Development of Protein Polysaccharide Complex for Stabilization of Oil-in-Water Emulsions

by

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A Thesis
presented to
The University of Guelph

In partial fulfilment of requirements
for the degree of
Doctor of Philosophy
in
Food Science

Guelph, Ontario, Canada

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ABSTRACT

DEVELOPMENT OF PROTEIN-POLYSACCHARIDE COMPLEX FOR
STABILIZATION OF OIL-IN-WATER EMULSIONS

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Soy whey protein isolate (SWPI) – Fenugreek gum conjugates were developed and their molecular characteristics and emulsifying properties were investigated. SWPI was extracted from soy whey of tofu processing. SWPI exhibited excellent emulsifying properties comparable to soy protein isolate. However, to improve the emulsifying properties of SWPI for some applications, it was conjugated to fenugreek gum. The extent of conjugation was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Fourier transform infrared (FTIR) and High performance size exclusion chromatography (HPSEC). The SDS-PAGE of the conjugates showed polydisperse bands at the top of the separating gel in the conjugates suggesting the formation of high molecular weight products. Refractive index spectrum of HPSEC profiles showed a reduction of protein peak of unconjugated mixture and shifted a peak to higher molecular weight of the conjugates. Ultraviolet spectrum of HPSEC showed an increase of protein peak intensity at polysaccharide region. FTIR spectrum showed an amide band I and II were still observed in the conjugates after the unreacted proteins were
removed. 1D NMR spectra showed that fenugreek gum was covalently bound to proteins through interaction between the reducing end of mannose residue and lysine.

The protein solubility of SWPI – Fenugreek gum conjugates improved as compared to SWPI and SWPI – Fenugreek gum mixture when assessed in the pH range 3 to 8 at 22°C, especially at isoelectric point of protein (pl). A 1:3 and 1:5 ratio of SWPI – Fenugreek gum gave rise to better emulsion stabilization compared to 1:1 ratio. Particle size analysis revealed that conjugation of SWPI – Fenugreek gum at 60°C for 3 days was enough to produce relatively small droplet sizes in oil-in-water emulsions. SWPI – Unhydrolyzed fenugreek gum conjugates exhibited better emulsifying properties compared to SWPI – Partially hydrolyzed fenugreek gum conjugates. The conjugates improved emulsifying properties of SWPI, particularly around the pl of protein. The emulsifying properties were greatly increased by heating the conjugates before emulsification. The conjugates also improved emulsion stability at high salt concentration compared to SWPI. In summary, incorporation of SWPI into fenugreek gum improved emulsifying properties of SWPI near the pl of protein and at high salt concentration.
ACKNOWLEDGMENTS

I am deeply indebted to my supervisors, Professor H. Douglas Goff and Professor Steve W. Cui for their help, suggestions and encouragement to complete this work. It is a great opportunity to study under their supervision and their support is highly appreciated.

I would like to express my appreciation to my colleagues, Ahmad Haniff Jaafar, Azizah Misran, Rashidah Sukor, Mohd Sabri Pak Dek, Firdaus Yusof, Dr. Ji Kang, Huihuang Ding, Xiaohui Xing, Dr. Junyi Yin and Liu Yanfang for their friendship and help during the course of study.

I would like to thank Dr. Qi Wang, Ms Cathy Wang, Dr. Guo Qingbin, John Nikiforuk, Marta Hernandez and other co-workers in Agriculture and Agri-Food Canada for their technical support.

Especially, I would like to give my special thanks to my parents and family whose patient love, support and encouragement which enabled me to complete this work.
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1 INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Protein-polysaccharide interactions play a significant role in the structure and stability of many processed foods. Dickinson (1998a) claimed that the best way to adsorb hydrocolloids onto interfaces is to link them to proteins. The high molecular weight glyco-conjugate is supposed to combine the properties of a hydrophobic protein, being firmly attached to the oil droplet surface, with the property of a hydrophilic polysaccharide, being highly solvated by the aqueous phase medium (Dickinson & Galazka, 1991). The complexation between proteins and polysaccharides at the emulsion droplet surface can improve steric stabilization. Droplet size can be smaller if the polysaccharide is present during homogenization and rate of creaming may be reduced so long as there is no bridging flocculation.

Covalent protein-polysaccharide conjugates suitable for stabilizing emulsions can be made in the laboratory by Maillard reaction of protein with polysaccharide under controlled dry heating conditions (Dickinson & Galazka, 1991). By optimizing the relative humidity and the heating time/temperature (e.g., 60°C for several days), it is possible to make soluble protein-polysaccharide conjugate with extremely good emulsifying properties. Maillard-type reactions of several proteins with polysaccharides, leading to covalent bond formation under benign reaction conditions, i.e., by gently heating of a dry biopolymer mixture over a period of few weeks, has been exploited by a number of workers in order to prepare hybrids with superior emulsifying properties compared to non-treated mixtures (Dickinson, 1993; Kato, Sasaki, Furuta, & Kobayashi, 1990; Kato, Shimokawa, & Kobayashi, 1991; Shepherd, Robertson, & Ofman, 2000). The attachment of polysaccharides to proteins can also lead to bigger improvements in solubility, thermal stability and emulsifying properties without loss of function and undesirable effects (Kato et al., 1990). Furthermore, Maillard reaction with polysaccharides may also mask
the epitope structure of the allergenic proteins (Arita, Babiker, Azakami, & Kato, 2001; Babiker, Hiroyuki, Matsudomi, Iwata, Ogawa, & Bando, 1998).

Soy proteins are important ingredients in many food formulations where they extend or replace the functionality of animal proteins in these systems. The soy whey proteins are functionally and nutritionally valuable because of their excellent solubility and because they contain some essential amino acids. If these proteins can be recovered, they will be a valuable source of protein. The loss of the soluble whey proteins and carbohydrates not only results in a water pollution problem, but also represents an economic penalty against the process because otherwise usable materials are being discarded.

Protein–polysaccharide conjugates, which are natural, non-toxic and have improved emulsifying properties, can be expected to have significant potential for use in the food and health industries (Akhtar & Dickinson, 2003). The Maillard reaction can be used as a simple and environmentally friendly way of recycling wastes into useful food ingredients. For more than two decades, the Maillard reaction has been employed in the preparation of functional glycoproteins between protein and carbohydrate. In contrast to chemical modifications, the application of the naturally occurring Maillard reaction provides safe conjugates with potential use in food systems. Based on the unique characteristics of fenugreek gum and the functional and nutritional value of soy protein we proposed fenugreek gum-soy whey protein isolate conjugates through Maillard reaction for stabilizing emulsions.

1.2 Protein and emulsion stabilization

Formation and stabilization of oil-in-water emulsion requires the presence of a surfactant that can effectively reduce the interfacial tension between the oil and aqueous phases such as lecithins, monoacylglycerol or macromolecules such as proteins (Damodaran, 2005). However,
proteins are generally less surface active than small surfactants due to their complex structural properties. Because of conformational constraints to properly orient the hydrophilic and hydrophobic groups at the interface and improper packing at the interface, proteins are unable to greatly reduce the interfacial tension. Although proteins are not highly effective in reducing the interfacial tension, protein-stabilized emulsions are generally more stable than those stabilized by small surfactants (Damodaran, 2005). Proteins form a gel-like film around oil droplets via non-covalent interactions (Dickinson, 2001). Conformational changes in the protein at the interface promote polymerization via the sulphydryl-disulfide interchange reaction (Dickinson & Matsumura, 1991; Monahan, McClements & Kinsella, 1993; Damodaran & Anand, 1997) These interactions, apart from making the protein irreversibly adsorbed to the interface, provide a highly viscoelastic film that resists coalescence (Dickinson, 1998b, 1999, 2001).

Proteins are good as emulsifying agents and stabilizers when solubility is good and the aqueous environment is suitable for steric and electrostatic stabilization (Dickinson & Stainsby, 1982). However, the emulsifying activity may be lost close to the isoelectric of a protein due to the precipitation or aggregation of protein (Damodaran, 1996). At this point protein has balanced positive and negative charges and hence the solubility of proteins is minimal. The emulsifying properties of proteins are also lost at high salt concentration due to charge shielding effects (Shepherd et al., 2000; Damodaran, 1996). At low concentrations (< 0.2 M), salts increase the water binding capacity of proteins. This is because hydrated salt ions bind (weakly) to charged groups on proteins. At this low concentration, binding of ions to proteins does not affect the hydration shell of the charged groups on the protein, and the increase in water binding essentially comes from water associated with the bound ions. However, at high salt concentrations, much of the existing water is bound to salt ions, and results in dehydration of the protein.
1.3 Polysaccharides

Polysaccharides have been proposed as the first biopolymers to have formed on earth. They are classified on the basis of their main monosaccharide components and the sequences and linkages between them, as well as anomeric configuration of linkages, the ring size (furanose or pyranose), the absolute configuration (D- or L-) and any other substituents present. Certain structural characteristics such as chain conformation and intermolecular associations will influence the physicochemical properties of polysaccharides.

Water soluble polysaccharides are often termed hydrocolloids or gums. They enhance viscosity and/or form gels in aqueous systems. Gum Arabic, locust bean gum, tragacanth, pectins and other naturally occurring molecules have been used for many years, in part because of their surface activity (Benichou, Aserin, & Garti, 2002). They are used in foods as stabilizers, thickening and gelling agents, crystallization inhibitors, and encapsulating agents. These polysaccharide gums occur in nature as storage materials, cell wall components, exudates, and extracellular substances from plants or microorganisms (Izydorczyk, Cui, & Wang, 2005).

1.4 Surface active polysaccharides

Polysaccharides are known to have significantly less surface activity in comparison to proteins (Dickinson & Stainsby, 1988). This inferiority is related to their pronounced hydrophilicity, low flexibility and monotonic repetition of the monomer units in the backbone (Darling & Birkett, 1987; Dickinson & Stainsby, 1982). A polysaccharide may show considerable surface activity, only if it contains auxiliary hydrophobic groups (methyl, acetyl, etc.) or if it is contaminated either with proteineous moieties attached to the biopolymer or with glycolipids. Chemically-modified polysaccharide derivatives such as highly substituted methyl
cellulose give surface activity at the oil–water interface similar to other food emulsifiers (Darling & Birkett, 1987).

Some polysaccharides have a good surface properties i.e., effective and efficient reduction of surface and interfacial tensions, and effective adsorption. Gum Arabic and fenugreek gum are two examples of polysaccharides that exhibit a good surface activity.

1.4.1 Gum Arabic

It is well documented that gum Arabic has excellent emulsification properties for oil-in-water emulsions. Stable oil-in-water emulsions with high opacity and low creaming are obtained when the gum is used at high concentration. About 12% (w/w) is required to give stable 20% (w/w) orange oil emulsions of small droplet size (Randall, Phillips, & Williams, 1988).

