05%, w/w) at pHs 5.6, 5.0, 4.0, 3.4, 3.0, and 2.0, respectively.

The peak maxima at 8.0 nm could be attributed to aggregates of BSA molecules, while diameters of 657 nm and 81 nm could be assigned to polysaccharides. The appearance of three separated peaks suggested no complex forming interactions between BSA and FG at pH 5.6. However it is possible that the polymers reacted in solution changing the particle distribution of both biopolymers. The absence of complexes was expected due to the negative charge of the biopolymers at pH 5.6. With a drop of pH to 5.0 > $pH_{\phi 1}$ (Figure 5.7D), two peaks were observed with the peak maximum diameters of 258 nm and 65 nm. Formation of complexes between BSA and FG is consistent with the particle size changes. Aggregation may be due to interactions between positive patches on the BSA and the carboxyl groups on the FG molecules or to altered dissociation equilibria induced by complex formation as discussed previously (Park, Muhoberac, Dubin, & Xia, 1992; Da Silva, Lund, Jonsson, & Akesson, 2006).

At pH 4.0, less than $pH_{\phi 1}$ (Figure 5.7E), only one peak with a peak diameter maximum of 221 nm was observed. This observation was consistent with formation of insoluble complexes. The formation of insoluble complexes reached maximum intensity at pH 3.4 = pH_{max} as shown in Figures 5.1 and 5.7F where the maximum diameter increased to 385 nm which was larger than observed at pH 4.0 (Figure 5.7E). The particle sizes increases occurred as the biopolymer mixture approached its isoelectric point during acid titration. With the drop of solution pH, the conformation of BSA and FG molecules change to expose more binding sites for interaction that, in turn, facilitates the formation of insoluble complexes. When pH reached 3.0, which was lower than pH_{max} (Figure 5.7G), insoluble complexes started to dissolve and dissociate due to progressive protonation of carboxyl groups on the FG structure. More peaks were found in the particle size distribution profile than at pH 4.0. For example, a peak indicating a maximum particle diameter of 15 nm was observed at pH 2.0, and this peak might be attributed to BSA. At this pH there were no interactions between BSA and FG due to the protonation of anionic groups (e.g. carboxyl groups) on polysaccharide structures and similar net charges carried by both biopolymers (Figure 5.7H). The increased particle size of BSA at pH 2.0 when compared to pH 5.6 might be attributed to the denaturation and unfolding of BSA at low pH (Vinayahan, Williams, & Phillips, 2010).

5.4.4 State Diagram

A phase diagram was established for the BSA-FG system with a C_T of 0.05% (w/w) at

room temperature by plotting the IEP values and critical pH transition points, including pH_c, $pH_{\phi 1}$, pH_{max}, and $pH_{\phi 2}$.

The diagram corresponds to phase-formation and loss during the acid titration of BSA-FG mixtures as a function of R based on turbidimetric analysis during the acid titration described above. As shown in Figure 5.8, $pH_{\phi 1}$, pH_{max}, and $pH_{\phi 2}$ increased as R increased from 1:15 to 15:1 (w/w). However, pH_c was constant with no significant changes as a function of R in agreement with Vinayahan, Williams, and Phillips (2010) who studied BSA-GA state diagrams. Between pH_c and $pH_{\phi 1}$, soluble complex formation between BSA and FG was indicated where positively charged patches on BSA surfaces were thought to be enable interactions even though the $pH_c >$ IEP of BSA. Between $pH_{\phi 1}$ and $pH_{\phi 2}$, insoluble complexes between BSA and FG were formed due to strong electrostatic interactions. The OD_{600} reached maximum at pH_{max} indicating the most significant interactions between the two biopolymers in solution. The pH_{max} were nearly coincident with the values of IEP of BSA-FG system with different R. ζ analysis confirmed the significance of charge neutralization in formation of soluble complexes. Where pH was less than $pH_{\phi 2}$, insoluble complexes were fully disassociated due to similar net charge of the biopolymers at that pH (pK_a of FG was 1.73) leading to a transparent solution. Yang, Anvari, Pan, and Chung (2012) observed this phenomenon in biopolymers that consisted of fish gelatin and GA. They reported the formation of soluble complexes when pH was below $pH_{\phi 2}$ of 2.5, which was higher than pK_a of GA (≈ 2.2). However, no complexes were observed in the aqueous mixtures of whey proteins and GA when the pH was lowered to $pH_{\phi 2}$ of 2.3 with the protonation of carboxyl groups on GA structure (Weinbreck, de Vries, Schrooyen, & de Kruif, 2003).

5.5 Conclusions

ζ and particle size measurement and turbidimetric analysis were conducted to investigate interactions between BSA and FG in solution. Either soluble or insoluble complex coacervates between BSA and FG can be formed as a function of solution pH and R. A phase diagram was developed by plotting critical structure-forming transitions (pH_c, $pH_{\phi 1}$, $pH_{\phi 2}$, and pH_{max}) and IEP against R. The most intensive complex coacervate formation occurred when charge-neutralization of the biopolymer mixture was reached based on the near coincidence of pH_{max} and IEP measured by ζ potentiometry. Soluble complex coacervates were formed in the region between pH_c and $pH_{\phi 1}$ due to binding of anionic FG to cationic patches on BSA surface.

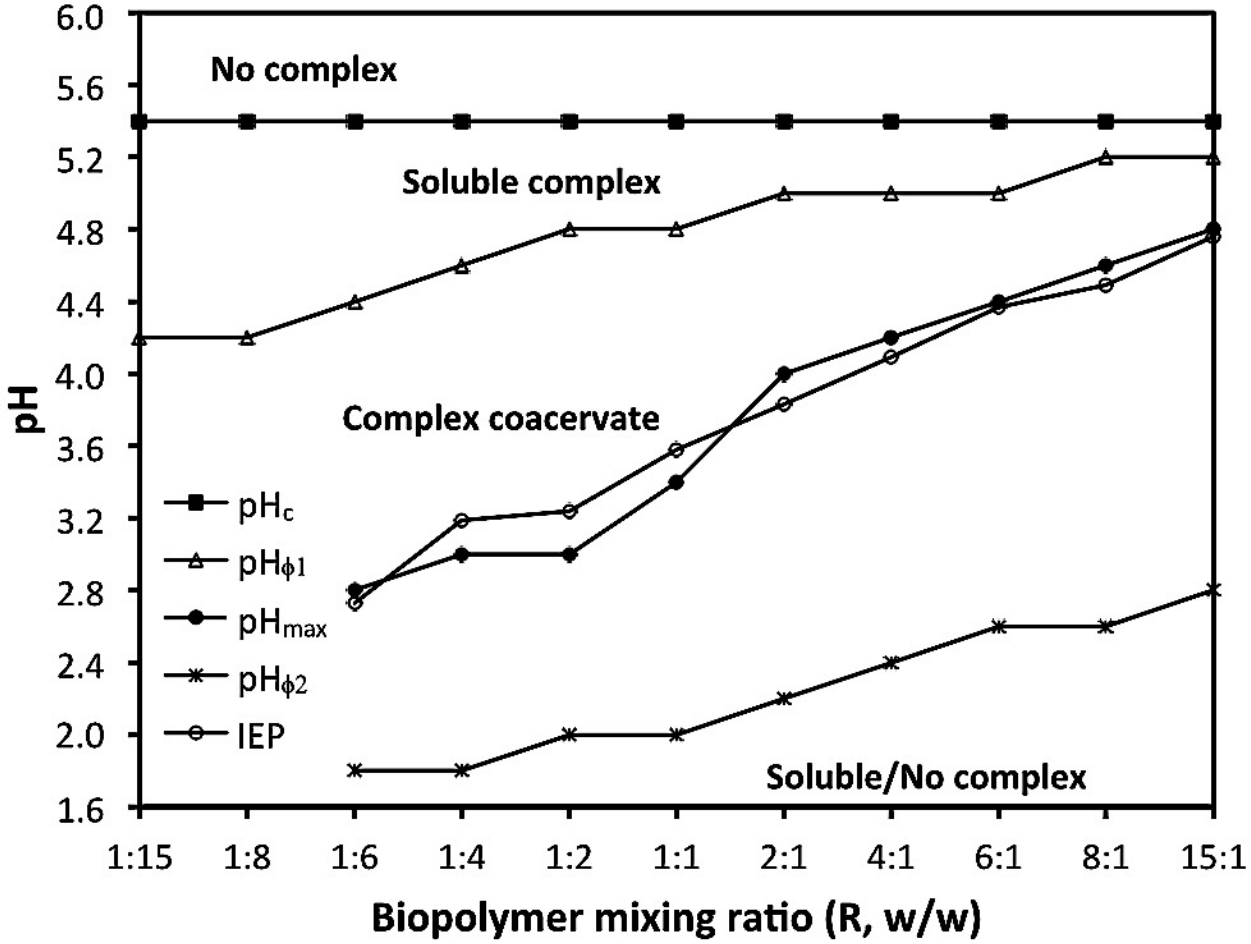

Figure 5.8 State diagram for BSA-FG mixture as a function pH and biopolymer mixing ratio (R, w/w) with C_T of 0.05% (w/w) obtained by turbidimetric analysis. IEP was the isoelectric point of BSA-FG mixture.

Insoluble complex coacervates were formed in the region between $pH_{\phi 1}$ and $pH_{\phi 2}$ where FG and BSA possessed opposite electrical charges, resulting in strong electrostatic attractive interactions. Beyond the region of $pH_{\phi 2}$, no complexes were formed due to the repulsive forces caused by similar electrical charges carried by biopolymers in solution. The presence of destabilization agents (NaCl and urea) suppressed the interaction intensity due to screening of biopolymer charges and disruption of electrostatic attractive forces and hydrogen bonds. Findings from this study demonstrate that electrostatic attractive forces primarily stabilized complex coacervate formation between BSA and FG while secondary stabilization is contributed by hydrogen bond formation. Results obtained from the present work provide essential background knowledge to introduce the application of protein-FG interactions in food, pharmaceutical, and cosmetic products.

5.6 Connection to Next Study

The previous study demonstrated that electrostatic interaction was the primarily driving force for interactions between BSA and FG in solution. Negatively charged functional groups on FG polysaccharide chains were determined the strength of the electrostatic interaction. The endogenous charge of FG derived from flaxseed is variable and not particularly intense as a relatively small number of FG sugar monomers are charge bearing galacturonic acid. In the next study, anionic functional groups, carboxymethyl, were introduced to the FG structure. This would modify both charged and neutral sugars and result in a FG that had "stronger" interactions between modified FG and food proteins. This could also increase the utility of FG for applications that require greater charge density.

CHAPTER 6

CARBOXYMETHYL DERIVATIVES OF FLAXSEED (*LINUM USITATISSIMUM* L.) GUM: CHARACTERIZATION AND SOLUTION RHEOLOGY

6.1 Abstract

Carboxymethyl ether moieties were introduced to flaxseed (*Linum usitatissimum* L.) gum (FG) by reacting gum with monochloroacetic acid (MCA). Effects of ether forming reaction conditions, temperature, NaOH concentration, and molar ratio of FG (anhydroxylose equivalent) to MCA, on the degree of substitution (DS) were investigated. Treatment at 70 °C, with 7.0 M NaOH, and molar ratio of MCA to FG of 10:1 over 3 h produced a polysaccharide with a DS of 0.824 ± 0.012. Both surface morphology and thermal behaviour were modified. Solutions of FG (0.5−4.0%, w/v) exhibited shear-thinning behaviour where viscosity decreased with decreased FG concentration. After carboxymethylation, modified polymer solutions also exhibited shear-thinning behaviour, where dynamic viscosity was lower than that of native FG solutions at the same concentration. The dynamic viscosities were lower for solutions of modified FG with higher DS and also lower for solutions made at lower FG concentrations (0.5−4.0%, w/v). A more liquid-like behaviour with increasing FG DS was indicated as storage modulus G′ and loss modulus G″ decreased and became more frequency dependent. The substitution of hydroxyl groups with carboxymethyl groups suppressed entanglement between FG polysaccharide chains by reducing intermolecular association.

Liu, J., Shim, Y. Y., Shen, J., & Reaney, M. J. T. (2016). Carboxymethyl derivatives of flaxseed (*Linum usitatissimum* L.) gum: characterization and solution rheology. *International Journal of Food Science & Technology, 51*, 530−541.