Gum Arabic has a complex structure with highly branched, polysaccharide carboxylic acids (2.5 x 10^5 Da). The surface activity of gum Arabic was related to its ability to form a thick viscoelastic film at oil–water interfaces (Shotton & Wibberley, 1959). It has been demonstrated that gum Arabic contains some levels of proteinaceous matter (Anderson, 1986). It consists of three principal fractions, i.e., an arabinogalactan (AG), an arabinogalactan–protein complex (AGP) (representing about 30%) and a glycoprotein (GP). The AGP fraction, where hydrophilic carboxylate blocks are linked to a main polypeptide chain forming a polysaccharide–polypeptide hybrid, enables strong adsorption at the oil–water interface and promotes emulsion stability. The AGP and GP components are denatured by heating, resulting in loss of emulsification efficiency and reduction in solution viscosity (Morris, 1990; Williams, Phillips., & Randall, 1990). The stabilization of oil-in water emulsions is mainly because of steric repulsive forces.
The arabinogalacto-protein complex is the fraction that provides the functionality of the gum Arabic as an emulsion stabilizer (Randall et al., 1988). Gum Arabic has been shown to consist of five distinct molecular mass components, each of which is present in varying proportions. Although the gum as a whole was shown to contain < 2% protein, it was found that most of this was present within one high molecular mass component, which constituted <10% of the total. Following emulsification it was found that only 1-2% of the gum Arabic adsorbed at the oil-water interface and that the portion that predominantly adsorbed was the high molecular mass, protein-rich fraction. It is evident, therefore, that only proteinaceous material adsorbs on to the oil droplets and that only a quarter of the total protein component is actually surface active under the conditions used. Surface activity and consequently emulsifying ability require the adsorbing species to contain both hydrophobic groups, which can reside at or penetrate inside the oil interface and hence anchor the molecule to the surface, and hydrophilic groups that can protrude out into the aqueous phase and give rise to steric stabilization. These emulsions also exhibited high surface viscosity, which do not change upon dilution (Dickinson & Stainsby, 1988, Dickinson, Elverson, & Murray, 1989; Connolly, Fenyo, & Vandevelde, 1988).

1.4.2 Fenugreek gum

Fenugreek (Trigonella foenum-graceum) is a leguminous plant grown in northern Africa, the Mediterranean, Western Asia, northern India, and currently cultivated in Canada. Fenugreek gum is a galactomannan where mannose constitutes the backbone and galactose units are attached at the 6-position at an average ratio of 1:1 (Bummer, Cui & Wang, 2003). The chemical composition of native extracted fenugreek gum consists of 2.9 wt.% protein, and 85.9 wt.% polysaccharides. Of the total carbohydrates, over 50% are water-soluble polysaccharides with surface activity. After extensive purification protein content was reduced to 0.95 wt.%, lipids to
0.4 wt.%, ash to 0.5 wt.%, moisture to 8.7 wt.% and carbohydrate content was increased to 89.7 wt.% (Garti, Madar, Aserin, & Sternheim, 1997).

Fenugreek polysaccharide is always accompanied by a proteinaceous matter and exhibits significant surface activity (Garti et al., 1997; Huang, Kakuda, & Cui, 2001). Unique surface activity of fenugreek gum makes it an effective stabilizer for o/w emulsions. Huang et al. (2001) reported that fenugreek gum exhibited the highest stabilizing properties among 11 commercial gums and five laboratory prepared gums in o/w emulsion model systems. Garti et al. (1997) found that stable emulsions with a relatively small droplet size (3 µm) could be formed using bipurified fenugreek gum (0.8% protein). The surface tension of the native gum is similar to those of guar and the interfacial tensions slightly higher (20 for guar versus 28 mN/m for fenugreek). However the emulsification capabilities of the gum fractions were very impressive. The emulsions could be aged for over 4 weeks at temperature ranges of 4-50°C without any significant droplet size change, which indicates that only slight coalescence took place.

The nature of this adsorption and whether it is related to certain ‘hydrophobic’ functional groups is not yet clear. Some may claim that these are proteinaceous moieties, chemically (hybrid) bound to the polysaccharide main structure (forming glycoproteins). Others may claim that they are hydrophobic lipophilic fatty acids, or lipophilic hydrocarbon skeletons attached to the polysaccharide (forming glycolipid structures). No strong evidence exists to support any one of these concepts. Brummer et al. (2003) reported that purified fenugreek gum that containing 0.6% residual protein showed lower surface activity compared to the unpurified gum. Moreover, Youssef, Wang, Cui, & Barbut (2009) found that protein free fenugreek (0.16% protein) significantly reduced the surface activity. The latter two studies suggest the present of protein in fenugreek gum played an important role in stabilizing o/w emulsions. It would be reasonable to
speculate that a surface active protein combined with polysaccharide would greatly improve the emulsification properties of polysaccharides.

1.5 Interaction between protein and polysaccharides

1.5.1 Electrostatic interactions

Electrostatic interactions between protein and polysaccharides may result in the formation of complex coacervation, soluble complex or an amorphous co-precipitate. In aqueous solution, complex coacervation takes place between two oppositely charged polymers. For instance, complexation between proteins and anionic polysaccharides occurs below the protein isoelectric point (pI) and at low ionic strengths. Complexation between two charged biopolymers is usually a reversible process depending on pH, ionic strength, protein to polysaccharide ratio, temperature and processing mixing (Benichou et al., 2002). Most food proteins (pI pr ~ 5) can form complex coacervates with anionic polysaccharides (pI po ~ 3) in the intermediate region of pH where the two macromolecules carry opposite net charges (pI po<pH<pI pr) (Dickinson, 1998a). Interaction between β-lactoglobulin and sodium alginate was studied by Harnsilawat, Pongsawatmanit, & McClements (2006) in the pH range 3-7. Depending on the pH and the relative charge density of biopolymers, no interaction at pH 6 and 7, weak interaction at pH 5 and strong interactions at pH 3 and 4 were observed.

The strength of complexation depends on a number of factors such as the distribution of ionizable groups on the surface of the protein, the ease of unfolding of its native structure, and the backbone flexibility and charge density of the polysaccharide (Dickinson, 1998a). The number of micro-ions present in the biopolymer solution is an important factor affecting complex coacervation. At low ionic strength, the micro-ion concentration has only a small effect
on coacervation. The number of charges present on the biopolymers is sufficient to allow electrostatic interactions. At high ionic strength (20 - 30 mM), the net charge carried by the biopolymers is reduced by interactions with microions, resulting in a decrease of the electrostatic attraction between macromolecules and hence the coacervation is suppressed. Figure 1.1 describes the effects of ionic strength on the coacervation of albumin with acacia gum at electrical equivalence pH (Schmitt, Sanchez, Banon, & Hardy, 1998). The influence of high salt concentration sharply reduced the coacervate yield. The optimum salt concentration is found to be around 10 mM. However, it was observed that complex coacervation is also limited by very low ionic strength. It was proposed that it could be due to the high charges of biopolymers under these environmental conditions, so that the molecule chains are in an extended configuration.

Figure 1-1 The effect of ionic strength on the coacervate yield of 1.0% (w/v) gelatin/acacia mixtures. (●)Type A gelatin/acacia (pH 3.8); (▼) Type B gelatin/acacia (pH 3.6). (Schmitt et al. 1998).
Soluble protein–polysaccharide complexes are generally produced when both the biopolymers carry a net negative charge (pH > pI_{pr}). In this case the attraction involves positively charged local patches on the protein interacting with the anionic polysaccharide. As an example, sulfated polysaccharides (carrageenans) and proteins can complex at pH values above the pl of the protein. Complex formation between carrageenan and a model protein is illustrated in Figure 1.2.

![Figure 1-2 Schematic illustration of ionic interactions between carrageenan and a model protein (Benichou et al. 2002).](image)

The main classes of positively charged residues in most proteins are the –NH$_3^+$ groups (pKa ~11). An increase in the net negative charge on the protein has two effects: (i) it produces an enhancement in the electrostatic protein–protein repulsion (i.e. an increase in A_{pr-pr}) and (ii) it reduces the protein–polysaccharide attraction by screening the interactions of the positively charged groups (thereby increasing A_{pr-po}). In terms of the relative importance of effects (i) and (ii), a distinction has to be made between carboxylated polysaccharides (e.g. alginate, pectin) and sulfated polysaccharides (e.g. carrageenan). The molecular attraction of protein bound -NH$_3^+$ groups for –OSO$_3^-$ groups is much stronger than for –CO$_2^-$ groups. This means that, for carboxylated polysaccharides, effect (i) is mainly predominant; so, at pH > pI of protein, the protein–polysaccharide complexation is very weak or non-existent, or the system exhibits thermodynamic incompatibility. In contrast, at low ionic strengths, sulfated polysaccharides of
relatively high charge density can form fairly strong reversible complexes with proteins, even at neutral or alkaline pH (i.e. well above pI of protein) (Dickinson, 1998a).

1.5.2 Covalent interaction

One modification recognized as being suitable for producing speciality ingredients for food applications is protein–polysaccharide conjugation via a Maillard-type reaction (Shepherd et al., 2000). The Maillard reaction can be divided into three stages; initial, intermediate and advanced. The initial stage involves the condensation of the carbonyl group of the reducing sugar with the available ε-amino groups (lysine been the primary reactive amino group) of the protein, resulting in an Amadori product being produced via the formation of a Schiff base with the release of water and the Amadori rearrangement (Ames, 1992). The intermediate stage involves the degradation of Amadori products resulting in a wide range of compounds. The final stage of the reaction results in extensively coloured, water insoluble, nitrogen containing polymeric compounds referred to as “melanoidins” (Friedman, 1996).

The initial stage of this reaction has been shown to be sufficient for conjugation to be achieved between the protein and the polysaccharide via the formation of a covalent bond, which has been shown to be very stable to changes in pH, ionic strength and temperature (Dickinson & Euston, 1991; Schmitt et al., 1998). These conjugates have been shown to possess improved functional properties, including enhanced emulsifying properties (Akhtar & Dickinson, 2007; Shepherd et al., 2000), increased solubility particularly around the isoelectric pH of the protein (Chevalier, Chobert, Popineau, Nicolas, & Haertlé, 2001; Jiménez-Castaño, López-Fandiño, Olano, & Villamiel, 2005) and increased heat stability compared to the protein itself (Aoki et al., 1999; Chevalier et al., 2001).
1.6 Protein-polysaccharide interaction and stabilization of emulsions

1.6.1 Electrostatic interaction

One important type of complexation arises from the electrostatic interactions between oppositely charged functional groups on protein and polysaccharides. Electrostatic complexation between proteins and charged polysaccharides onto the surface of protein-coated oil droplets, leading to the enhancement of emulsion stabilization by the bulky secondary layer of charged polysaccharide molecules, could be achieved via combined electrostatic and steric stabilization mechanisms (Ettelaie, Dickinson & Murray, 2005). However, whether such protein–polysaccharide complexation actually produces enhanced emulsion stability depends in practice on the types and relative concentrations of the biopolymers, the solution conditions, and also the method of emulsion preparation (Guzey & McClements, 2006; Jourdain, Leser, Schmitt, Michel, & Dickinson, 2008; McClements, 2005a). Two separate instability mechanisms may be involved: (i) bridging flocculation, when the polysaccharide content is so low that droplet collisions occur faster than the rate of polysaccharide saturation of the protein-coated droplet surface; and (ii) depletion flocculation, when the excess non-adsorbed polysaccharide concentration exceeds a certain critical value (Dickinson, 2008; McClements, 2005b).