6.2 Introduction

FG is comprised of high molecular weight (MW) carbohydrate heteropolymers. Similar heteropolymers are among the most widely used industrial materials (Mirhosseini & Amid, 2012). These materials are used extensively in food, industrial and pharmaceutical products, and they have advantages over synthetic polymers, such as greater sustainability, biocompatibility, biodegradability, and biosafety. These materials are used as solutes to induce a range of physical and chemical properties to solution (Prajapati, Jani, Moradiya, & Randeria, 2013). However, natural gums may exhibit undesirable properties, including uncontrolled rates of hydration, pH dependent solubility, thickening, viscosity loss with storage, and microbial contamination. Unpredictable performance of natural polysaccharide gum can restrict use of these materials in specific applications (Rana et al., 2011). Functional properties of biopolymers, including FG, may be improved by physical modification approaches such as micro-fluidization, extrusion, freeze-thaw cycling and/or chemical modification approaches such as graft co-polymerization, oxidation, thiolation, and carboxymethylation (Ahuja, Singh, & Kumar, 2013).

Carboxymethylation is widely used *O*-alkylation reaction for modifying natural polysaccharide gums and producing products with novel properties (Biswal & Singh, 2004). The carboxymethylation reaction involves two steps based on Williamson synthesis (Figure 6.1). In this reaction, polysaccharide alkoxide is reacted with monochloroacetic acid (MCA) to substitute protons of alcohol groups with carboxymethyl moieties (Gong et al., 2012). This reaction is employed for industrial modification of polysaccharide gums. The reaction is efficient, proceeds under mild conditions, utilizes low cost reactants and produces products with little toxicity (Kumar & Ahuja, 2012; Verraest, Peters, Batelaan, & Vanbekkum, 1995). Polysaccharide carboxymethyl derivatives are made with different starting polymers, including cellulose (Biswal & Singh, 2004), starch (Stojanovic, Jeremic, Jovanovic, & Lechner, 2005; Tijsen, Kolk, Stamhuis, & Beenakers, 2001; Bhattacharyya, Singhal, & Kulkarni, 1995), chitin (Kittur, Prashanth, Sankar, & Tharanathan, 2002), gellan (Ahuja, Singh, & Kumar, 2013), pullulan (Glinel, Sauvage, Oulyadi, & Huguet, 2000), and inulin (Akin, Oner, Bayram, & Demadis, 2008). Polyelectrolyte reaction products have better water solubility, increased hydrophilicity and solution clarity when compared to natural gums (Silva et al., 2004).

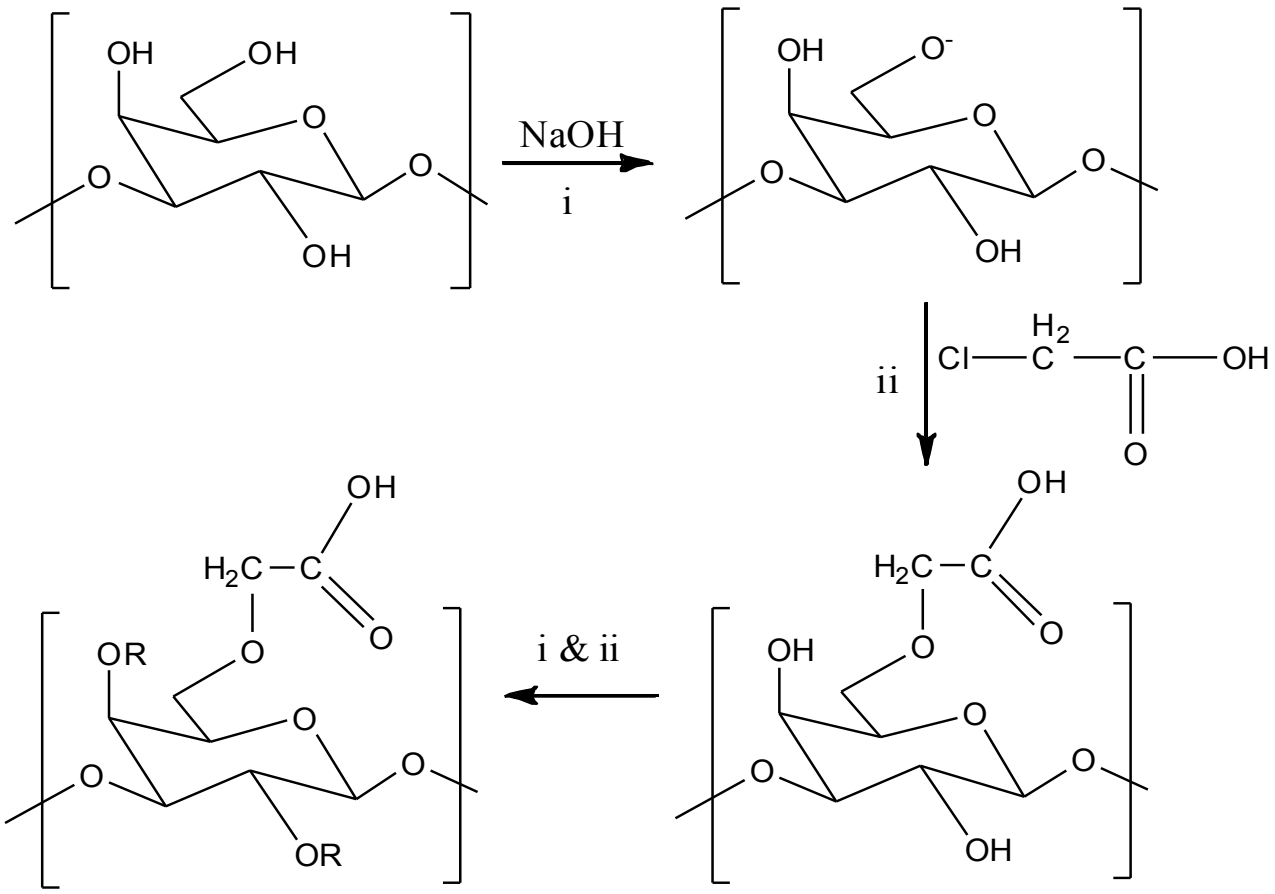

Figure 6.1 Schematic representation of the reaction of monochloroacetic acid with carbohydrate (R: H or $–CH_2–COOH$).

Flaxseed (*Linum usitatissimum* L.) gum, is a potential by-product of the flax oil industry that is present mainly in the outermost layer of flaxseed hulls and, thus, is readily released from whole or ground seed when soaked in water. FG is comprised of at least two distinct polysaccharides: a neutral arabinoxylan (75%) and an acidic rhamnogalacturonan (25%) (Cui, Mazza & Biliaderis, 1994). The neutral, uronic acid-free fraction is primarily arabinoxylans with β-D-(1,4)-xylan backbones and a molecular weight (MW) of 1,200 kDa. The acidic fraction of rhamnogalacturonan can be further separated into two sub-fractions with MWs of 650 and 17 kDa, which contribute 3.8 and 21.3% of FG mass, respectively (Warrand et al., 2005a). However, Qian, Cui, Wu, and Goff (2012) reported that a fraction of FG with a MW of 1,470 kDa that was thought to be neutral contained a small amount of uronic acid (1.8%). Through methylation analysis and 1D/2D nuclear magnetic resonance (NMR) spectroscopy the structure of the acidic fraction from flaxseed hulls was partially elucidated as a rhamnogalacturonan-I (RG-I) backbone that features diglycosyl repeating unit of [→2)-α-L-Rha*p*-(1→4)-α-D-Gal*p*A-(1→] (Qian, Cui, Nikiforuk & Goff, 2012). D-xylose, L-arabinose, D-glucose, L-galactose, D-galacturonic acid, and L-rhamnose were reported as major constituent FG monomers and uronic acid was also found after acid hydrolysis (Cui & Mazza, 1996; Warrand et al., 2005a). Due to both good swelling and high viscosity in aqueous solution, FG shows marked water-holding capacity (Chen, Xu, & Wang, 2006). Rheological properties of FG extracts are dependent on genetics (Cui, Kenaschuk, & Mazza, 1996). FG showed shear-thinning (pseudoplastic) flow behaviour with weak gel-like properties that are dependent on FG sugar monomer composition (Cui, Mazza, & Biliaderis, 1994). In addition, FG rheological properties are influenced by concentration, pH, temperature, electrolytes, extraction conditions, and drying methods (Oomah, Kenaschuk, Cui, & Mazza, 1995; Wang et al., 2008). The 'Weak-gel' like property of FG make it suitable for use as a replacement for gums used in non-gelling food and non-food applications (Chen, Xu, & Wang, 2007; Singh, Mridula, Rehal, & Barnwal, 2011).

FG has undesirable characteristics including slow dissolution rate in cold water, dull colour in solution, and low storage stability. Properties of other polysaccharides have been modified and improved by formation of carboxymethyl ethers. There are no literature reports of the synthesis and properties of FG carboxymethylation products. In the present study, FG was treated with reagents to afford carboxymethylated flaxseed gum (CMFG). Fourier transform infrared (FTIR) spectroscopy, nuclear magnetic resonance (NMR) spectroscopy,

differential scanning calorimetry (DSC), and scanning electron microscopy (SEM) were used to characterize these modified gum products. The effects of reaction conditions on the degree of carboxymethyl substitution were determined using titrimetric methods. Rheological behaviours of FG with different levels of modification were studied to determine product uniformity and performance.

6.3 Materials and Methods

6.3.1 Materials

Whole flaxseed (var. CDC Bethune, 2011) used for extraction of FG was provided as a generous gift from Dr. G. Rowland and harvested from Floral (SK, Canada). MCA, sodium hydroxide (NaOH, ≥ 97.0%), phenolphthalein, and potassium bromide (FTIR grade) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Deuterium oxide (D_2O) was obtained from VWR International (Radnor, PA, USA). Anhydrous ethanol (≤ 0.10% water by volume) was purchased from Commercial Alcohols Inc. (Brampton, ON, Canada) and hydrochloric acid (37% by weight) was obtained from Fisher Scientific (Ottawa, ON, Canada). All other chemicals were of analytical grade and used as received. A Milli-Q deionization reversed osmosis (RO) system (Millipore, Bedford, MA, USA) was used to prepare deionized RO water with a resistivity of greater than 18.2 MΩ•cm at 25 °C.

6.3.2 Extraction of FG from Whole Flaxseed

An aqueous process was used to extract FG used in this work. Whole flaxseed (1.0 kg) was washed in deionized RO water for 1 min at 21–23 °C to remove surface particulate matter, and then soaked with deionized water (mass ratio of 1: 10 = seed: water) for 24 h at 60 °C while stirring gently with a Teflon® coated magnetic bar (300 rpm) as suggested by Wang et al. (2009b) with small modifications. Extracts were then filtered through cheesecloth to remove seed then the filtrate was centrifuged at 12,700 × g for 20 min at 4 °C to settle insoluble particles. FG was precipitated from the supernatant by thoroughly mixing extracts with one volume of anhydrous ethanol. After centrifugation (12,700 × g, 20 min, 4 °C) precipitates were collected and re-dissolved in deionized water before freeze-drying (LABCONCO, Kansas City, MO, USA). The freeze-dried FG was kept in a desiccator for subsequent studies.

6.3.3 Preparation of CMFG

FG was reacted with MCA under alkaline conditions as reported earlier for gellan gum with some small modifications (Ahuja, Singh, & Kumar, 2013). To form the carboxymethyl products, FG (1.0 g) was first dispersed in ice-cold NaOH solution (40 mL) with stirring over 30 min. MCA solutions (75%, w/v) were added to each of these solutions with constant stirring. The mixtures were then heated to the desired temperature (Table 6.1) and held at the reaction temperature for 3 h. After each reaction, mixtures were cooled to 21−23 °C and neutralized (pH 7.0) with hydrochloric acid solutions (4 M). The resulting CMFG products were dialyzed (MW 3,500 Da) with Spectra/Por® molecular porous membrane tubing (Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) against distilled water for 72 h at 4 °C with gentle stirring (150 rpm) and freeze-dried for subsequent analyses.

6.3.4 Degree of Substitution

The degree of substitution (DS) was determined using the titrimetric method of Stojanovic, Jeremic, Jovanovic, and Lechner (2005). Freshly prepared CMFG (400 mg) was dispersed in 10 mL of 2 M hydrochloric acid reagent with 70% (v/v) ethanol as the solvent for 3 h under continuous stirring to convert the sodium form (Na-CMFG) to the hydrogen form (H-CMFG). The H-CMFG was precipitated and washed with 95% (v/v) ethanol to remove free acid. H-CMFG collected after washing was oven dried at 60 °C until it reached a constant weight.