In electrostatic complex system, the method of emulsion preparation along with types and concentration of polysaccharide, pH and ionic strength play an important role in emulsion stability (Dickinson, 1998a; Jourdain et al., 2008). The effect of addition of the anionic polysaccharide dextran sulphate (DS) on the properties of oil-in-water emulsions containing sodium caseinate has been studied under different DS concentration, pH and ionic strength conditions (Jourdain et al., 2008). Two types of methods were used for preparation of emulsions, namely ‘bilayer’ and ‘mixed’ emulsions. In bilayer emulsions, the dextran sulfate was added to a
sodium caseinate-stabilized emulsion after emulsification while in mixed emulsions, the oil was directly emulsified into the mixed biopolymer solution.

![Figure 1-3 Effect of concentration of DS on the mean particle size $d_{43}$ of emulsions prepared in 20 mM imidazole buffer at pH 6 with 20 vol% oil, 0.5 wt% sodium caseinate and different concentrations of DS: ♦, mixed emulsions, □, bilayer emulsions (Jourdain et al., 2008).](image)

The effects of DS concentration on the particle size $d_{43}$ of emulsions of mixed emulsions and bilayer emulsions at pH 6 are shown in Figure 1.3. In the mixed emulsions, the value of $d_{43}$ was found to be essentially independent of polysaccharide content. No bridging flocculation was observed and the oil droplets were nicely dispersed, even at low DS concentration. In the “bilayer” emulsion, without any DS present in the emulsion, the mean diameter of the droplets was small but, when just 0.1 wt% DS was present in the bilayer systems, a great increase in particle size was observed. Increasing DS concentration to 0.5 and 1 wt%, decreased the droplet size indicating that enough DS molecules are available to cover all the casein-coated droplets.
properly, and so the droplets are effectively stabilized by combined steric and electrostatic interactions. The greater aggregation in the 2 wt% DS emulsions can be attributed to the much higher viscosity of the more concentrated DS solution and the resulting inhomogeneous stirring during the preparation of the emulsions. It might be presumably due to depletion flocculation due to excess of unadsorbed polysaccharide.

The influence of pH and DS concentration on the particle size $d_{43}$ of emulsions of mixed emulsion and bilayer emulsions are shown in Figure 1.4. In mixed emulsions, the mean particle size increased drastically when the pH was lowered from 6 to 2 in the emulsion containing 0.1 wt% DS (Figure 1.4A). However, increasing concentration of DS decreased the particle size when pH was decreased.

It can be suggested that, at low DS concentration, the sodium caseinate-stabilized droplets are not fully covered by DS. When the pH is lowered towards acidic values, the charges on the sodium caseinate fragments are neutralized and aggregation occurs in the same way as for sodium caseinate-stabilized emulsion. At higher DS concentration, the polysaccharide forms a protective layer around the protein-coated droplets. The positive patches formed on sodium caseinate when the pH is lowered are neutralized by the large excess of negative charge on DS, and the net charge on the surface of the droplets remains negative. Hence the system is protected against acid-induced precipitation.

In bilayer emulsions (Figure 1.4B), the emulsion system appeared to be more aggregated under acidic conditions than did the mixed emulsion system. This could be due to the slightly higher amount of DS bound to sodium caseinate in the mixed emulsion. A higher concentration of DS would be needed for the bilayer emulsions to be protected against acid-induced precipitation. It is also suggested that, in the mixed emulsions, the structure of the species at the
interface is rather condensed, i.e., more resembling a complex coacervate, whereas in a bilayer emulsions a more stratified layer is probably formed. The mixed emulsion might therefore be expected to be more resistant to changes in pH.

Figure 1-4 Effect of pH on the mean particle size $d_{43}$ of (A) mixed emulsions and (B) bilayer emulsions prepared in 20 mM imidazole buffer with 20 vol% oil, 0.5 wt% sodium caseinate and different concentrations of DS: ♦, zero; □, 0.1 wt%; ▲, 0.5 wt%; O, 1 wt%; ◊, 2 wt%. The pH was reduced by addition of HCl (Jourdain et al., 2008).
Figure 1-5 Effect of salt concentration on the mean diameter $d_{43}$ in (A) mixed emulsion and (B) bilayer emulsions prepared in 20 mM imidazole buffer at pH 6 and containing 20 vol% oil, 0.5 wt% sodium caseinate and different concentrations of DS. Sets of data points correspond to different NaCl concentrations; ♦, zero; Δ, 0.1 M; O, 0.5 M (Jourdain et al., 2008).

The effect of ionic strength on stability of bilayer and mixed emulsions is shown in Figure 1.5. The mean particle size $d_{43}$ of the bilayer emulsions increased when 0.1 wt% DS was
added after emulsion formation, which indicative of bridging flocculation. In contrast, for the mixed emulsion, no significant change in $d_{43}$ was observed at 0.1 or 0.5 M NaCl when 0.1 wt% DS was present in the emulsion. For both the bilayer and the mixed emulsions, some serum phase separation was observed at high DS concentrations, presumably corresponding to depletion flocculation due to excess of unadsorbed polysaccharide.

1.6.2 Covalent interaction

It is now well-recognized that impressive improvements in protein solubility and interfacial functionality can be achieved via the complexation and covalent linking of proteins to polysaccharides (Ledward, 1994; Samant, Singhal, Kulkarni, & Rege, 1993; Schmitt et al., 1998; Syrbe, Bauer, & Klostermeyer, 1998). In particular, for the stabilization of emulsions and foams, it has been demonstrated that Maillard-type conjugates produced by the dry-heating of a mixture of these two kinds of biopolymers can have excellent functional properties (Akhtar & Dickinson, 2003; Chevalier et al., 2001; Dickinson & Galazka, 1991, 1992; Dickinson & Izgi, 1996; Dickinson & Semenova, 1992; Diftis & Kiosseoglou, 2003; Dunlap & Côté, 2005; Einhorn-Stoll, Ulbrich, Sever, & Kunzek, 2005; Kato, 1996; Kato et al., 1990, 1992; Mishra, Mann, & Joshi, 2001; Morris, Sims, Robertson, & Furneaux, 2004; Nagasawa, Takahashi, & Hattori, 1996; Neirynck, van der Meeren, Bayari Gorbe, Dierckx, & Dewettinck, 2004; Shepherd et al., 2000). The formation of these high molecular-weight glycopolypeptide conjugates combines the characteristic property of proteins to adsorb strongly to the oil–water (or air–water) interface with the characteristic property of the polysaccharide for solvation by the aqueous phase medium (Dickinson & Galazka, 1991). Such studies have shown that the effectiveness of the conjugate as an emulsifier/stabilizer in model systems is dependant inter alia on the protein/polysaccharide ratio and the polysaccharide molecular weight. This kind of
conjugate, which is ‘natural’, non-toxic and relatively simple to prepare, has for some time been recognized (Dickinson, 1993) as having significant potential for exploitation in food-related emulsification applications.

Several researchers have found that selecting a protein-polysaccharide conjugate as the emulsifying agent can markedly improve emulsion stabilization. O’Regan & Mulvihill (2009) reported the conjugation between sodium caseinate (NaCN) and maltodextrin (Md). Two types of maltodextrin were used in the experiment (Md40 and Md100) with a DE of 4-7 and 9-12 respectively. When assessed in the pH range 2.0-8.0 at 20°C and 50°C, conjugate had improved solubility compared to NaCN, particularly around the isoelectric point of protein especially the NaCN-Md100 conjugates. The NaCN-Md100 conjugate was entirely soluble over the entire pH range studied. The emulsifying properties of NaCN-Md conjugates were assessed in oil-in water emulsions. It was observed that the fat globule size distribution and mean globule size of model emulsions stabilized by NaCN, NaCN-Md conjugates and mixtures were similar immediately after emulsification. After 20 days of storage at 45°C a shift in fat globule size to larger fat globules was observed for all emulsions. However, the fat globule size of NaCN-Md conjugate stabilized emulsions was much smaller compared to NaCN and NaCN-Md mixture stabilized emulsions. The improved stability of the conjugate stabilized emulsions is attributed to the conjugate protein molecule forming a bulkier polymeric layer than the non-conjugated protein on the droplet surface, with the Md portion extruding outwards into the continuous phase providing better steric stabilization, thus preventing droplet aggregation and coalescence (Akhtar & Dickinson, 2007). The NaCN-Md mixture stabilized emulsions were the least stable of the model emulsions. This may be attributed to the presence of non-absorbed Md in the continuous phase of
the emulsion causing attractive forces between droplets resulting in destabilization of the emulsions by depletion flocculation (de Kruif & Tuiner, 1994; Dickinson & Galazka, 1991).

The emulsifying behaviour of glyco-protein complexes of the non-ionic polysaccharide dextran (500 kDa) with whey protein isolate (WPI) have been investigated in systems containing 20 vol.% medium chain triglyceride oil (Akhtar & Dickinson, 2003) under acidic condition (pH 3.2) and high ionic strength (0.2 M). Emulsions were made with WPI and whey protein isolate-dextran (WPI-DX) conjugate. Stability was followed by determining changes in average droplet size and extent of serum separation with time. Gum Arabic (GA) was chosen as reference emulsifier. Both GA and WPI systems exhibited poor stability under acidic conditions in terms of retention of average droplet size on extended storage. The average droplet sizes after 800 h were $d_{43} > 14 \mu m$ and $d_{43} \sim 3 \mu m$ for WPI and GA stabilized emulsions respectively. Droplet sizes of emulsion made with WPI alone were found to increase steadily over the storage period of 34 days. However, emulsification with the WPI-DX conjugates generates much smaller droplets ($d_{43} < 0.5 \mu m$) than with either GA or with protein alone at pH 3.2. The retention of a constant low droplet size during extended storage indicates that the WPI-DX conjugates are extremely efficient in producing stable emulsions under these conditions.

1.7 Research objectives

Polysaccharides are known to modify liquid properties by increasing viscosity and formation of a gel, which could be used to stabilize an emulsion. Fenugreek gum has been shown to exert better emulsifying properties compared to other gums. Proteins are known to exert surface activity that can stabilize emulsions. Our hypothesis is by conjugating fenugreek gum and soy whey protein isolate, the new material will exert better functional properties including improved emulsification
properties. If the new product is successful, it could be very useful in food industry. The objectives of the current research are:

1. To develop protein-polysaccharide conjugate with improved functional properties
2. To characterize and elucidate the structure of the protein-polysaccharide conjugate
3. To apply the protein-polysaccharide conjugate in oil-in-water emulsion model system
4. To understand the structure function relationship of protein-polysaccharide conjugates
2. PHYSICOCHEMICAL AND EMULSIFICATION PROPERTIES OF SOY WHEY PROTEIN POWDERS

2.1 Introduction

Tofu is one of the most important food products made from soybean protein. Tofu is very popular in East and South Eastern Asian countries and has become popular in western countries due to good nutrition and health benefits to humans. It is cholesterol-free, low in saturated fat and high in protein. In the United States, the consumption of tofu and soymilk products is increasing at a rate of 15% per year (R. Sinner Brothers & Bresnahan Co., Casselton, ND, pers. comm.).