Dried H-CMFG (200 mg) was dissolved in 20 mL of standardized NaOH solution (0.1 M) and the solution stirred for 3 h, to allow complete dissolution, before analysis of DS. Excess NaOH was then back titrated with standardized hydrochloric acid solution (0.05 M) using phenolphthalein as an indicator. The following equations were used to calculate the degree of carboxymethyl substitution:

$$DS = \frac{150\ \mathrm{N}}{\mathrm{M} - 58\ \mathrm{N}} \tag{6.1}$$

$$N = \mathrm{C}_{NaOH}\mathrm{V}_{NaOH} - \mathrm{C}_{HCl}\mathrm{V}_{HCl} \tag{6.2}$$

Where C_{NaOH} and C_{HCl} were the molar concentration of NaOH and hydrochloric acid solutions used for titration, respectively. V_{NaOH} was the volume of NaOH (20 mL) and V_{HCl} was the volume of hydrochloric acid used for the back titration of the excess of NaOH. M was

Table 6.1 Reaction conditions used for the carboxymethylation of FG.

Sample	Temperature (°C)	NaOH concentration (mol/L)	n_{MCA}/n_{FG} [a]	DS [b]
CMFG 1	30	5.0	7	0.114
CMFG 2	40	5.0	7	0.316
CMFG 3	50	5.0	7	0.379
CMFG 4	60	5.0	7	0.491
CMFG 5	70	5.0	7	0.573
CMFG 6	80	5.0	7	0.551
CMFG 7	70	3.0	7	0.477
CMFG 8	70	4.0	7	0.524
CMFG 9	70	6.0	7	0.586
CMFG 10	70	7.0	7	0.617
CMFG 11	70	8.0	7	0.602
CMFG 12	70	7.0	6	0.405
CMFG 13	70	7.0	8	0.669
CMFG 14	70	7.0	9	0.738
CMFG 15	70	7.0	10	0.862
CMFG 16	70	7.0	11	0.754

[a] n_{MCA}/n_{FG} is the molar ratio of MCA to FG.

[b] DS is the degree of substitution of carboxymethylated FG.

the mass of H-CMFG used for titration and 150 g/mol was used as the molar mass of the anhydroxylose units.

All titrations were conducted in duplicate and results are expressed as means. One-way analysis of variance (ANOVA) was conducted and Duncan's multiple range test was used for mean comparisons. *P* values of < 0.05 were regarded as significant.

6.3.5 Characterization of CMFG

Fourier-transform infrared spectroscopy (FTIR). FG and CMFG samples were ground with solid potassium bromide (KBr) powder and mixtures were pressed into pellets for FTIR spectroscopy (Bio-Rad FTS-40, Bio-Rad Laboratories, Philadelphia, PA, USA). Sample transmittance spectra were recorded between 4000 and 400 cm^{-1} (16 scans), with a resolution of 4 cm^{-1}. All spectra were collected and analyzed using *Win-IR* software (Version 3.0, Bio-Rad Laboratories, Philadelphia, PA, USA).

Differential scanning calorimetry (DSC). DSC thermograms of FG and CMFG samples were recorded using a TA-Q2000 differential scanning calorimeter (TA Instruments, New Castle, DE, USA). Samples (5–8 mg) were placed in a standard aluminum pan which was crimped then heated at a rate of 20 °C/min from 20 to 400 °C under a dry nitrogen atmosphere. Platinum™ software (TA Instruments, New Castle, DE, USA) was used for data analysis.

NMR Spectroscopy. The solution state ^{13}C-NMR spectroscopy of FG and CMFG was performed using a Bruker Avance spectrometer (Bruker BioSpin Ltd., Milton, ON, Canada) at 125.721 MHz and 298 K. Results were analyzed by XWIN-NMR 3.5 software (Bruker BioSpin Ltd., Milton, ON, Canada). Samples were dissolved in D_2O and transferred into 5 mm OD tube with a concentration of 5.0% (w/v). The ^{13}C-NMR spectral peaks were assigned based on data for comparable compounds (Glinel, Sauvage, Oulyadi, & Huguet, 2000; de Paula, Heatley, & Budd, 1998).

Scanning electron microscopy (SEM). The surface morphology of freeze-dried FG and CMFG were observed using SEM (JSM-6010 LV, JEOL, Ltd., Tokyo, Japan). Prior to analysis, samples were coated with gold and mounted in a sample holder. Photomicrographs of each sample were obtained at an accelerating voltage of 5.0 kV with magnification shown in each figure.

6.3.6 Rheological Properties of FG and CMFG

All rheological measurements of FG and CMFG were conducted using an AR2000ex rheometer (TA Instruments Ltd., Crawley, UK) equipped with Peltier system to control temperature. Before measurements, FG and CMFG dispersions were prepared by dissolving an accurately weighed amount of dried sample in deionized RO water. Dispersions were stirred gently (300 rpm) for 3 h at RT, and kept at 4 °C for 24 h to ensure hydration of FG and CMFG polysaccharides.

All dispersions were centrifuged at 2,500 g for 2 min to collapse air bubbles before making measurements. Samples were then loaded onto the bottom plate and equilibrated for 2 min and a solvent trap cover was applied to limit solvent evaporation and mitigate interference. The flow curves were obtained under continuous shear over the shear rate range of 0.1–100 s^{-1} with an acrylic plate geometry (60 mm diameter, 500 μm gap). The apparent viscosity of each sample was obtained by fitting the experimental flow curves to the Power-law model (Eq. 6.3).

$$\eta = \mathrm{k}\gamma^{n-1} \tag{6.3}$$

Where *k* is the consistency coefficient ($Pa \cdot s^n$), γ is the shear rate (s^{-1}), and *n* is the fluid behaviour index (Rao, 1999). The viscoelastic behaviour (storage modulus G′ and loss modulus G″) of FG before and after modification by carboxymethyl group addition were examined by two different types of oscillatory measurements: strain sweep measurement and frequency sweep measurement. Prior to frequency sweep tests, strain sweep measurements were conducted at 0.01 to 100% strain with a constant frequency of 1.0 Hz (6.28 rad/s) and temperature of 25 °C. A linear viscoelastic region (LVR), where dynamic G′ and G″ are independent of strain amplitude (Peng et al., 2011), were determined based on the results of stain sweep measurements. From this region, constant strain amplitude of 0.1% strain was selected for subsequent frequency sweep measurements over an angular frequency range of 0.1–100 rad/s.

All measurements were performed in duplicate and expressed as means. Storage modulus (G′), loss modulus (G″), and tan δ of all samples were recorded for further data analysis by TA Rheology Advantage Data Analysis software V 5.4.7 (TA Instruments Ltd., Crawley, UK).

6.4 Results and Discussion

6.4.1 Effects of Reaction Parameters on DS Values of CMFG

CMFG is obtained by reacting FG with MCA in solution. Two steps are involved in carboxymethylation reaction of FG: (1) alkoxidation of FG by NaOH (Eq. 6.4) where hydroxyl groups of FG molecules are activated; and (2) S_N2 reaction of FG alkoxide with MCA (Eq. 6.5). An undesirable side reaction (Eq. 6.6) also occurs, resulting in the formation of sodium glycolates between NaOH and MCA. The side reaction competes with production of CMFG (Tijsen, Scherpenkate, Stamhuis, & Beenackers, 1999).

$$\text{FG-OH} + \text{NaOH} \rightarrow \text{FG-O}^{-}\text{Na}^{+} + \text{H}_2\text{O} \quad (6.4)$$

$$\text{FG-O}^{-}\text{Na}^{+} + \text{ClCH}_2\text{COOH} \rightarrow \text{FG-O-CH}_2\text{COOH} + \text{NaCl} \quad (6.5)$$

$$\text{NaOH} + \text{ClCH}_2\text{COOH} \rightarrow \text{HOCH}_2\text{COOH} + \text{NaCl} \quad (6.6)$$

In this study, reaction parameters that might affect DS of CMFG were investigated including: temperature, NaOH concentration, and mole ratio of MCA to FG (n_{MCA}/n_{FG}). Reaction parameters were optimized by varying a parameter while keeping the other constant (Table 6.1). Each optimized parameter is used for subsequent studies of other reaction parameters.

The effect of temperature (30−80 °C) on CMFG DS was investigated in reactions where the ratio n_{MCA}/n_{FG} was held constant at 7.0 with a NaOH concentration of 5.0 M (Table 6.1). A reaction at 70 °C resulted in a DS of 0.573 while the same reactants achieved just 0.114 DS at 30 °C. During carboxymethylation reactions, higher reaction temperatures favoured diffusion, mixing of reaction molecules, and polysaccharide swelling (Sharma, Kumar, Soni, & Sharma, 2003). This improves the effective collision between reactants (etherifying agents and FG). However, a further rise of temperature to 80 °C led to a decrease of DS from 0.573 to 0.551, possibly due to competitive reaction of glycolate formation (Eq. 6.5) prevailing over carboxymethylation and/or degradation of FG polymers. Similar effects of temperature on carboxymethylation of *Cassia occidentalis* seed gum has been reported where DS was increased from 0.023 to 0.464 when the reaction temperature increased from 30 to 80 °C and DS decreased at higher temperatures (Gupta, Sharma, & Soni, 2004). DS of carboxymethylated *Cassia angustifolia* seed gum increased from 0.033 to 0.474 as reaction temperature increased from 30 to 75 °C. Further increases in reaction temperature causes polysaccharide polymer degradation (Rajput, Pandey, & Joshi, 2015).

The effect of NaOH concentration (3.0−8.0 M) on FG carboxymethylation at n_{MCA}/n_{FG} (7.0) and temperature (70 °C) was investigated. The DS of CMFG increased from 0.477 to 0.617 with increasing NaOH concentration from 3.0 to 7.0 M (Table 6.1). The DS increase indicated that carboxymethylation reactions provided in Eq. 6.4 & 6.5 prevail over the competitive side reactions (Eq. 6.6). Greater NaOH solution concentration increased DS by increasing the opportunity for nucleophilic activation (Peng et al., 2011). NaOH also served as a swelling agent during reactions facilitating diffusion and penetration of the etherifying agent to FG polysaccharide molecules. Above 7.0 M NaOH concentration DS decreased. For example, DS was 0.602 at 8.0 M NaOH concentration. The decline of DS was due to FG glycolate formation by a competitive reaction of NaOH with MCA (Silva et al., 2004). Similarly, a significant increase in DS has been observed during reaction of *Cassia tora* gum with MCA in the presence of NaOH (0.125 M). Above 0.125 M, formation of glycolate increased, and products with lower DS were produced (Sharma, Kumar, Soni, & Sharma, 2003). The effects of NaOH concentration on DS have also been reported for other gums (Verraest, Peters, Batelaan, & Vanbekkum, 1995; Gupta, Sharma, & Soni, 2004; Stojanovic, Jeremic, & Jovanovic, 2000).

MCA concentration also influenced DS of CMFG. For a constant reaction temperature (70 °C) and NaOH concentration (7.0 M) an increase in n_{MCA}/n_{FG} from 6.0 to 10.0 led to an increase in DS from 0.405 to 0.862 (Table 6.1). The greater ratio of MCA to FG enhances the availability of MCA in the vicinity of FG hydroxyl groups, thereby, facilitating a higher extent of *O*-alkylation. However, when n_{MCA}/n_{FG} exceeded 10.0, a drop of DS was observed as the side reaction (Eq. 6.6) between NaOH and MCA consumes NaOH to form sodium glycolate.

Glycolate formation decreases reaction efficiency (Ren, Sun, & Peng, 2008). Similar findings were reported regarding the effects of MCA concentration on DS of *Cassia tora* gum (Sharma, Kumar, Soni, & Sharma, 2003), tarmind kernel powder (Goyal, Kumar, & Sharma, 2007), and *Cassia occidentalis* seed gum (Gupta, Sharma, & Soni, 2004). In our study we produced CMFG with a maximum DS value of 0.862 by reaction conditions that included an n_{MCA}/n_{FG} of 10.0, a NaOH concentration of 7.0 M and a temperature of 70 °C for 3.0 h. This result was verified by replicating the synthesis three times from fresh FG samples. The DS for CMFG products of these syntheses was 0.824 ± 0.012, which was consistent with the DS of 0.862 at the 95% confidence interval (IBM SPSS Statistics version 21, IBM Corporation, Armonk, NY, USA).

6.4.2 FTIR Spectroscopy

The infrared spectra (4000–400 cm^{-1}) of native FG and a representative CMFG (DS = 0.862) are presented (Figure 6.2). Native FG has a broad absorbance at 3357 cm^{-1} that is attributed to stretching vibration of aliphatic alcohol O–H groups. An absorbance observed at 2934 cm^{-1} is due to $–CH_2–$ symmetrical stretching vibrations. C–O–C symmetrical stretching vibrations appeared at 1241 cm^{-1} and a strong absorbance at 1042 cm^{-1} is ascribed to asymmetrical stretching vibrations in C–O–C linkages. Skeletal stretching vibrations of FG produce spectral absorbance lines at 898 and 825 cm^{-1}. Absorbance at 1620 cm^{-1} in spectrum A of Figure 6.2 was characteristic of C=O stretching vibration of glucuronic acid in native FG while $–CH_2$ scissoring and –OH bending vibrations contributed absorbance lines of 1414 and 1345 cm^{-1}, respectively (de Paula, Heatley, & Budd, 1998). After carboxymethyl modification of FG, the vibration frequency of hydroxyl groups shifted from lower (3345 cm^{-1}) to higher wave numbers (3357 cm^{-1}). Meanwhile, the intensity of hydroxyl group absorbance decreased with replacement of hydroxyl groups in FG with ether bonds. Additionally, substantial increases in band intensities caused by introduction of new carboxylic groups into the FG structure were observed at 1609, 1414, and 1324 cm^{-1} (Figure 6.2B). These spectral changes confirm that hydroxyl group protons of FG were substituted with carboxymethyl groups (Yuen, Choi, Phillips, & Ma, 2009).

6.4.3 ^{13}C-NMR Spectroscopy

^{13}C-NMR spectra of native FG and a representative CMFG with a DS of 0.862 are shown in Figure 6.3. ^{13}C=O of glucuronic acid moieties produces resonance at 176 ppm in ^{13}C-NMR spectra of native FG (Figure 6.3A). This is similar to observations by Cui, Mazza, & Biliaderis (1996) and consistent with the FTIR absorbance at 1620 cm^{-1}. FG has two major polysaccharides: a neutral arabinoxylan, which is substantially free of uronic acid, and acidic pectic-like polysaccharide (Cui & Mazza, 1996). However, Qian, Cui, Wu, and Goff (2012) reported that a FG neutral fraction with MW of 1,470 kDa also contained uronic acid. Carbon atoms participating in monosaccharide anomeric bonds contributed ^{13}C-NMR resonances between 95–105 ppm (Figure 6.3A). Carbons 2 and 5 of monosaccharides contributed resonances between 65–85 ppm. The primary carbons (C-6) of FG were observed around 60 ppm. After reaction with MCA, ^{13}C-NMR spectra (Figure 6.3B) of CMFG (DS = 0.862) included new resonances of approximately 178 ppm.

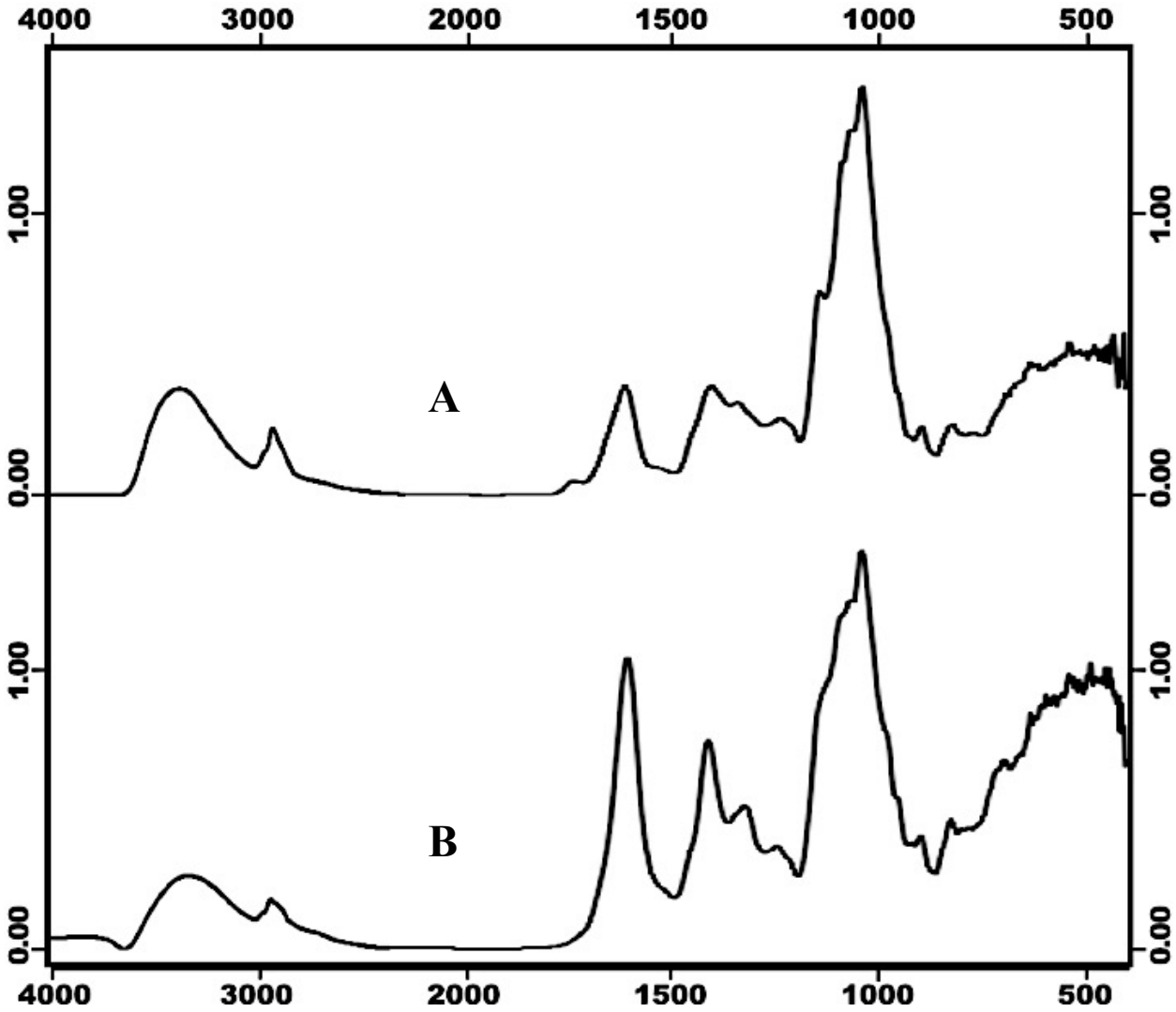

Figure 6.2 FTIR spectroscopy of FG (A) and CMFG (B, DS = 0.862).

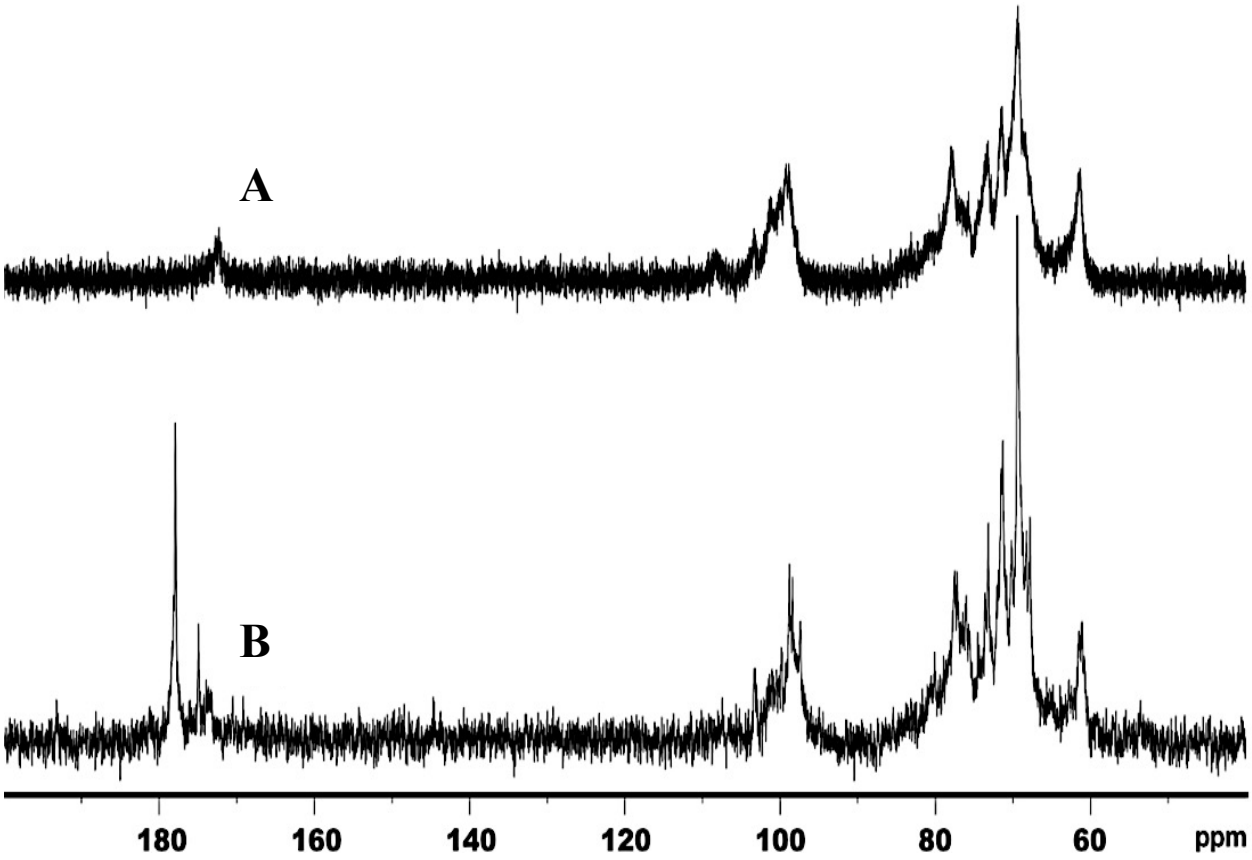

Figure 6.3 ^{13}C-NMR spectra of FG (A) and CMFG (B, DS = 0.862).

These ^{13}C resonances were contributed by carboxylate anion ($–COO^-$) from carboxymethyl substituents on FG polysaccharides. Similarly, prominent resonances were observed at 178 ppm in ^{13}C-NMR spectra of kappa-carrageenan after modification with carboxymethyl ether groups (Tranquilan-Aranilla, Nagasawa, Bayquen, & Dela Rosa, 2012). Collectively these spectra show nonselective carboxymethyl substitution onFG in agreement with previously published reports of similar reaction conditions with other polysaccharides (Xu et al., 2009; Yang et al., 2011).

6.4.4 DSC

The thermal behaviour of FG and CMFG were evaluated using DSC analysis while heating in an inert atmosphere. A broad endotherm occurred at 166.8 °C with heat flow of 128.9 J/g in the FG thermogram, whereas, for CMFG an endotherm was observed at 145.4 °C with heat flow of 236.9 J/g (Figure 6.4). Higher temperature exothermic heat release was observed at 271.6 °C with a heat flow of 201.2 J/g and 288.2 °C with a heat flow of 206.0 J/g for native FG and CMFG, respectively. Shifts in endo- and exo-thermic behaviour including variations in heat flow were probably induced by the substitution of hydroxyl group protons with carboxymethyl groups. The thermograms of polysaccharides are highly dependent on structural features. The carboxymethyl polysaccharides may have fewer intermolecular and intramolecular hydrogen bonds (Xu et al., 2009). Results of DSC analysis provided additional evidence of carboxymethyl ethers in the structure of modified FG.

6.4.5 SEM

Discrete sheet-like structures separated from each other were present in freeze-dried native FG (Figure 6.5A). After reaction with MCA (Figure 6.5C), gum structure was polyporous with many alveolate holes in the “sheets”. Alkaline conditions during the reaction induced this structure and insured access of *O*-alkylation agents to FG polysaccharide molecules (Wang et al., 2010a). At higher magnification of “sheets” (Figures 6.5B & 6.5D), CMFG exhibited a more smooth surface morphology than native FG.