Tofu processing involves boiling the soy milk and then treating with a coagulant. Calcium sulfate and/or glucono-δ-lactone (GDL) are mostly used coagulants for the production of tofu. The 7S and 11S globulins are two major components of soy protein that are responsible for the gelation process during tofu processing (Fukushima, 1991; Nishinari et al., 1991; Yoshida, Kohyama, & Nishinari, 1992; Kohyama, Yoshida, & Nishinari, 1992; Kohyama & Nishinari, 1992, 1993). Two steps of gel formation involve protein denaturation and hydrophobic coagulation for the formation of tofu. At first, the hydrophobic regions of the protein molecules in the native state are located inside and are exposed to the outside by heat denaturation as shown by fluorescence studies (Koshiyama, Hamano, & Fukushima, 1981; Matsudomi, Mori, Kato, & Kobayashi, 1985). Since the denatured soybean protein is negatively charged (Kohyama & Nishinari, 1993), the formation of protons induced by GDL or calcium ions neutralizes the net

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Note: To be published as Kasran, M., Cui, S. W., & Goff, H. D. (2013). *Journal of Food Science.*
charge of the protein in the second step. As a result, the hydrophobic interaction of the neutralized protein molecules becomes more predominant and induces the aggregation.

Tofu whey is a by-product of tofu processing. Tofu whey is a good source of carbohydrates, mainly sucrose, raffinose and stachyose, as well as protein and magnesium (from the coagulant agent) (Ounis, Champagne, Makhlouf, & Bazinet, 2008). The tofu whey proteins are functionally and nutritionally valuable because of their excellent solubility and contain a greater proportion of some essential amino acids (such as methionine). The tofu whey has the advantage of being low cost since it has little market value (Nguyen, Champagne, Lee, & Goulet, 2003) and its disposal actually constitutes an environmental and industrial problem (Peñas, Préstamo, Polo, & Gomez, 2006). Tofu wastes deteriorate very quickly because of their high water content and their high content of nutritious substances for bacteria.

Recently, tofu whey has been proposed as a potential alternative to soy milk as a substrate for the production of starters for the fermentation of soy products. Nguyen et al. (2003) have evaluated tofu whey as a growth medium for a *Lactobacillus paracasei* ssp. *paracasei*. Their results demonstrated that relatively high viable counts of these lactobacilli could be obtained in tofu whey and that this medium was a better substrate than cow milk for the production of a *L. paracasei* strain. Ounis et al. (2008) reported the utilization of tofu whey pre-treated by electromembrane process as a growth medium for *Lactobacillus plantarum* LB17. Electromembrane (electrodialysis for salt recovery and electroacidification with bipolar membranes for protein recovery) process allowed the recovery of almost 45% and 54% of the initial protein and mineral contents respectively. The protein recovered could be reused in tofu process to increase yield.
Recovery of proteins from tofu whey not only solves the environmental and industrial problem but also a way of recycling wastes into useful food ingredients. Proteins are efficient emulsifying agents and stabilizers of food oil-in-water emulsions under conditions where solubility is good and the aqueous phase environment is suitable for steric and electrostatic stabilization (Dickinson & Stainsby, 1982). Since soy protein are important ingredients in many food formulations and can replace or extend the functionality of animal proteins, the recovery of proteins from tofu whey will be giving benefit to food industries.

The aim of this work was to extract tofu whey proteins and evaluate their physicochemical and emulsifying properties. The whey powder was extracted by means of dialysis and either precipitated at pH 4.5 using HCl or direct freeze drying of the tofu whey after being concentrated in rotary evaporator. The physicochemical properties include proximate analysis, amino acids analysis, SDS-PAGE analysis, High Performance Size Exclusion Chromatography (HPSEC), Fourier Transform Infrared Spectroscopy (FTIR) and surface tension analysis. The emulsifying properties were tested using 10% volume of canola oil and the stability was evaluated by measuring the average droplet size (d_{43}) as a function of storage time from 0 – 14 days at 25^\circ C.

2.2 Materials and Methods

2.2.1 Materials

Soybean was obtained from Goudas Food Products Co. Ltd., Concord, ON, Canada L4K 2I8. All chemicals used in this study were of analytical grade unless otherwise specified. Canola oil was purchased from the local supermarket.
2.2.2 Preparation of soy protein isolate

Soybeans (50 g) were soaked in water overnight, dehulled and ground in a mixer (IKA, A10 Basic, Willmington, NC). The ground soybeans were freeze dried and further ground to 100 mesh using a laboratory mill (Retsch, MM2000 Ball mill). The soy flour was defatted by soaking the soy flour in hexane (1:10 ratio, soy flour to hexane) for 24 hr. After removing the hexane from soy flour, the residual hexane was removed by heating the defatted soy flour at 65°C for 30 min. Soy protein isolates were prepared by suspending the defatted soy flour in water at 1:10 ratio (w/v, soy flour:water) for 2 h at 25°C. The solution was gradually added with 0.5 M NaOH until the pH constant at pH 8. Soluble proteins present in the solution were separated by centrifugation at 12,000 g for 30 min at 4°C. The supernatant was adjusted to pH 4.5 with 2 N HCl enabling the precipitation of protein. The precipitate was separated by centrifuging at 12,000 g for 30 min at 4°C and the supernatant was discarded. The precipitate was washed with water and re-solubilized in deionized water and the final pH was adjusted to pH 7.0 with 2 N NaOH. The protein was dialyzed overnight at 4°C and freeze dried.

2.2.3 Extraction and physicochemical characterization of soy whey protein powder (SWPP)

2.2.3.1 Extraction of soy whey from tofu processing

The process for tofu making was based on Wang (1984). Soybeans (500 g) were soaked in water overnight and ground in a mixer (Black & Decker, Black & Decker Corporation, Maryland, USA) for 10 min at 24°C. At the same time about 5 L of water was boiled in a 10 L pot. The ground soybean was then poured into a pot at a temperature of 95°C. This corresponded to raw bean to water ratio of 1:10 (w/v) for extracting solids from soybean into raw soymilk. The solution was stirred for another 20 min, cooled and the soymilk was filtered using muslin cloth.
from the fibrous part. The soy milk was heated to 75°C and calcium sulfate (3.12 g/L) or 22.9 mM was slowly added into soy milk solution until tofu was coagulated. This amount is in the range of coagulant needed for the coagulation of tofu i.e. 16.5 – 27.2 mM (Cai & Chang, 1998). The coagulated tofu was centrifuged at 15,180 g at 20°C for 10 min to separate tofu from the whey. The tofu is a solid curd and the whey solution (supernatant) contains the protein which was used in section 2.2.3.2 for extraction of biomass from soy whey.

2.2.3.2 Extraction of biomass from soy whey

Three methods were applied to extract biomass from soy whey, namely undialyzed, dialyzed and isoelectric precipitation at pH 4.5. In the undialyzed method, the soy whey was concentrated by using rotary evaporator to one third of original volume and freeze dried. For dialyzed method, instead of direct freeze drying the concentrated whey was dialyzed using 6-8 kDa molecular weight cut off (MWCO) for 3 days at 4°C to remove small molecules prior to freeze drying. The third method was extraction at isoelectric point to make soy whey protein isolate (SWPI). The whey was adjusted to pH 4.5 using 2 M HCl. The supernatant was discarded and the precipitated protein was collected. The pellet was then washed with ultrapure water and subsequently centrifuged at 15,180 g at 20°C for 5 min. The precipitated protein was resolubilized in ultrapure water and the final pH was adjusted to pH 7.0 with 2 M NaOH. The protein was dialyzed (6 – 8 kDa molecular weight cut off) overnight at 4°C and freeze dried.

2.2.3.3 Physicochemical characterization of SWPPs

2.2.3.3.1 Chemical analysis

Ash and moisture content was determined according to AACC (2000) methods 08-01 and 44-40, respectively. Protein content was determined from nitrogen content (N X 6.25) using a
ThermoQuest Italia S.P.A. EA/NA 1110 Automatic Elemental Analyzer (Strada Rivoltana, Milan, Italy). Total carbohydrate content was determined using the method of Dubois, Gilles, Hamilton, Rebers, & Smith (1956). Lipid was determined by soxhlet extraction using petroleum ether (AACC method, 2000). Individual amino acids were determined by another independent lab (University of Jiangnan, China).

2.2.3.3.2 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970) using 12%, w/v, acrylamide separating gel and a 4%, w/v, stacking gel both containing 0.1%, w/v, SDS. SPI (0.25%, w/v) and SWPPs {SWPI (0.25%, w/v), dialyzed (0.25%, w/v) and undialyzed (1.5%, w/v)} were prepared in 0.0625 M Tris-HCl buffer (pH 6.8) containing 2%, w/w SDS, 5%, w/w, mercaptoethenol and 0.012%, w/w, bromophenol blue and heated at 95°C for 4 min prior to loading in the gel slots. Electrophoresis was carried out at a constant voltage (200 V) for 45 min using a tris glycine buffer (pH 8.3) containing 0.125%, w/w, SDS. The gel was removed from the electrophoresis unit, stained with Coomassie brilliant blue R-250 and destained with 50:10:40 methanolic:acetic acid:water. Following staining, the gels were photographed with Synoptic digital camera and the photographs were analyzed using SynGene GeneTools (Version 3.06.04, Perkin) scanning densitometer software to determine the amount of each protein band.

2.2.3.3.3 High Performance Size Exclusion Chromatography (HPSEC)

The molecular weight distribution of the samples was determined using (HPSEC), which included the following detectors: refractive index, viscosity (Model 250, Viscotek, Houston, TX, USA), ultraviolet (280 nm) and right angle laser light scattering (RALLS, Viscotek). Two
columns, connected in series (Shodex OHpak KB-806 M, Showa Denko K.K. Tokyo, Japan; and Ultrahydrogel Linear, Waters, Milford, MI), were kept at 40°C during measurements. A Shimadzu SCL-10Avp pump unit (Shimadzu Scientific Instruments Inc., Columbia, MD, USA) was used with Viscotek Triple detectors. The mobile phase consisted of 0.1 M NaNO₃ using a flow rate of 0.6 mL/min and an injection volume of 100 μL. The samples were dissolved in ultrapure water (overnight), and filtered through a 0.20 μm filter prior to injection onto the column.

2.2.3.3.4 Fourier Transform Infrared (FTIR)

FTIR spectra were carried out on a Golden-gate Diamond single reflectance ATR in an FTS 7000 FT-IR spectrometer with a DGTS detector (DIGILAB, Randolp, MA). The spectra were recorded at absorbance mode from 1200 to 800 cm⁻¹ at a resolution of 4 cm⁻¹ with 128 co-added scans.