6.4.6 CMFG Solution Rheological Properties

Hydrocolloids and their derivatives are generally used in the form of solutions. Thus, knowledge about rheological properties of hydrocolloid solution is necessary to provide basic information for different applications. In this study, steady state shear properties of native

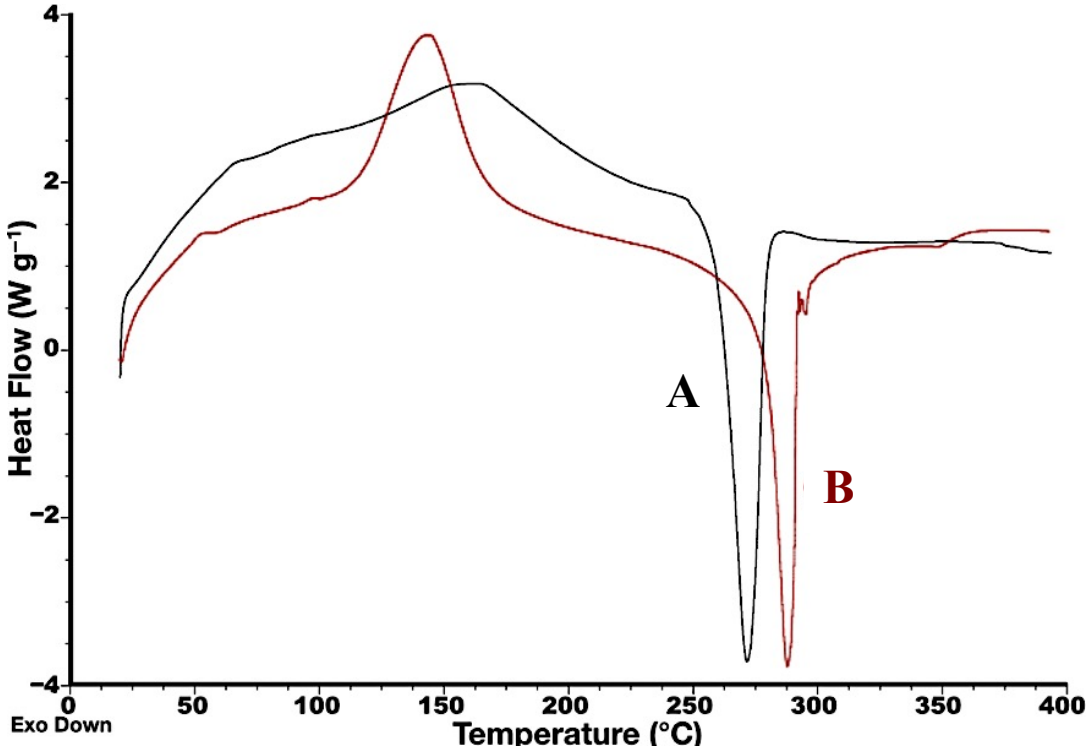

Figure 6.4 DSC thermograms of FG (A) and CMFG (B, DS = 0.862).

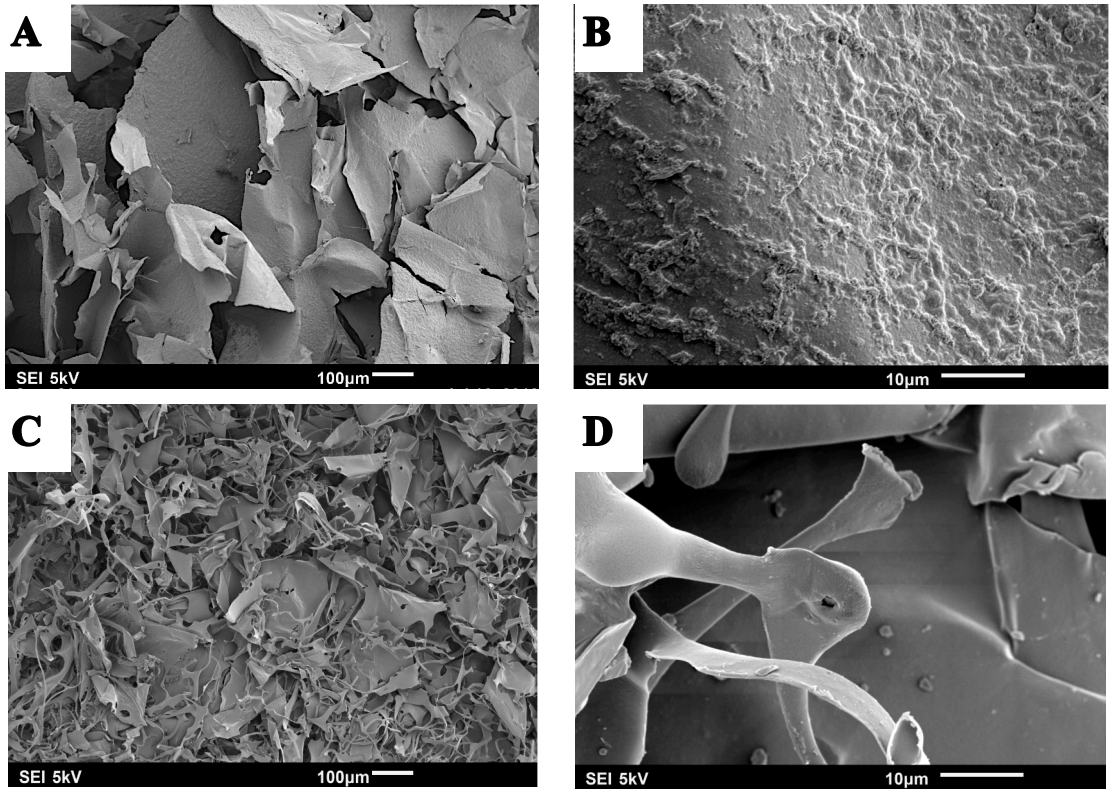

Figure 6.5 SEM pictures of FG (A and B) and CMFG (C and D, DS = 0.862) with different magnification.

FG and CMFG solutions at different concentration and DS were examined over a broad range of shear rates (0.1–100 s^{-1}) at 25 °C. The steady state shear flow curves of FG solutions at concentrations from 0.5 to 4.0% (w/v) are shown in Figure 6.6A. Typical pseudo-plastic or shear-thinning flow behaviour was observed for FG solutions with all concentrations tested. During the shear, small non-Newtonian regimes formed by alignment and deformation of transiently elongated polymer chains in the direction of flow. Here the rate at which polymer entanglements were disrupted by externally imposed movement was greater than that of formation of new entanglements. This depleted the cross-link density of FG in solution and decreased viscosity. Warrand et al. (2005b) reported the presence of large aggregates in 2.0% (w/v) FG solutions during filtration. Intermolecular associations *via* hydrogen bonds are thought to form, leading to shear-thinning flow behaviour. Shear-thinning behaviour of FG solutions above 1.0% has also been observed over a shear rate range of 0.1−1000 s^{-1} by Cui, Mazza, and Biliaderis (1994). The typical shear-thinning behaviour of FG solution was attributed to the high molecular mass of neutral FG arabinoxylans. Goh, Pinder, Hall, and Hemar (2006) determined that the hydrodynamic radius of FG in solution was in excess of 100 nm, which contributed to its high intrinsic viscosity of 1030 ± 20 mL/g. However, Newtonian-like properties were observed with FG neutral fractions when concentration was lower than 0.3% (w/v). Newtonian like flow curves were observed for FG acidic fractions at all concentrations examined. This phenomenon could be explained by the much smaller molecular size of its constituent polysaccharides (Cui, Mazza, & Biliaderis, 1994). Viscosity of FG is related to the flaxseed genotype. Gums extracted from yellow flaxseed exhibited stronger rheological properties than those from brown flaxseed (Cui, Kenaschuk, & Mazza, 1996).

Steady state shear flow curves of CMFG solutions at concentrations from 0.5 to 4.0% (w/v) with a DS of 0.862 are shown in Figure 6.6A. The apparent viscosity of CMFG solution (DS = 0.862) increased with increasing concentration (0.5−4.0%, w/v). At higher concentration, entanglements of macromolecular chains of CMFG increased due to strengthened hydrogen bonds, which resulted in increased viscosity (Peng et al., 2011; Kobayashi, Tsujihata, Hibi, & Tsukamoto, 2002). When the concentration was higher than 3.0% (w/v), typical pseudo-plastic or shear-thinning behaviour was observed for CMFG solutions, and apparent viscosity of CMFG solution decreased gradually with increasing shear rate (Figure 6.6A). At low shear rates intertwined CMFG molecular chains formed aggregates. These resisted flow and produced

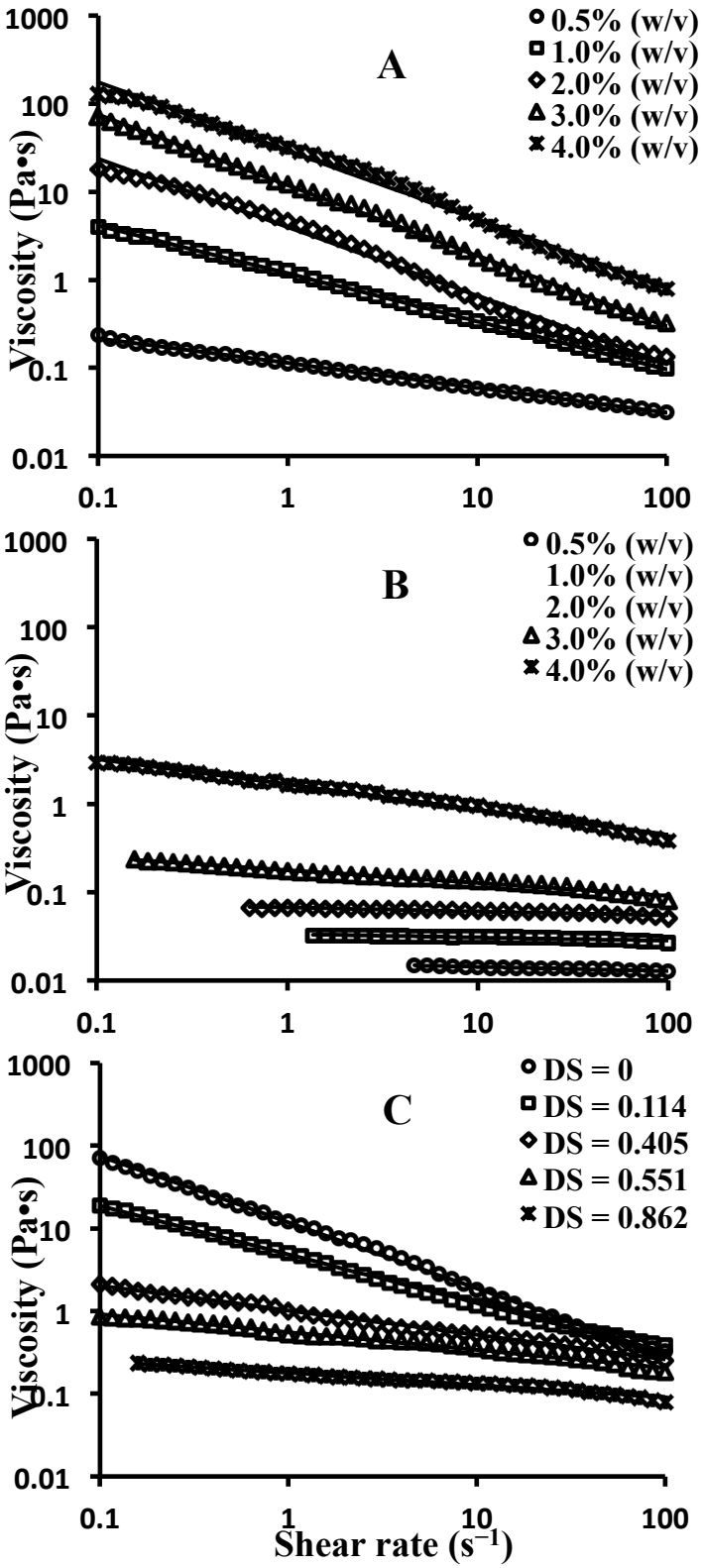

Figure 6.6 Shear rate dependence of viscosity for FG (A), CMFG as a function of concentration (B), and DS (C).

higher viscosity. With increasing shear rate, aggregates were disassembled and molecular entanglements disrupted. The CMFG molecular chains align with direction of flow producing a solution with decreased flow resistance and solution viscosity. However, a more Newtonian-like flow behaviour was observed when the CMFG concentration was lower than 2.0% (w/v), where apparent viscosity of CMFG solution was independent of shear rate. Fewer macromolecular entanglements were formed in diluted CMFG solutions. In addition, deformation of entanglements caused by imposed external shear can be rapidly replaced, and no obvious viscosity reduction is observed (Kobayashi, Tsujihata, Hibi, & Tsukamoto, 2002).

Flow curves of FG solutions were fitted to the Power-law model. The relationship between viscosity and shear rate data (Eq. 6.3) are illustrated by continuous lines in Figure 6.6A. The coefficients of determination (R^2) were higher than 0.993 for FG solutions as a function of concentration (0.5−4.0%, w/v), representing a good fit of FG solution flow behaviour to the Power-law model (Table 6.2). The Power-law parameter of flow behaviour index (*n*) was less than 1, indicating a pseudoplastic (shear-thinning) nature of FG solutions under experimental conditions. The value of flow behaviour index, *n*, decreased from 0.729 to 0.199 when FG solution concentration increased from 0.5 to 4.0% (w/v). Conversely, the consistency coefficient, k, increased from 0.113 to 29.229 Pa•s^n when FG solution concentration increased from 0.5 to 4.0% (w/v). Effects of concentration on dynamic viscosity of CMFG (DS = 0.862) in solution were tested and fitted to a Power-law model as illustrated by continuous lines in Figure 6.6B. Within the range of CMFG concentration (0.5−4.0%, w/v) tested, the coefficients of determination (R^2) were higher than 0.906, indicating a good fit of CMFG solution flow behaviour to the Power-law model (Table 6.2). A pseudoplastic (shear-thinning) behaviour of CMFG within the shear rate range tested was observed as the Power-law parameter of flow behaviour index (*n*) was less than 1. The value of flow behaviour index, n, decreased from 0.956 to 0.714 when CMFG concentration increased 2.0 to 4.0% (w/v). When CMFG concentrations were lower than 2.0% (w/v), the flow behaviour index value approached 1 ($n \geq 0.952$), indicating more Newtonian-like flow behaviour. The consistency coefficient, k, increased from 0.016 to 1.667 Pa•s^n when CMFG solution concentration increased from 0.5 to 4.0% (w/v).

The effect of DS on CMFG viscosity was also investigated at 3.0% (w/v) and fitted to the Power-law model (Figure 6.6C). A good fit was observed for all the tested samples with the coefficient of determination (R^2) higher than 0.974. With increasing of DS from 0 (native FG) to

Table 6.2 Power-law parameters for CMFG solutions as functions of concentration and DS.

Treatment		Model parameter		
Concentration (%, w/v)	DS	*n*	k ($Pa•s^n$)	R^2
0.5	0.862	0.952	0.016	0.959
1.0	0.862	0.963	0.034	0.898
2.0	0.862	0.956	0.067	0.906
3.0	0.862	0.856	0.178	0.974
4.0	0.862	0.714	1.667	0.987
3.0	0.000	0.199	11.862	0.998
3.0	0.114	0.411	4.835	0.997
3.0	0.405	0.704	1.021	0.995
3.0	0.551	0.783	0.557	0.986

0.862, the flow behaviour index (*n*) increased from 0.199 to 0.856 (Table 6.2), while the consistency coefficient (k) decreased from 11.862 to 0.178 $Pa•s^n$ (Table 6.2). These represented the non-Newtonian flow behaviour of test samples within the range of DS investigated. With decreasing DS the flow behaviour of CMFG solution departed from Newtonian behaviour as indicated by lower values for flow behaviour indices and higher values for consistency coefficient (Table 6.2). Similar results were reported for carboxymethylated guar (Gong et al., 2012), and xanthan gums (Ahuja, Kumar, & Singh, 2012). It is well documented that polysaccharides with high molecular weight and rigid conformations exhibit more pronounced shear-thinning behaviour (Izydorczyk & Biliaderis, 1992). After carboxymethyl substitution, new anionic groups were introduced to the polymer backbone chains, which decreased viscosity by improving columbic repulsion and prevented entanglement. In addition, alkaline reaction conditions and high temperature during carboxymethylation reactions lowered FG MW. This was, in part, responsible for decreased shear-thinning behaviour associated with CMFG solutions (Peng et al., 2011).

Typical oscillatory flow curves of CMFG solutions (3.0%, w/v) as a function of DS range from 0.862 to 0 (native FG) at 25 °C (Figures 6.7A−6.7D). Both the storage modulus (G′) and loss modulus (G″) of all samples were frequency dependent as is typical of entangled networks of disordered polymer coils (Medina-Torres, Brito-De La Fuente, Torrestiana-Sanchez, & Katthain, 2000). The viscoelastic moduli (G′ and G″) proportionally decrease as DS of CMFG in solution increases. For native FG solution (DS = 0, 3.0%, w/v), G′ was higher than G″ at higher frequency as the oscillation rate exceeded the time scale for molecular rearrangement (Figure 6.7A), indicating weak gel-like properties. This was in agreement with a published study that showed dispersions of crude dialyzed FGs and neutral fractions all exhibited “weak gel” properties (Cui et al., 1994). However, Qian, Cui, Wu, and Goff (2012) reported dispersions of FG, 2.0% (w/v), showed liquid-like behaviour, as the loss modulus (G″) exceeded the storage modulus (G′) over the entire frequency range investigated. A solid-like property of FG solution was found with concentrations of 1.0% (w/v) (Wang et al., 2009b). Discrepancies may be due to the source of raw material, extraction procedures, and variations in post extraction treatments (Cui, Kenaschuk, & Mazza, 1996; Wang et al., 2009; Wu et al., 2010; Wang, Li, Wang, & Xue, 2011). With increasing DS, a predominantly viscous behaviour was observed with G″ higher than G′ throughout the frequency range studied (Figures 6.7B−6.7D). This indicated a more liquid-like rheological behaviour of CMFG solution when compared to unmodified FG.

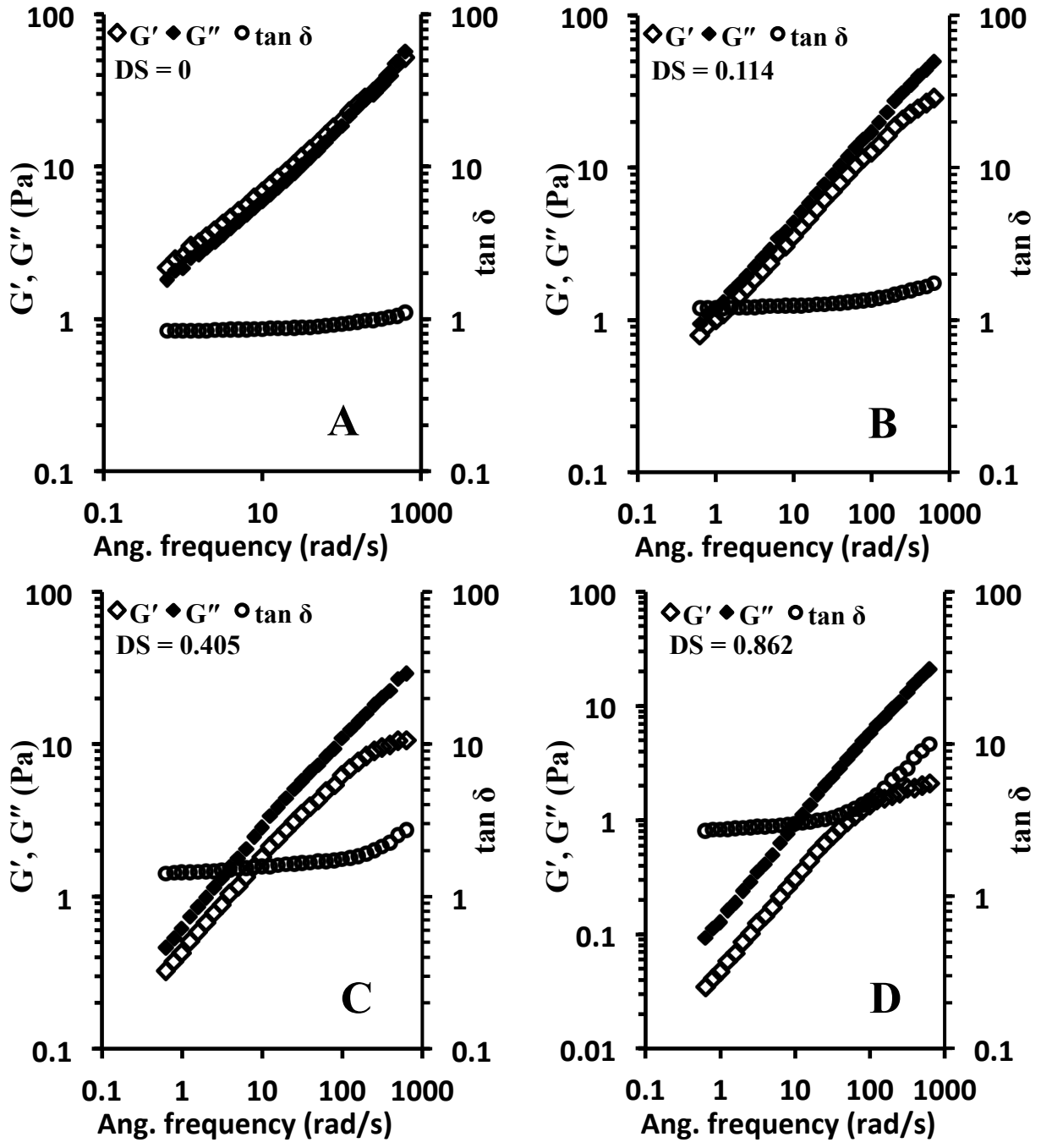

Figure 6.7 Frequency dependent of storage modulus (G′) and loss modulus (G″) for CMFG as a function of DS.

This was consistent with observed tan δ, a useful measurement of energy dissipation (Garcia-Cruz, Rodriguez-Ramirez, Lagunas, & Medina-Torres, 2013). Polymer solutions with predominantly elastic behaviour demonstrate tan $\delta < 1$, while a tan $\delta > 1$ indicates predominantly viscous behaviour of polymer solutions (Steffe, 1996). Tan δ increased with increasing DS throughout the frequencies tested, indicating fewer bonds or entanglements between macromolecular chains in polymer solutions. FG solution, with DS = 0, tan δ fell in the range of 0.84 to 1.10 over the sweep frequency tested, which indicated a weak elastic gel-like property (Figure 6.7A). With increasing DS, from 0.114 to 0.862, the tan δ was increased and was higher than 1 (Figures 6.7B−6.7D). This was in agreement with viscoelastic modulus measurements that indicated predominantly viscous behaviour of FG solutions prepared with CMFG. Overall these observations might be explained by decreases in FG MW during carboxymethyl product formation. The lower MW products exhibit less polymer chain entanglement than unmodified FG. Furthermore, substitution of FG hydrogen groups with carboxymethyl groups might also decrease both moduli through reduction of intermolecular association in solution. Similar results were reported by Peng et al. (2011) who observed lower elastic modulus and loss modulus in carboxymethyl hemicellulose than unmodified hemicellulose.

FG has been proposed for use as a hydrocolloid in foods, such as salad dressing, cloudy carrot juice, and dairy desserts (Stewart & Mazza, 2000; Qin, Xu, & Zhang, 2005). It can also increase thermal stability of salt-soluble meat protein as well as water holding capacity of porcine myofibrillar protein (Sun, Li, Xu, & Zhou, 2011). Moreover, FG has nutritional value as a dietary fibre, which plays a role in reducing diabetes and coronary heart diseases risk, preventing colon and rectal cancer, and decreasing the incidence of obesity (Thakur, Mitra, Pal, & Rousseau, 2009). The above beneficial properties arise from the inherent physicochemical properties of FG, especially rheological properties. However, low dissolution rate in cold water, dull colour of gum solution, and low storage stability etc. limit FG utilization. Results from this study provide an approach, through modification FG structure, to increase applications of FG in foods and related fields. For example, lower viscosity CMFG could be favoured in dietary fibre fortified foods that improve sensory attributes without leading to over-texturization. This is important when significant concentrations of fibre are required to impart health benefits (Ibrügger, Kristensen, Mikkelsen, & Astrup, 2012).

6.5 Conclusions

In the present study, FG carboxymethyl ethers were prepared with MCA as the etherifying agent. Structure of CMFG was confirmed by FTIR spectroscopy and NMR spectroscopy. Surface smoothness of substituted CMFG (observed by SEM) increased and thermal behaviour was also affected. Reaction parameters were optimized with a maximum DS value of 0.824 ± 0.012 achieved by reaction conditions that included a n_{MCA}/n_{FG} of 10.0, a NaOH concentration of 7.0 M and a temperature of 70 °C for 3.0 h. Solutions of FG exhibited shear-thinning behaviour over a concentration range of 0.5−4.0% (w/v). Shear-thinning behaviour was also observed with CMFG solutions (DS = 0.862, 0.5−4.0%, w/v) at the same concentration with apparent viscosity being significantly reduced. These phenomena were DS dependent. A more liquid-like property was observed with FG after carboxymethylation, probably due to suppressed entanglement of FG polysaccharide chains and reduced intermolecular association. Results of this study provide basic information to help expand the potential application of gum from flaxseed in food or related fields.

CHAPTER 7
GENERAL DISCUSSION

Flaxseed (*Linum usitatissimum* L.) is of Mesopotamic origin has been used for food and textile fibre over 5000 years (Carraro et al., 2012). It contains a portfolio of functional components such as polyunsaturated fatty acids (α-linolenic acid), proteins, lignans (secoisolariciresinol diglucoside), orbitides, and soluble polysaccharides. Those functional components in flaxseed determine the value of flaxseed (Oomah & Mazza, 1998; Shim et al., 2014). Flaxseed soluble polysaccharides, usually referred as FG, are a largely water-soluble dietary fibre that contains some protein. FG can provide considerable functional properties to food and enhance nutritional value. When included in aqueous solutions, FG can improve solution viscosity, and readily form emulsions, gels, and foams (Chen, Xu, & Wang, 2006; Singh, Mridula, Rehal, & Barnwal, 2011). As a source of dietary fibre, flaxseed can reduce diabetes and coronary heart diseases risk, mitigate the risk of colon and rectal cancer, and decrease the incidence of obesity (Cunnane et al., 1993; Thakur, Mitra, Pal, & Rousseau, 2009). These benefits might arise from interactions of FG with food in the gut. Similar biological effects are observed with other viscous high molecular weight polysaccharide sources. Based on these properties, FG has been proposed to be used in salad dressing, sausage, carrot juice, and dairy desserts (Stewart & Mazza, 2000; Zhou et al., 2010).