2.2.3.3.5 Surface tension

Surface tension was measured by the Du Nouy ring method using a semi-automatic model 21 Surface Tensiomat (Fisher Scientific, Toronto, Canada) at 23°C. Deionized water was used to calibrate the tension meter for surface tension measurements. The various concentrations of samples (0.045, 0.09, 0.18, 0.27 and 0.36% protein) were prepared in 20 mL distilled water. All samples were prepared by dissolving the samples in distilled water with constant stirring for 2 h. The samples were kept standing at 23°C for 2 h before analysis being carried out.

2.2.4 Emulsifying properties of SWPPs

2.2.4.1 Emulsion preparation
SPI and SWPPs dispersion were prepared by dissolving in Milli-Q water and stirred using magnetic stirrer with 0.01 wt.% sodium azide added as an antimicrobial agent. The pH of the solution was adjusted to pH 7.0 by adding a few drops of 0.5 N NaOH. Oil-in-water (O/W) emulsion (10% vol. of canola oil) was prepared by pre-homogenising the mixture with Polytron (Kinematica GmbH, Brinkman Instruments, Rexdale, ON) at power level 5 for 5 min at room temperature. The pre-homogenized mixture was homogenized for three passes with laboratory scale jet homogenizer (Nano DeBee, B.E.E. International Inc., Easton, MA) working at the operational pressure of 35 MPa.

2.2.4.2 Particle size measurement

The fat globule size distribution was measured by static light scattering analyzer (Mastersizer 2000S, Malvern Instruments Inc. Westborough, MA). The optical parameters selected were a dispersed phase refractive index of 1.466, a droplet absorbance of 0 and a continuous phase refractive index of 1.333. The average droplet size was characterized by two mean diameters, surface weighted mean diameter, \( d_{32} \) and volume weighted mean diameter, \( d_{43} \). The \( d_{32} \) value was used to estimate the specific surface area of freshly made emulsions, and the \( d_{43} \) value was used to monitor changes in droplet-size distribution on storage.

2.3 Results and discussion

2.3.1 Physicochemical characterization of SWPPs

2.3.1.1 Mass yield of SWPPs extracted by three different methods

An amount of 59.27 ± 1.84 g of SWP (11.9% w/w, dry basis) was extracted with the undialyzed method from 500 g of soybean while 17.60 ± 0.85 g (3.5% w/w, dry basis) was extracted using dialyzed method. Mass yield obtained of SWPI was 5.87 ± 0.25 g (1.2% w/w, dry basis).
2.3.1.2 Chemical composition of SWPPs

Proximate composition of SWPPs is shown in Table 1. SWPI powder contained the highest amount of protein, followed by dialyzed SWP. In contrast, total sugar and ash content were highest in undialyzed SWPP compared to dialyzed SWP and SWPI powder. The total proximate composition of undialyzed SWPP was only 78.50% compared to 97.03 and 95.56% for dialyzed SWP and SWPI respectively. The lower value of overall proximate composition for undialyzed SWP might be due to the presence small molecules such as amino acids, peptides and organic acids, which contribute high proportion of the total mass. Jackson, StawiarSKI, Wilhelm, Goldsmith, & Eykamp (1974) reported that soy whey contained 20% of amino acids, peptides and organic acids on dry weight basis.

**Table 2-1 Proximate composition of SWPI, undialyzed and dialyzed SWPPs**

<table>
<thead>
<tr>
<th></th>
<th>Protein (%)</th>
<th>Moisture (%)</th>
<th>Fat (%)</th>
<th>Ash (%)</th>
<th>Total sugar (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undialyzed SWPP</td>
<td>17.88 ± 0.73</td>
<td>4.87 ± 0.21</td>
<td>0.08 ± 0.02</td>
<td>23.66 ± 0.09</td>
<td>32.01 ± 2.03</td>
</tr>
<tr>
<td>powder</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dialyzed SWPP</td>
<td>66.52 ± 0.35</td>
<td>7.15 ± 0.35</td>
<td>1.38 ± 0.03</td>
<td>3.33 ± 0.53</td>
<td>18.65 ± 1.32</td>
</tr>
<tr>
<td>powder</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SWPI powder</td>
<td>79.68 ± 1.25</td>
<td>5.84 ± 0.24</td>
<td>1.15 ± 0.08</td>
<td>2.85 ± 0.25</td>
<td>6.04 ± 0.19</td>
</tr>
</tbody>
</table>

*Data are the average of three determinations*

Table 2.1 also shows that undialyzed SWPP has significantly less protein and more sugars. The proteins might have been coagulated during the formation of tofu, which reduced their amount in the whey. However, most of the sugars were leached out during tofu formation that contributed to their higher amount in the whey. In dialyzed SWPP, during dialysis, small molecules such as amino acids, peptides, organic acids, minerals and sugars were dialyzed out thus enabling concentration of protein.
The amino acids composition (g/100 g of protein) of SPI, SWPI, dialyzed and undialyzed SWPPs are shown in Table 2.2. All SWPs were rich in glutamic acid, aspartic acid, serine, arginine, proline, lysine and leucine. In comparison, the total amino acids of SWPPs were lower than that of SPI (USDA National Nutrient Database for Standard Reference 2011). The proportions of essential amino acids as well as hydrophobic amino acids were also lower compared to SPI (USDA National Nutrient Database for Standard Reference 2011).
Some of the amino acids were totally or partially lost during acid hydrolysis. Tryptophan was totally lost during acid hydrolysis while methionine, threonine, serine and cysteine are progressively destroyed during acid hydrolysis (Smith, 2010). These contributed to only 79% of amino acids being calculated in the samples. Asparagine and glutamine were converted to aspartic acid and glutamic acid during acid hydrolysis, which contributed to higher amount of aspartic acid and glutamic acid in the samples (Smith, 2010).

2.3.1.3 SDS-PAGE analysis

Figure 2.1 shows the SDS-PAGE electrophoretogram of SWPI, SPI, undialyzed and dialyzed SWPPs. The protein components of SPI composed mainly of two major fractions: \( \beta \)-conglycinin (7S) and glycinin (11S) (Thanh & Shibashaki, 1976). \( \beta \)-conglycinin consisted of \( \alpha' \), \( \alpha \) and \( \beta \) subunits (Thanh & Shibasaki, 1977; Davies, Coates, & Nielsen, 1985) while glycinin consisted of acidic and basic subunit (Nielsen, 1985). \( \beta \)-conglycinin and glycinin fractions account for 36.84% and 41.01% respectively (Table 3). Depending on seed genotype, these two fractions account for 65-80% of the total seed protein (Keerati-U-Rai & Corredig 2009a).

The protein band of SDS-PAGE for SWPPs either undialyzed, dialyzed or SWPI show a slight difference from SPI. The amount of \( \alpha' \), \( \alpha \) and \( \beta \) of \( \beta \)-conglycinin for SWPI, undialyzed and dialyzed SWPPs were much lower compared to SPI (Table 2.3). The amount of \( \alpha' \) subunit was 2.29 and 2.93% in undialyzed and dialyzed SWPPs respectively compared to 12.93% in SPI while \( \alpha' \) subunit present in negligible amount in SWPI. Meanwhile, \( \alpha \) subunit of \( \beta \)-conglycinin constituted 2.51, 4.02 and 4.64% in SWPI, undialyzed and dialyzed SWPPs respectively compared to 13.61% in SPI. \( \beta \) subunit of \( \beta \)-conglycinin present in negligible amount in
undialyzed SWP, while SWPI and dialyzed SWP contained 6.12 and 3.05% β subunit of β-conglycinin respectively. The amount of β subunit of β-conglycinin in SPI was 10.3%.

![Image of SDS-PAGE electrophoretogram]

Figure 2-1. SDS-PAGE electrophoretogram of SWPPs. The labeled lanes are (M) protein molecular weight standards; (1) undialyzed SWP; (2) dialyzed SWP; (3) SWPI; and (4) SPI.

The amount of acidic subunit of glycinin fraction in SWPI, undialyzed and dialyzed SWPPs were 23.12, 15.51 and 16.02% respectively. The amount was much lower compared to its amount in SPI, which contained 30.87% of acidic subunit. In contrast to SPI, SWPPs (undialyzed, dialyzed and SWPI) exhibited a pronounced protein band at molecular weight between 20 – 30 kDa. These protein constituents contributed 43.17, 43.68, and 46.32% for SWPI, undialyzed and dialyzed SWPPs respectively. In addition, the amount of β amylase was higher in undialyzed and dialyzed SWPs with an amount of 18.40 and 13.53% respectively. β amylase content in SWPI and SPI was 3.0 and 2.33% respectively.
Table 2-3 Protein composition (%) of SWPI, SPI, undialyzed and dialyzed SWPPs a

<table>
<thead>
<tr>
<th>Protein constituent</th>
<th>Undialyzed SWPP (%)</th>
<th>Dialyzed SWPP (%)</th>
<th>SWPI (%)</th>
<th>SPI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7S α’ sub.</td>
<td>2.29</td>
<td>2.93</td>
<td>-</td>
<td>12.93</td>
</tr>
<tr>
<td>7S α sub.</td>
<td>4.02</td>
<td>4.64</td>
<td>2.51</td>
<td>13.61</td>
</tr>
<tr>
<td>7S β sub.</td>
<td>-</td>
<td>3.05</td>
<td>6.12</td>
<td>10.30</td>
</tr>
<tr>
<td>11S Acidic sub. (A1, A2, A3, A4, A5)</td>
<td>15.51</td>
<td>16.02</td>
<td>23.12</td>
<td>30.87</td>
</tr>
<tr>
<td>11S Basic sub. (B1, B2, B3, B4)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10.14</td>
</tr>
</tbody>
</table>

Other protein constituents

<table>
<thead>
<tr>
<th></th>
<th>Undialyzed SWPP (%)</th>
<th>Dialyzed SWPP (%)</th>
<th>SWPI (%)</th>
<th>SPI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipoxygenase</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3.24</td>
</tr>
<tr>
<td>β amylase</td>
<td>18.40</td>
<td>13.53</td>
<td>3.00</td>
<td>2.33</td>
</tr>
<tr>
<td>γ of γ conglycinin</td>
<td>7.17</td>
<td>7.06</td>
<td>4.31</td>
<td>5.68</td>
</tr>
<tr>
<td>21-30 kDa</td>
<td>43.68</td>
<td>46.32</td>
<td>43.17</td>
<td>4.96</td>
</tr>
</tbody>
</table>

aData are the average of three determinations

As shown in Figure 2.1 and Table 2.3 there is a significant protein bands at ~30-20 kDa for undialyzed, dialyzed and SWPI. During tofu processing soy milk was heated up to 95°C and then heated up again at 75°C before calcium sulfate was added for the formation of tofu. Heating of soy protein solution has a great effect on soy protein components which caused the appearance of a significant amount of these protein bands. Heating dilute solution of water extractable proteins aggregates 11S and 15S fractions plus a part of 7S fraction; this was indicated by ultracentrifugation and gel filtration (Saio, Wakabayashi, & Watanabe, 1968; Watanabe & Nakayama, 1962). Wolf & Tamura (1969) reported more detailed studies on the effects 11S soy protein. Heating 0.5% solutions of 11S protein at 100°C (pH 7.6, 0.5 ionic strength) caused them to become turbid, and protein precipitation was observed. The 11S component disappeared after heating for less than 5 minutes, and a soluble protein aggregate of 80S-100S appeared. Upon continued heating, the soluble aggregate was observed to increase in size prior complete precipitation after about 7 minutes. Disappearance of the 11S component was accompanied by formation of a slow-sedimenting 3S-4S component and the transient appearance
of a 7S intermediate. The concentration of the 3S-4S fraction reached a maximum after 5-7 minutes heating and appeared to be stable for 30 minutes of heating.

2.3.1.4 HPSEC analysis

The molecular weight distribution of undialyzed, dialyzed and SWPI powder are shown in Figure 2.2. Refractive index chromatogram of HPSEC distribution showed that soy whey powder had two groups of molecular weight. One group was at lower molecular weight region and the other group was a little bit higher. Dialyzed soy whey powder contained both high and low molecular weight proteins (retention volume 17 mL to 21 mL) while undialyzed soy whey powder only contained low molecular weight protein (retention volume around 20 mL to 21 mL). SWPI powder has a higher molecular weight protein (retention volume 16.8 mL to 19.8 mL).

Ultraviolet (UV) spectrum of HPSEC was used to confirm the presence of protein in the SWPs. As shown in Figure 2.3, the protein was clearly seen in UV chromatogram. A broad protein spot was clearly seen in UV chromatogram of dialyzed SWPP as parallel to the broad peak in RI detector. UV spectrum of undialyzed SWPP is located at low molecular weight region, while the UV spectrum of SWPI is located at high molecular weight region. High molecular weight protein was not seen in the UV spectrum of undialyzed SWPP. As the amount of biomass in SWPI, which contained high molecular weight proteins was only 10% of undialyzed SWPP, hence, the proportion of high molecular weight protein in undialyzed soy whey powder was much lower, which could not be seen in UV spectrum.
Figure 2-2. Refractive index spectrum of HPSEC of SWPI, dialyzed and undialyzed SWPPs.

Figure 2-3. UV spectrum of HPSEC of undialyzed SWPP (A), dialyzed SWPP (B) and SWPI (C).
2.3.1.5 FTIR analysis

The amide bands that arise from the vibration of the peptide groups provide information on the secondary structure of polypeptides and proteins (Haris & Severcan, 1999). Analysis of the peptide group vibration in model compounds and in polypeptide systems allows assignment of these characteristic bands. The polypeptide and protein repeat unit give rise to nine characteristic IR absorption bands, namely, amide A, B and I-VII (Krimm & Bandekar, 1986; Susi & Byler, 1986; Surewich & Mantsch, 1988). The amide I band (1700-1600 cm\(^{-1}\)), which is the most sensitive spectral region to the protein secondary structural components arises almost entirely from the C=O stretching vibration of the peptide group. The amide II band (1480-1575 cm\(^{-1}\)) is primarily N–H bending and a contribution from C–N stretching vibrations (Krimm & Bandekar, 1986). The amide III band (1229-1301 cm\(^{-1}\)) also derives mainly from NH bending and CN stretching vibration. The amide III absorption is normally very weak in the infrared.

The amide I and II overlapped in a broad spectrum of undialyzed SWPP at wavenumber 1480-1700 cm\(^{-1}\) (Figure 2.4A) while small peak was observed at wavenumber 1229-1301 cm\(^{-1}\) for amide III. The amide I, II and III are more pronounced in dialyzed and SWPI powders (Figure 2.4B and 2.4C). It was due to the higher protein concentration in dialyzed and SWPI powders that contributes to the stronger amide bands. One IR marker of sugars is located within the fingerprint region of carbohydrate, between 1000 and 1200 cm\(^{-1}\) (Khajehpour, Dashnau, & Vanderkooi, 2006). At this region, absorbance arises from composite modes of vibration of sugar ring. An intense peak was observed in finger print region of undialyzed SWPP as well as dialyzed SWPP indicating the presence of sugar molecules or oligosaccharides. As confirmed by proximate analysis both powders contained higher amount of sugar. The intensity in finger print
region of undialyzed SWP is higher compared to dialyzed SWPP. Due to low sugar content in SWPI powder the intensity at finger print region is low.

![FTIR spectrum of undialyzed SWPP (A), dialyzed SWPP (B) and SWPI (C).](image)

**Figure 2-4. FTIR spectrum of undialyzed SWPP (A), dialyzed SWPP (B) and SWPI (C).**

2.3.1.6 Surface tension

The concentration dependence of surface tension for SPI, SWPI, dialyzed and undialyzed SWPs is presented in Figure 2.5. The measurement of surface tension was carried out at the same protein concentration and 2 h after the solution was kept standing to enable the orientation of protein molecules at the interface. All SWPPs demonstrated the ability to reduce the surface tension of pure water. As the protein concentration of SWPPs is increased, the surface tension falls until it reaches a relatively constant level at protein concentration of 0.18% and no more reduction of surface tension was observed on increasing the concentration of SWPPs. At this
concentration the interface has become saturated with the solutes. However, with increasing
concentration of undialyzed SWPP, the surface tension was further reduced.

In comparison, SPI reduced the surface tension to slightly lower values than SWPI,
which indicates better surface activity. As shown in Table 2.2, SWPI contained lower amount of
hydrophobic amino acids as compared to SPI. Dialyzed and undialyzed SWPPs reduced the
surface tension of pure water to much lower values as compared to SPI and SWPI. The ability of
undialyzed and dialyzed SWPPs to reduce the surface tension lower than SPI and SWPI might be
due to the presence of low molecular weight proteins in the dialyzed and undialyzed SWPPs and
trace amount of saponins. As shown in UV spectrum of HPSEC (Figure 2.3), undialyzed SWPP
contained low molecular weight proteins and dialyzed SWPP contained both low and high
molecular weight proteins. During dialysis, most of the minerals and some sugars were leached
out enabling the concentration of proteins.

Based on theoretical considerations, it has been pointed out that pure proteins cannot
decrease the surface tension of water below 50 dynes/cm (Damodaran, 2004). This prediction is
borne out by the fact that the minimum surface tension that even the most surface active protein
β-casein can achieve is only about 50 dynes/cm. The inability of protein to greatly reduce the
interfacial tension is related to their complex structural properties (Damodaran, 2005). However,
in another study, Kitabatake & Doi (1982) reported that ovalbumin, soybean protein, bovine
serum albumin and casein have different capabilities of reducing surface tension. The surface
tension values of ovalbumin, soybean protein, bovine serum albumin and casein were 44.0, 42.2,
48.6 and 46.5 dynes/cm respectively at 1% protein concentration.
2.3.2 Emulsifying properties of SWPPs

To optimize the concentration of soy whey powder for emulsion stability, a different concentration of SWPI was used to prepare the oil in water emulsions (10% canola oil). A typical scan of the average droplet size profiles of SWPI emulsions as a function of emulsifier concentration is shown in Figure 2.6. As expected, increasing the concentration of SWPI decreases the average droplet size. At the concentration of 0.8%, fine O/W emulsions of canola oil can be made with average droplet size, $d_{43}$ of about 1.21 μm. There was no significant difference of average droplet size, $d_{43}$ on storage for up to 14 days at 25°C.
Figure 2.6. Average droplet size (d_{43}) of canola oil (10%) emulsified with SWPI at 0.1%, 0.2%, 0.4% and 0.8% concentration at pH 7.0 as a function of storage time from 0 - 14 days at 25°C. Data are the average of three determinations.

Figure 2.7 shows the average droplet size, d_{43} of 10% canola oil emulsified with SPI, SWPI, undialyzed and dialyzed SWPPs on storage up to 14 days at the same protein concentration (0.72%). It was noted that, the average droplet size, d_{43} of oil globules emulsified with undialyzed soy whey powder shows the highest average droplet size, d_{43} on storage from 1 up to 14 days. As indicated by the highest average droplet size, undialyzed SWPP is the weakest emulsifier compared to the dialyzed and SWPI powder. The results were parallel to the finding by Chove, Grandison, & Lewis (2001) who reported that soy whey was found to have poor emulsification properties. Emulsions stabilized by dialyzed SWPP showed a smaller droplet size as compared to undialyzed SWPP but slightly bigger than stabilized by SWPI. SWPI was the best emulsifier as indicated by the lowest average droplet size on storage up to 14 days at 25°C.
There was no significant difference of average droplet size for SWPI and SPI as a function of storage time from 0 to 14 days of storage at 25°C.

**Figure 2-7.** Average droplet size ($d_{43}$) of canola oil (10%) emulsified with 0.72% protein of SPI, SWPI, undialyzed and dialyzed SWPPs at pH 7.0 as a function of storage time from 0 - 14 days at 25°C. Data are the average of three determinations.

Despite the observation that dialyzed and undialyzed SWPPs could reduce the surface tension much lower than SPI and SWPI, there is a big difference of the capability of the protein powders in stabilizing oil-in-water emulsions. It might be due to a difference in adsorption of protein at air-water and oil-water interfaces. In the current study, the variation in protein content and molecular weight may have contributed to the variations in emulsion stability. As shown in Figure 2.3, the undialyzed SWPP mainly contained low molecular weight proteins; the dialyzed SWPP contained both high molecular weight and low molecular weight proteins while the SWPP isolate mostly contained high molecular weight proteins. Our understanding is that the low
surface tension in undialyzed and dialyzed SWPs were mainly coursed by the presence of trace amount of saponins and low molecular weight proteins; however, both did not contribute to the long term emulsion stabilization of the oil/water emulsions in the present study. It was the high molecular weight protein fraction found in SWPI contributed to the long term stability of the emulsions.

The stability of an oil/water emulsion is influenced by many factors, including the composition and physicochemical properties of both the oil and aqueous phase (Phipps, 1985; Walstra & Smulder, 1998). The aqueous phase of an emulsion may contain a wide variety of components, including minerals, acids, bases, biopolymers, sugars, alcohols, and gas bubbles. Many of these components will alter the size of the droplets produced during homogenization because of their influence on rheology, interfacial tension, coalescence stability, or adsorption kinetics (McClements, 2005b). Proximate composition (Table 2.1) showed that undialyzed SWPP contained the highest amount of ash (minerals) and sugars. Dialyzed SWPP also showed a considerable amount of sugars. In many food emulsions, droplet coalescence may be promoted by the presence of solid particles or crystals due to their ability to disrupt the thin film separating the droplets, for example fat, ice, sugar, or salts crystals (McClements, 2005b).