Physicochemical and functional properties of FG were highly affected by extraction conditions, drying processes, shear (high-pressure homogenization, and extrusion), as well as flaxseed genetics (Cui, Kenaschuk & Mazza, 1996; Wang et al., 2009b; Wang et al., 2010b; Wang, Li, Wang, & Xue, 2011). The influence of flaxseed genetics on FG yield and functional properties was widely investigated for breeding purpose (Bhatty, 1993; Diederichsen, Raney, & Duguid, 2006; Fedeniuk & Biliaderis, 1994; Oomah, Kenaschuk, Cui, & Mazza, 1995; Wannerberger, Nylander, & Nyman, 1991; Pavlov et al., 2014). However, limited information is available on cultivar dependent FG functional properties and composition. The potential

utilization of FG requires the standardization of FG in cultivars to achieve consistent performance or identity preservation to select cultivars that produce uniform FG extracts. Six registered Canadian flaxseed cultivars (CDC Bethune, CDC Sorrel, CDC Arras, CDC Glas, Vimy, and Flanders) were selected for FG preparation. Canada is the world's largest producer of flaxseed, which accounts for nearly 80% of the global trade in flaxseed (Singh, Mridula, Rehal, & Barnwal, 2011). Thus, cultivars selected for these studies would represent the majority of globally traded commercial flaxseed (Saskatchewan Flax Development Commission). Significant variations were observed in FG yield (9.33 ± 0.14 to 14.45 ± 0.49 g/100 g seeds), neutral sugar content (367 ± 27 to 592 ± 84 mg/g FG, D-xylose equivalent), acidic sugar content (89 ± 25 to 181 ± 17 mg/g FG, D-galacturonic acid equivalent), protein content (56.6 ± 5.1 to 90.8 ± 4.7 mg/g FG, BSA equivalent), as well as ζ of FG solution (−27.4 ± 0.6 to −16.4 ± 0.6 mV) among the seed, gum, and solutions of selected flaxseed cultivars. FG solution rheological properties were also cultivar dependent with typical shear-thinning behavior being observed for all FG solutions. FG solution apparent viscosity was positively correlated with neutral sugar content but negatively correlated with both acidic sugar and protein content. FG solution rheological properties responded to temperature (15−45 ºC), NaCl concentration (50−200 mM), and sucrose concentration (5.0−20%, w/w), but were not sensitive to solution pH (3.0−9.0). Due to the cultivar dependence of FG composition variations observed in solution properties and response to solution conditions were cultivar specific. FG solution emulsification properties EAI (41.30 ± 0.71 to 59.63 ± 0.95 m^2/g) and ES (37.39 ± 0.95 to 52.03 ± 1.39%) were also cultivar dependent. Both interfacial tension reduction effects caused by protein fraction in FG and steric and mechanical stabilization effects of FG polysaccharide chains contributed to FG solution emulsification properties. Knowledge of genotypic variation in FG solution rheological and emulsification properties will prove useful in the design of FG products with consistent properties for targeted utilization as food thickeners or emulsifiers. For example, FG extracts from CDC Glas and CDC Bethune might be superior for use as food thinkers in beverages and dairy products when compared to FG prepared other flaxseed cultivars. FG prepared from CDC Glas and CDC Bethune demonstrated higher apparent viscosity thus smaller amount of FG would be needed to induce the desired mouth-feel, texture, and stability to food products when compared with FG from other cultivars. CDC Glas had considerably more total gum yield than CDC Bethune and thus this would be a preferred source of this product. Flanders FG extracts

would be more suitable to serve as an emulsifier for salad dressing as higher EAI and ES were observed for Flanders FG solution than FG solutions prepared from other cultivars. It also could be possible to tune FG physicochemical and functional properties through mixing FG extracts from different flaxseed cultivars (e.g. CDC Glas and Flanders). As FG solution rheological and emulsification properties were highly correlated with FG composition (sugar and protein content), blends of CDC Glas and Flanders FG extracts could demonstrate both good solution thickening and emulsification properties.

FG can stabilize oil-in-water emulsions like most hydrocolloids, and FG proteins are thought to play an important role in emulsification properties (Bhatty, 1993; Wang et al., 2010a). FG protein can help to increase FG solution surface activity, while FG solution emulsion stability was reduced after protein was removed by protease treatments, regardless of polysaccharide molecular mass, chain flexibility and rheological properties (Qian, Cui, Wu, & Goff, 2012). Other natural food hydrocolloids, including sugar beet pectin (Funami et al., 2007), gum Arabic (Yadav, Igartuburu, Yan, & Nothnagel, 2007), corn fibre gum (Yadav, Nunez, & Hicks, 2011), and soy soluble polysaccharide (Nakamura et al., 2004a & 2004b; Nakamura, Yoshida, Maeda, & Corredig, 2006), demonstrated similar emulsification properties. The protein fraction in these natural food hydrocolloids can adsorb on oil-water interfaces while the highly branched polysaccharide structure stabilizes emulsions through steric and mechanical effects (Funami et al., 2007; Yadav, Nunez, & Hicks, 2011). FG protein was purified through deglycosylation with TMFS then the proteins were separated by 2D-SDS-PAGE. Two protein gel spots were observed with estimated MW of 10−11 kDa and 11−12 kDa, respectively, and estimated IEP of 6.5 and 7.5, respectively. The two protein gel spots were excised, digested with trypsin, and the tryptic fragments were analyzed using MALDI-TOF-MS. For protein gel spot 1, matched peptides corresponded to a flax hypothetical protein (NCBI accession number CAC94010) with a protein score of 394 and coverage of 41% of the sequence. Protein gel spot 2 matched a protein (NCBI accession number CAC94011) with a protein score of 395 and sequence coverage of 36%. CAC94010 and CAC94010 match the sequence of conlinin, a 2S storage protein of flaxseed. Therefore, conlinin was identified as the major protein in both excised protein gel spots and the major protein in FG. When FG was subjected to protease treatment to deplete FG solution proteins both of EAI and ES were significantly decreased compared with the original FG solution. Untreated FG solution induced comparable EAI and ES

to gum Arabic solutions of the same concentration. Findings here suggest that conlinin proteins are part of the underlying mechanism of FG solution emulsification properties.

FG is a mixture of anionic polysaccharides and proteins. FG dissolved in water can produce solutions that are naturally occurring coacervates of negatively charged polysaccharide chains and positively charged albumins. This coacervate scatters light and induces the dull appearance associated with FG solutions. This finding suggests that further studies of coacervate formation between FG and other food proteins might also prove important in understand FG utility. In formulated foods, protein and polysaccharide coacervate formation is of great importance in determining structure and physical properties (Schmitt et al., 1998; Strauss & Gibson, 2003). Coacervate formation phenomenon was first systematically studied by Tiebackx (1911) using fish gelatin and gum Arabic. Since then, many polysaccharides, including pectin, alginate, and carboxymethyl cellulose, were noted to form coacervates with protein (de Kruif, Weinbreck, & de Vries, 2004). Coacervate properties introduce a wide range of properties with related potential applications, such as encapsulation, protein separation and recovery, enzyme immobilization, gelation, emulsification, and foam stabilization (Navratil & Sturdik, 2000; Roy & Gupta, 2003; Thimma & Tammishetti, 2003; Zhao et al., 2014). Bovine serum albumin (BSA) from cow whey was used here as a model protein to interact with FG as it is an ingredient in many food products and its properties are well characterized. Complex coacervates formed between BSA and FG were investigated by turbidimetric analysis, ζ and particle size measurements as a function of pH (6.0−1.4), biopolymer mass ratio (R = 1:15 to 15:1, w/w), salt concentration (NaCl, 0−100 mM), and urea concentration (0−150 mM). For BSA-FG mixture (R = 1:1) with C_T = 0.05% (w/w), critical pH dependent phase transitions of pH_c, $pH_{\phi 1}$, and $pH_{\phi 2}$ associated with formation of soluble and insoluble complexes were observed at pH 5.4, pH 4.8, and pH 2.0, respectively. pH_{max}, indicating the maximum interaction between BSA and FG as evidenced by the highest optical density (OD_{600}), was found at pH 3.4. In the absence of destabilization agents, the maximum coacervate formation at pH_{max} (OD_{600} = 0.818 ± 0.005) occurred at R = 2:1. As R increased from 1:15 to 15:1 the critical phase transition pH also increased ($pH_{\phi 1}$ from 4.2 to 5.2, and $pH_{\phi 2}$ from 1.8 to 2.8) with no influence on pH_c. Electrostatic interaction was found to be the primarily determining force in stabilizing BSA-FG coacervates as pH_{max} was consistent with the isoelectric point of BSA-FG mixtures found by electrophoretic mobility measurements. The most intensive complex coacervate formation occurred when

biopolymer mixture charge-neutralization was achieved. Furthermore, NaCl addition suppressed BSA-FG coacervate formation due to biopolymer charge screening and disruption of electrostatic attractive forces. In the presence of urea, the turbidity curve of BSA-FG coacervate formation was shifted to more acidic pH. While urea suppressed the maximum OD_{600} this effect was less than induced by NaCl. Based on the phase diagram developed by plotting critical structure-forming transitions (pH_c, $pH_{\phi 1}$, $pH_{\phi 2}$, and pH_{max}) and IEP against R, soluble complex coacervates were formed in the region between pH_c and $pH_{\phi 1}$ due to binding of anionic FG to cationic patches on BSA surfaces. Insoluble complex coacervates were formed in the region between $pH_{\phi 1}$ and $pH_{\phi 2}$ where FG and BSA possessed opposite electrical charges, resulting in strong electrostatic attractive interactions. Beyond the region of $pH_{\phi 2}$, no complexes were formed due to repulsive forces caused by similar electrical charges carried by solution biopolymers. Hydrogen bonding also stabilizes complex coacervates formed between BSA and FG. Interaction intensity of the BSA-FG system decreased with increasing urea concentration, as would be expected with the disruption of hydrogen bonds. The knowledge obtained through these studies of interactions between FG and proteins will help to expand FG utilization as a functional food component or a pharmaceutical additive or a cosmetic product. As an example, interactions between FG and proteins could be controlled for selective binding and utilized for protein purification. Protein-FG can form biopolymer complexes with better emulsification properties than achievable with each ingredient used independently. Knowledge of interactions between FG polysaccharides and food proteins will help to design food systems with desired structure and extended shelf-life.

Natural gums are widely used in food, industrial and pharmaceutical industries as they demonstrate advantages over synthetic polymers such as greater sustainability, biocompatibility, biodegradability, and biosafety. However, intrinsic undesirable properties that could limit utilization include uncontrolled hydration rates, pH dependent solubility, thickening, viscosity loss with storage, and microbial contamination. Similarly, FG dissolves slowly in cold water, produces dull colour solutions, and yields products with low storage stability. The introduction of carboxymethyl groups is widely employed for industrial modification of polysaccharide gums, including cellulose (Biswal & Singh, 2004), starch (Stojanovic, Jeremic, Jovanovic, & Lechner, 2005; Tijsen, Kolk, Stamhuis, & Beenakers, 2001; Bhattacharyya, Singhal, & Kulkarni, 1995), chitin (Kittur, Prashanth, Sankar, & Tharanathan, 2002), gellan (Ahuja, Singh, & Kumar, 2013),

pullulan (Glinel, Sauvage, Oulyadi, & Huguet, 2000), and inulin (Akin, Oner, Bayram, & Demadis, 2008). CM modified gums usually demonstrate better water solubility, increased hydrophilicity and solution clarity as alcohol group protons of natural gums are substituted with more polar carboxymethyl moieties (Gong et al., 2012; Silva et al., 2004). FG was modified through reaction with MCA as the etherifying agent. CMFG was successfully prepared and the modifications were confirmed by FTIR and NMR spectroscopy. After modification of FG with carboxymethyl groups the hydroxyl group absorbance (3345 cm^{-1}) intensity decreased with replacement of FG hydroxyl groups with ether bonds. Meanwhile, substantial increases in band intensities, caused by introduction of new carboxylic groups into the FG structure, were observed at 1609, 1414, and 1324 cm^{-1}. New resonances of approximately 178 ppm in CMFG ^{13}C-NMR spectra, contributed by carboxylate anion ($–COO^-$) from carboxymethyl substituents on FG polysaccharides, were observed after reaction with MCA. Parameters affecting CMFG DS were varied to optimize the level of modification. A maximum DS value of 0.824 ± 0.012 was achieved by reaction conditions that included a n_{MCA}/n_{FG} of 10.0, a NaOH concentration of 7.0 M and a temperature of 70 °C for 3.0 h. As CM groups were introduced to the FG structure, surface smoothness increased compared to unmodified FG and thermal behavior (DSC) was also changed. Shear-thinning behavior was also observed with CMFG solutions (DS = 0.862, 0.5−4.0%, w/v) when compared with FG solutions of the same concentration. Similarly apparent viscosity was significantly reduced. Meanwhile, CMFG apparent viscosity decreased as DS increased due to suppressed entanglement of FG polysaccharides and reduced intermolecular association. CMFG with decreased viscosity might be favoured for use as a dietary fibre in fortified foods with improve sensory attributes but without over-texturization. This is important when significant fibre concentration is required to impart health benefits (Ibrügger, Kristensen, Mikkelsen, & Astrup, 2012). Importantly, it is expected that CMFG and proteins would react strongly as there are substantially more anionic groups on CMFG compared with native FG.