2.4 Conclusion

SWPI powder contained the highest amount of protein, followed by dialyzed SWPP. The protein band of SDS-PAGE showed that SWPPs were slightly different from SPI. SWPI powder has a higher molecular weight protein, undialyzed SWPP contained low molecular weight protein while dialyzed SWP contained both high and low molecular weight protein. SPI and SWPI exhibited better emulsifying properties compared to undialyzed and dialyzed SWPPs.
3. CONJUGATION AND VERIFICATION OF FENUGREEK GUM – SOY WHEY PROTEIN ISOLATE

3.1 Introduction

Protein-polysaccharide interactions play a significant role in the structure and stability of many processed foods. The most permanent form of protein-polysaccharide interaction that occurs during complex formation is covalent bonding. This kind of interaction is often achieved by the chemical reaction of amino groups from the proteins and carbonyl groups from the polysaccharides, to give an amide covalent bond (Stainsby, 1980). One of the most promising methods is to apply the naturally occurring Maillard reaction (Shepherd et al., 2000). Maillard reaction can be divided into three stages; initial, intermediate and advanced. The initial stage involves the condensation of the carbonyl group of the reducing sugar with the available ε-amino group of the protein, resulting in an Amadori product being produced via the formation of a Schiff base with the release of water and the Amadori rearrangement (Ames, 1992).

The initial stage of this reaction has been shown to be sufficient for conjugation between protein and polysaccharide via the formation of a covalent bond, which has been shown to be very stable to changes in pH, ionic strength and temperature (Dickinson & Euston, 1991; Schmitt et al., 1998). These conjugates have been shown to possess improved functional properties, including enhanced emulsifying properties (Akhtar & Dickinson, 2007; Shepherd et al., 2000), increased solubility particularly around the isoelectric pH of the protein (O’Regan & Mulvihill, 2009; Chevalier et al., 2001) and increased heat stability compared to protein itself (Aoki et al., 1999; Chevalier et al., 2001).

The aim of this work was to conjugate soy whey protein isolate (SWPI) to fenugreek gum (partially hydrolyzed and unhydrolyzed) and to verify the extent of conjugation by means of SDS-PAGE analysis, High Performance Size Exclusion Chromatography (HPSEC) and Fourier Transform Infrared (FTIR). The effect of hydrolysis on monosaccharide composition using High Performance Anion-exchange with Pulsed Amperometric Detection (HPAEC-PAD) was also investigated.

3.2 Materials and methods

3.2.1 Materials

Soybean was obtained from Goudas Food Products Co. Ltd., Concord, ON, Canada L4K 2I8. Crude fenugreek gum (Canafen Gum®) was supplied by Emerald Seed Products, Avonlea, SK, Canada. Ethanol was obtained from Commercial Alcohols Inc., Montreal, QC, Canada. All chemicals used in this study were of analytical grade unless otherwise specified. Canola oil was purchased from the local supermarket.

3.2.2 Purification of fenugreek gum

Crude fenugreek gum (5 g) was dissolved in 1 L of water at 80°C in a water bath for 6 h, followed by stirring at room temperature overnight. The dissolved gum was centrifuged (Sorvall RC5C Plus; Mandel Scientific Co., Ltd., Edmonton, AB, Canada) for 15 min, at 15,180 g. Fenugreek gum was precipitated by adding 2.3 L of 100% ethanol into the supernatant. The mixture was centrifuged for 15 min, at 15,180 g, and the supernatant decanted. The residue was re-dissolved in ultrapure water and freeze dried. The resulting gum was termed purified fenugreek gum.
3.2.3 Isoelectric precipitation of protein from the whey of tofu processing (SWPI)

Isoelectric precipitation of proteins was carried out as described in section 2.2.3.1 and 2.2.3.2.

3.2.4 Preparation of the conjugates

3.2.4.1 Partial hydrolysis of fenugreek gum

Fenugreek gum solution (0.3%) was partially hydrolyzed using 0.05 M HCl at 90°C for 10 min (HD10), 30 min (HD30) or 50 min (HD50). The partially hydrolyzed fenugreek gum was then neutralized with 0.5 M NaOH to pH 7 after which it was dialyzed (6 – 8 kDa molecular weight cut off) for 24 h at 4°C to remove small molecule sugars. After dialysis the partially hydrolyzed fenugreek gum was subjected to High Performance Size Exclusion Chromatography (HPSEC) analysis to confirm the removal of small molecule sugars.

3.2.4.2 Preparations of soy whey protein isolate (SWPI) – Fenugreek gum conjugates

Protein and polysaccharide were brought into contact by dissolving the SWPI and fenugreek gum (hydrolyzed and unhydrolyzed) in distilled water at a weight ratio of 1:3. SWPI was chosen to be conjugated to fenugreek gum since SWPI exhibited the best emulsifying properties compared to dialyzed and undialyzed SWPPs. The solution was then freeze dried, milled to make a powder and sieved (80 mesh). A desiccator containing saturated NaCl was placed in the oven at 60°C for 30 min to achieve equilibrium temperature and relative humidity (75%). The dried polymer admixture was incubated at 60°C for 3 days after which the conjugates were freeze dried and stored at 4°C until further use. After incubation at 60°C for 3 days, a light brownish color appeared due to non-enzymatic browning.
3.2.5 Removal of unreacted protein from SWPI – Fenugreek gum conjugates

About 0.4 g conjugates were dispersed in 100 mL distilled water and stirred for 2 h. The dispersion was adjusted to pH 4.5 with 2 N HCl enabling the precipitation of unreacted protein. The precipitate was separated by centrifugation at 17,210 g for 30 min at 4°C and the supernatant was collected. The supernatant was adjusted to pH 7.0 with 2 N NaOH. The solution was dialyzed overnight at 4°C and freeze dried. The unreacted protein from SWPI – Fenugreek gum conjugates was further removed by precipitating conjugate solution in ethanol at different concentration. Conjugates (0.1 g) were dissolved in 100 mL distilled water. Ethanol was added to make a final concentration of ethanol in solution of about 40, 60 and 80%. The solution was stirred for 2 h and stored at 4°C overnight to precipitate the high molecular polymer and centrifuged at 17,210 g for 30 min. The supernatant was discarded and the precipitate was re-dissolved in ultrapure water, stored at -20°C overnight and freeze dried.

3.2.6 Verification of the conjugates

3.2.6.1 SDS-PAGE analysis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970) using 12% (w/v), acrylamide separating gel and a 4% (w/v), stacking gel both containing 0.1% (w/v), SDS. SWPI – Unhydrolyzed fenugreek mixture and conjugate or SWPI-partially hydrolyzed mixture and corresponding conjugates (1.5%, w/v) were prepared in 0.0625 M Tris-HCl buffer (pH 6.8) containing 2% (w/v), SDS, 5% (w/v), 2-mercaptoethanol and 0.012% (w/v), bromophenol blue and heated at 95°C for 4 min prior to loading in the gel slots. Electrophoresis was carried out at a constant voltage (200 V) for 45 min using a tris glycine buffer (pH 8.3) containing 0.125% (w/v), SDS.
The gel was removed from the electrophoresis unit, stained with Coomassie brilliant blue R-250 and de-stained in 50:10:40 methanolic:acetic acid:water.

3.2.6.2 Determination of protein content

Protein content was determined from nitrogen content (N X 6.25) using a ThermoQuest Italia S.P.A. EA/NA 1110 Automatic Elemental Analyzer (Strada Rivoltana, Milan, Italy).

3.2.6.3 HPSEC analysis

HPSEC analysis was carried out as described in section 2.2.3.3.3.

3.2.6.4 FTIR analysis

FTIR analysis was carried out as described in section 2.2.3.3.4.

3.2.7 Monosaccharide composition

Monosaccharide composition of unhydrolyzed and hydrolyzed fenugreek gum was determined by hydrolyzing samples in 1 M H₂SO₄ at 100°C for 3 h followed by HPAEC-PAD analysis. The samples were hydrolyzed by 1 M H₂SO₄ at 100°C for 3 h and diluted 10 times. The diluted samples were passed through a 0.45 μm filter and injected to a high-performance anion-exchange with pulsed amperometric detection (HPAEC-PAD) (Dionex-550, Dionex Corporation, Canada). Separations were achieved with isocratic eluent (100 mM NaOH) on a CarboPac A1 column (250 x 4 mm I.D., Dionex Corporation, Canada) and a guard column (3 x 25 mm, Dionex Corporation, Canada) at a flow rate of 1.0 mL/min. The column system was cleaned after each analysis with 300 mM NaOH for 30 min. A post-column delivery system of 600 mM NaOH with a flow rate of 1.0 mL/min was added to the HPAEC-PAD system. The
instrument was controlled and data was processed using Dionex A1 450 software (Dionex Corporation, Canada).

3.3 Results and discussion

3.3.1 SDS-PAGE profiles of the conjugates

In order to establish that covalent coupling of SWPI to fenugreek gum had indeed occurred during the heat treatment, SDS-PAGE was conducted as previously reported in studies of other protein-polysaccharide conjugate systems (Akhtar & Dickinson, 2007; O’Regan and Mulvihill, 2009; Yadav, Parris, Johnston, Onwulata, & Hicks, 2010). The protein band of SDS-PAGE for SWPI showed a slight difference from SPI (Kasran, Cui & Goff, 2012, submitted) where the amount of α’, α and β of β-conglycinin for SWPI was much less compared to soy protein isolate (SPI). In addition, SWPI only contained acidic subunit of glycinin fraction. However, SWPI exhibited a pronounced protein band at molecular weight between 20 – 31 kDa.

Figure 3-1 SDS-PAGE electrophoretogram of SWPI, SWPI – Fenugreek gum mixture (1:3) and SWPI-Fenugreek gum conjugate (1:3), 60°C, 3 days. The labeled lanes are (M) protein molecular weight standards; (1) SWPI – Fenugreek gum mixture; and (2) SWPI – Fenugreek gum conjugate.
The SDS-PAGE of SWPI-fenugreek gum mixture and conjugate, stained for protein with Coomassie brilliant blue showed polydispersed bands at the top of the separating gel in the conjugate suggesting the formation of high molecular weight products (Figure 3.1, lane 2). A portion of the conjugate also remained immobile in the beginning of the stacking gel, which suggested the presence of high molecular weight conjugated protein that was too large to penetrate the gel. The characteristics of 7S fraction disappeared on conjugation with fenugreek gum, with a distinct shift to a higher molecular weight. The acidic subunits of the 11S fractions also disappeared. The amount of protein at molecular weight 30 kDa of the SWPI-fenugreek gum conjugate remained unchanged as compared to SWPI-fenugreek gum mixture (Table 3.1). However, in parallel with the disappearance of protein band at molecular weight 21 and 24 kDa, the amount of protein at these molecular weights was much less compared to SWPI-fenugreek mixture.