CHAPTER 8
SUMMARY AND CONCLUSIONS

Flaxseed (*Linum usitatissimum* L.) gum (FG), a water-soluble dietary fibre, occurs mainly at the seed's outermost layers. FG is of special research interest as it imparts marked functional properties when dissolved in solutions such as enhanced viscosity, emulsification properties, gelation and foaming properties, as well as proposed nutritional values. In order to design useful FG products for utilization, it is critical to develop methods to produce FG materials with consistent physicochemical and functional properties. This can be realized through flaxseed genotype selection, FG characterization and optimization, and FG structure modification. In this study, six registered Canadian flaxseed cultivars (CDC Bethune, CDC Sorrel, CDC Arras, CDC Glas, Vimy, and Flanders) were collected and FG was prepared from each of them. Genotype was associated with large differences in the FG solution physicochemical and functional properties that were examined. FG yield (9.33 ± 0.14 to 14.45 ± 0.49 g/100 g flaxseed), neutral sugar content (367 ± 27 to 592 ± 84 mg/g FG), acidic sugar content (89 ± 25 to 181 ± 17 mg/g FG), protein content (56.6 ± 5.1 to 90.8 ± 4.7 mg/g FG), and solution zeta potential (ζ) (−16.4 ± 0.6 to −27.4 ± 1.5 mV) were all cultivar dependent. FG solution rheological properties were also determined by the genotype with typical shear-thinning behaviour observed for all FG solutions within the concentration range tested (0.5−3.0%, w/v). CDC Glas FG solution (2.984 ± 0.204 Pa•s) had the highest apparent viscosity while the lowest observed was for CDC Sorrel FG solution (0.048 ± 0.001 Pa•s) (temperature 25 ºC, shear rate 1.0 s^{-1}, and 1.0%, w/v). FG solution apparent viscosity was positively correlated with neutral sugar content but negatively with acidic sugar and protein content. Cultivar dependent FG solution rheological properties were also observed with changes in solution temperature (15−45 ºC), solution pH (3.0−9.0), NaCl concentration (0−200 mM), and sucrose concentration (0−20%, w/v). FG solution rheological properties were affected by increased solution temperature (decreased apparent viscosity), but not sensitive to solution pH. NaCl demonstrated charge screening effects on FG anionic carboxyl groups, resulting in reduced

apparent viscosity with increased NaCl concentration. Sucrose could form specific interactions with FG molecules and increased sucrose content increased apparent viscosity. Cultivar dependent FG solution emulsification properties were also observed using emulsion activity index (EAI) and emulsion stability (ES) as indicators. Both EAI and ES of FG solution prepared from FG extracts of selected cultivars varied significantly. The highest EAI and ES were observed for Flanders FG solution (71.8 ± 2.8 m^2/g) and CDC Arras FG solution (52.0 ± 1.4%), respectively, and the lowest were for CDC Sorrel FG solution (41.3 ± 0.7 m^2/g) and CDC Bethune FG solution (37.4 ± 1.0%), respectively. The hydrophilic FG polysaccharide chains were thought to contribute to FG solution emulsification properties of FG solution by forming thick charged layers around emulsion droplets that prevent aggregation through steric and mechanical stabilization effects. FG protein might reduce interfacial properties between oil and water phases and play an important role in FG solution emulsification properties. However, composition and identity of FG protein have never been reported. Here, FG was prepared from CDC Bethune (FG_B), the most grown flaxseed cultivar in Canada. Protein fraction in FG_B was enriched through deglycosylation with trifluoromethanesulfonic acid (TFMS). When the FG proteins were subjected to 2D-SDS-PAGE only two protein spots were observed with estimated molecular weights (MW) of 10–11 kDa and 11–12 kDa. The two protein spots were excised from the gel, and digested with trypsin. The tryptic digestion fragments were analyzed by matrix-assisted laser ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and peptide MS was compared to MS of peptides from gene models available through National Center for Biotechnology Information (NCBI) database. A low-molecular-mass 2S storage flaxseed protein, conlinin, was identified as the major FG protein constituent. The contribution of FG proteins to FG_B solution emulsification properties was also examined. Protease hydrolysis was employed to deplete FG_B protein content. After protease treatment both EAI and ES decreased from 98.7 ± 5.4 to 59.9 ± 3.2 m^2/g and from 66.4 ± 1.1 to 42.1 ± 2.0%, respectively, thus confirming the contribution of protein fraction to emulsification properties of FG_B solution. The hydrophobic polypeptide chains of protein fraction in FG_B would anchor the FG_B polysaccharide chains onto oil droplet surfaces and stabilize oil-in-water emulsions.

Interactions between FG and food protein was investigated using bovine serum albumin (BSA) as a model. The formation of BSA-FG coacervates was investigated by turbidimetric analysis, zeta potentiometry, and dynamic light scattering as a function of pH (6.0−1.4),

biopolymer mass ratio (R = 1:15 to 15:1, w/w), salt concentration (NaCl, 0−100 mM), and urea concentration (0−150 mM). The association and disassociation processes during complex coacervate formation in the BSA-FG system was monitored by particle size distribution. A phase diagram was developed by plotting critical structure-forming transitions (pH_c, $pH_{\phi1}$, $pH_{\phi2}$, and pH_{max}) and IEP against R. When R increased from 1:15 to 15:1 the critical phase transition pH values of $pH_{\phi1}$ and $pH_{\phi2}$ increased from 4.2 to 5.2 and from 1.8 to 2.8, respectively. Also pH_{max} shifted from 2.80 to 4.80, which was consistent with isoelectric point of BSA-FG mixtures. The pH_c was independent of R. The maximum coacervate formation was observed at pH_{max} (OD_{600} = 0.818 ± 0.005) of R = 2:1, where charge-neutralization of biopolymer mixtures was achieved based on the near coincidence of pH_{max} and IEP measured by ζ potentiometry. Addition of NaCl, significantly suppressed BSA-FG interactions. Critical phase transition pHs (pH_c, $pH_{\phi1}$, and pH_{max}) decreased while $pH_{\phi2}$ increased. The addition of urea shifted the turbidity curve towards a more acidic pH but this effect was less pronounced than with the addition of NaCl. Findings here demonstrated that BSA-FG coacervates were primarily stabilized by electrostatic attractive forces between polysaccharide anionic carboxyl groups and cationic amide groups on proteins. FG is a naturally occurring coacervate between negatively charged polysaccharide chains and positively charged albumins. This coacervate would scatter light and induce the dull colour of FG. Anionic CM ether moieties were introduced to FG through ether forming reactions with monochloroacetic acid (MCA). Reaction conditions were optimized with a highest degree of substitution (DS) observed at a temperature of 70 °C, with 7.0 M NaOH, and a molar ratio of MCA to FG of 10:1 over 3 h reaction. The CMFG exhibited both modified surface morphology and thermal behaviour. Shear-thinning behaviour was observed with CMFG (0.5−4.0%, w/v) with decreased apparent viscosity when compared with solutions of native FG. Apparent viscosity of CMFG was also decreased with increased DS. A more liquid-like behaviour was observed for CMFG with higher DS as indicated by decreased more frequency dependent storage modulus G' and loss modulus G″. This probably was caused by suppressed entanglement of FG polysaccharide chains and reduced intermolecular association. In planned research the interaction between CMFG and food protein will be investigated for applications in food, pharmaceutical, and cosmetic products as texture modifiers, emulsifiers, or encapsulation vehicles.

CHAPTER 9
OUTLOOK AND PERSPECTIVES

FG might have unrealized potential to be used as an additive that imparts functional properties to food and enhances food nutritional properties. Clearly FG is substantially water-soluble and addition of FG to aqueous solutions can improve solution viscosity and thereby enable the production of emulsions, gels, and foams (Chen, Xu, & Wang, 2006; Singh, Mridula, Rehal, & Barnwal, 2011). FG is also a source of dietary fibre which might interact with food components in the gut to help to mitigate the risk of type II diabetes, coronary heart diseases, colon and rectal cancer, and decrease the incidence of obesity (Cunnane et al., 1993; Thakur, Mitra, Pal, & Rousseau, 2009). Both FG functional and nutritional properties make it possible to consider FG as replacement for most non-gelling gums for food and non-food applications (Cui & Mazza, 1996; Chen, Xu, & Wang, 2006). Prior to commercialization FG product properties and performance should be well defined. Producing defined flaxseed products would require additional future research work that goes well beyond the current study.

FG functional properties are dependent on FG structural features. FG obtained from different flaxseed cultivars has highly varied structural features that impart varied functional properties. It is necessary to understand flaxseed cultivar effects if FG is to be used as a source. Specific FG attributes including monosaccharide composition, ratio of neutral and acidic polysaccharides, and FG protein content determine FG properties and utility. Narrowly defined FG attributes would enhance opportunities for FG utilization, as consistent performance would be required in a commercial application. For example, CDC Glas and CDC Bethune would be preferred for use as thickeners in food products due to relatively higher neutral sugar content. Higher FG protein content induced higher EAI and ES, therefore, FG solutions prepared from Flanders would be superior for use as food emulsifiers. Applications of any hydrocolloid require that it provide consistent often application specific properties. This requires application specific testing.

Efforts need to be paid to test FG performance in appropriate foods. For example, when FG was used to prepare salad dressing, solute concentration, solution ionic strength, temperature, and pH were all controlled. For protein enriched food products, such as yogurt, FG may interact with milk protein. Interactions between FG and proteins might lead to the formation of precipitates and decreased product stability. When FG is included in gluten-free food products, it might interact with other food components to affect food quality. Thus, more research is needed to address FG solution and matrix functional properties and mechanism involved in producing those properties. Studies of simulated foods could help to determine how FG performance in specific applications. Knowledge obtained would enable the design of FG with consistent performance and introduce FG enhanced food products with suitable properties.

Long-term consumption of flaxseed lowers cholesterol in human subjects. FG likely contributes part or all of this health benefit. However, more evidence is needed to demonstrate if FG consumption reduces blood cholesterol itself. Viscous FG solutions would likely reduce cholesterol transport and absorption in the gut and FG protein might absorb cholesterol thereby lowering its effective concentration. FG from different flaxseed cultivars is a variable material in both its structure and the rheological properties it imparts. Any cholesterol-lowering effect of flaxseed should be associated with a specific composition and composition is largely determined by flaxseed genetics. Cholesterol lowering effects might be cultivar specific and recommended consumption levels might also be specific. Just as FG solutions were demonstrated to be natural coacervates of polysaccharides and proteins, FG might also interact with food proteins in the gut to form alternate complexes with unknown effects on serum cholesterol. Future studies of cholesterol mobility in the gut should be conducted and the effect of FG to lower cholesterol without the addition of whole seed should be studied. If 40 g of flaxseed effectively lowers cholesterol does 4 g of FG have the same effect?

Price and supply are the other major concerns for FG utilization and commercialization. For FG to compete with other commercially available gum products, such as gum Arabic, the cost of FG production should competitive. Rational design of FG processes, including extraction, purification, blending etc. enable cost reduction while enhance FG yield and quality. Extraction of FG from whole flaxseed with water would require the use of large amounts of water and significant energy inputs for drying FG solutions. In addition, FG is effectively precipitated with ethanol to prepare FG concentrates. Food-grade ethanol is expensive and alternate methods of

concentration and precipitation could improve the economics of FG production. FG production quality might also be determined by the starting material where whole flaxseed, flaxseed hull, or flaxseed meal might all produce useful gums. FG price might also be elevated if the supply of suitable flaxseed is limiting. Farmers should be encouraged to plant flax with improved composition for total utilization of flaxseed oil, meal and FG byproducts.

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