**Table 3-1 Protein composition (%) of SWPI-Fenugreek gum (1:3) mixture and conjugate (60°C, 3 days)**

<table>
<thead>
<tr>
<th>Protein constituents</th>
<th>SWPI-Fenugreek gum mixture (%)</th>
<th>SWPI-Fenugreek gum conjugate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High Mwt. protein</td>
<td>3.78 ± 0.88</td>
<td>44.32 ± 0.37</td>
</tr>
<tr>
<td>7S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α'</td>
<td>2.58 ± 0.35</td>
<td>0</td>
</tr>
<tr>
<td>α</td>
<td>4.38 ± 0.35</td>
<td>0</td>
</tr>
<tr>
<td>β</td>
<td>1.91 ± 0.03</td>
<td>0</td>
</tr>
<tr>
<td>β Amylase</td>
<td>4.31 ± 0.32</td>
<td>11.67 ± 0.93</td>
</tr>
<tr>
<td>11S acidic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A₁</td>
<td>4.56 ± 0.78</td>
<td>0</td>
</tr>
<tr>
<td>A₄</td>
<td>3.29 ± 0.06</td>
<td>0</td>
</tr>
<tr>
<td>A₁a₂, A₁b₂, A₂</td>
<td>9.87 ± 0.57</td>
<td>0</td>
</tr>
<tr>
<td>11S basic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B₁a, B₁b, B₂, B₃, B₄</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 kDa</td>
<td>24.43 ± 0.44</td>
<td>24.55 ± 1.35</td>
</tr>
<tr>
<td>24 kDa</td>
<td>15.95 ± 0.18</td>
<td>4.07 ± 0.44</td>
</tr>
<tr>
<td>21 kDa</td>
<td>7.40 ± 0.40</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 3-2 SDS-PAGE stained for protein with Coomassie brilliant blue R-250. The labeled lanes are (M) molecular weight marker; (1) SWPI – Fenugreek (1:3) mixture; (2) SWPI – Fenugreek (1:3) conjugate; (3) SWPI – HD10 (1:3) mixture; (4) SWPI – HD10 (1:3) conjugate; (5) SWPI – HD30 (1:3) mixture; (6) SWPI – HD30 (1:3) conjugate; (7) SWPI – HD50 (1:3) mixture; (8) SWPI – HD50 (1:3) conjugate.

The SDS-PAGE of SWPI-partially hydrolyzed and unhydrolyzed fenugreek gum mixture and conjugates, stained for protein with Coomassie brilliant blue R-250, is shown in Figure 3.2. The SDS-PAGE patterns show polydispersed bands at the top of the separating gel in the conjugate suggesting the formation of high molecular weight products (lane 2, 4, 6 and 8). Comparing unhydrolyzed and partially hydrolyzed fenugreek gum, it was observed that the intensity of polydispersed band at the top of the separating gel was more pronounced in the partially hydrolyzed fenugreek gum particularly after 50 min of hydrolysis. The band at molecular weight 30 kDa of the unhydrolyzed fenugreek gum gradually disappeared as the
hydrolysis time was increased although it did not fully disappear. Some unreacted proteins were still observed.

### 3.3.2 HPSEC

To further confirm that conjugation had occurred, HPSEC of SWPI-fenugreek gum conjugates was performed. The RI and UV spectrum were used to confirm if there were any differences before and after conjugation. Figure 3.3 shows an example of RI spectrum of HPSEC profiles for SWPI – Partially hydrolyzed fenugreek gum (90°C, 30 min) mixture and conjugates. The chromatogram shows a reduction of protein peak of unconjugated mixture and shifted a peak to a higher molecular weight of the conjugates on conjugation between SWPI and fenugreek gum.

![Figure 3-3 Refractive index (RI) spectrum of HPSEC profiles for SWPI – HD30 (1:3) mixture and conjugate (60°C, 3 days).](image)
Figure 3.4 shows the UV spectrum of HPSEC profiles for SWPI – Fenugreek gum (1:3) mixture and conjugates of hydrolyzed and unhydrolyzed fenugreek gum. The differences of UV spectrum were observed at retention volume of 14 mL to 17.5 mL of the chromatogram. The peaks at retention volume 17.5 mL to 21 mL were protein peaks that were not interacted to polysaccharide. The chromatograms showed that after conjugation, the peak intensity at retention volume 14 mL to 17.5 mL was slightly increased indicating that conjugation had taken place. The intensity of the peaks increases from chromatogram A to D. The results indicate that as the hydrolysis time is increased more reaction between SWPI and fenugreek gum is observed. Hydrolysis reduces the chain length of fenugreek gum and produces more reducing end, which
then interacts with protein. The intensity of the peak was more pronounced after 50 min of hydrolysis.

![Figure 3-5 UV and RI spectrum of HPSEC of SWPI – Unhydrolyzed fenugreek gum conjugate (A) without precipitation with ethanol, (B) precipitated at 40% ethanol, (C) precipitated at 60% ethanol and, (D) precipitated at 80% ethanol.](image)

As shown in Figure 3.4 some proteins were not reacted to fenugreek gum during Maillard reaction. These unreacted proteins should be removed to determine the amount of proteins reacted to polysaccharide. The unreacted proteins were removed by dispersing the conjugates in ethanol at different ethanol concentration. Figure 3.5 shows the UV spectrum of HPSEC of the conjugates after ethanol treatment. It was observed that at 40 and 60% ethanol concentrations the unreacted proteins was totally removed. However, at 80% ethanol concentration, the unreacted proteins were not removed and remained in the conjugates. Table 3.2 shows the protein content
of the conjugates before and after the proteins were removed. SWPI – Fenugreek gum unconjugated mixture contained about 23% proteins. After precipitation at pH 4.5, the SWPI – Fenugreek gum conjugates contained 12.93% protein. After treatment of the conjugates in 40 and 60% ethanol, the proteins were further reduced to 10.37 and 10.42% respectively. These values equivalent to 45% of the proteins interacted to polysaccharide. However, there was no significant difference of protein content precipitated at 80% ethanol as compared to the conjugates without ethanol precipitation.

Table 3-2 Protein content of SWPI – Unhydrolyzed Fenugreek gum mixture and SWPI – Unhydrolyzed fenugreek gum conjugates with and without ethanol precipitation

<table>
<thead>
<tr>
<th>Protein content (%)</th>
<th>SWPI – Fenugreek gum unconjugated mixture</th>
<th>SWPI – Fenugreek gum conjugate without ethanol precipitation</th>
<th>SWPI – Fenugreek gum conjugate (precipitated at 40% ethanol)</th>
<th>SWPI – Fenugreek gum conjugate (precipitated at 60% ethanol)</th>
<th>SWPI – Fenugreek gum conjugate (precipitated at 80% ethanol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>23.09 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.93 ± 0.21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.37 ± 0.16&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10.42 ± 0.22&lt;sup&gt;c&lt;/sup&gt;</td>
<td>12.82 ± 0.32&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are the average of three determinations. Means with different letter are significantly different (P < 0.05).

The molecular weight of SWPI – Fenugreek gum mixture and conjugates of hydrolyzed and unhydrolyzed fenugreek gum is shown in Table 3.3. Comparing the molecular weight of unhydrolyzed and partially hydrolyzed fenugreek gum, as the time of hydrolysis is increased the molecular weight decreased. The results also showed that the molecular weight of SWPI – Unhydrolyzed fenugreek gum conjugate was also decreased. The decrease of molecular weight might be due to the degradation of fenugreek gum during heating. Although there was evidence that protein had conjugated to fenugreek gum, the amount of protein that had conjugated to
fenugreek gum was too small compared to the degraded fenugreek gum. The molecular weight of SWPI – Fenugreek gum conjugate was 6.43 x 10^5 Da compared to 1.56 x 10^6 Da of the unconjugated SWPI – Fenugreek gum. However, incubating SWPI with hydrolyzed fenugreek gum increased the molecular weight of the conjugates. The intrinsic viscosity and hydrodynamic radius of SWPI – Unhydrolyzed fenugreek gum conjugate was significantly decreased as compared to their corresponding mixture. The intrinsic viscosity of SWPI – Partially hydrolyzed fenugreek gum conjugates were also decreased but not significantly different from their corresponding mixtures. Meanwhile, the hydrodynamic radius of SWPI – Partially hydrolyzed fenugreek gum conjugates were slightly increased but not significantly different from their corresponding mixtures.

Table 3-3 Molecular weight, intrinsic viscosity and hydrodynamic radius of unhydrolyzed and hydrolyzed fenugreek gum [0.05 M HCl, 90°C for 10(HD10), 30(HD30) and 50(HD50) min] – SWPI mixture and conjugates (60°C, 3 days)

<table>
<thead>
<tr>
<th></th>
<th>Molecular weight (Daltons)</th>
<th>Intrinsic viscosity (dl/g)</th>
<th>Hydrodynamic radius (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SWPI – Fenugreek gum 1:3 mixture</td>
<td>1.56 x 10^6 ± 41719^a</td>
<td>7.95 ± 0.34^a</td>
<td>56.09 ± 0.33^a</td>
</tr>
<tr>
<td>SWPI – Fenugreek gum 1:3 conjugate</td>
<td>6.43 x 10^5 ± 39904^b</td>
<td>4.26 ± 0.11^b</td>
<td>33.41 ± 1.05^b</td>
</tr>
<tr>
<td>SWPI – HD10 1:3 mixture</td>
<td>2.49 x 10^5 ± 18572^d</td>
<td>1.94 ± 0.04^e</td>
<td>17.85 ± 0.61^c</td>
</tr>
<tr>
<td>SWPI – HD10 1:3 conjugate</td>
<td>3.10 x 10^5 ± 2458^c</td>
<td>1.91 ± 0.04^e</td>
<td>19.00 ± 0.71^c</td>
</tr>
<tr>
<td>SWPI – HD30 1:3 mixture</td>
<td>1.08 x 10^5 ± 6146^f</td>
<td>0.88 ± 0.04^d</td>
<td>10.10 ± 0.25^d</td>
</tr>
<tr>
<td>SWPI – HD30 1:3 conjugate</td>
<td>1.71 x 10^5 ± 14.85^e</td>
<td>0.84 ± 0.08^d</td>
<td>11.64 ± 0.15^d</td>
</tr>
<tr>
<td>SWPI – HD50 1:3 mixture</td>
<td>6.79 x 10^4 ± 815^h</td>
<td>0.63 ± 0.03^e</td>
<td>8.12 ± 0.12^e</td>
</tr>
<tr>
<td>SWPI – HD50 1:3 conjugate</td>
<td>8.31 x 10^4 ± 3912^g</td>
<td>0.62 ± 0.07^e</td>
<td>8.29 ± 0.04^e</td>
</tr>
</tbody>
</table>

Means with the different letters are significantly different (P < 0.05).
3.3.3 FTIR

Infrared spectroscopy is one of the oldest and well established experimental techniques for the analysis of secondary structure of polypeptides and protein. It can be used to establish the presence of protein-polysaccharide conjugation through Maillard reaction. The initial stage of this reaction has been sufficient for conjugation to be achieved between the protein and polysaccharide via the formation of covalent bond. During the reaction, an amide bond is formed through condensation between the amino group of amino acids (terminal and intrastitial amines) or protein and the carbonyl group of reducing sugars (Shepherd et al., 2000).

![FTIR spectrum of SWPI – Fenugreek gum conjugates](image)

**Figure 3-6 FTIR spectrum of SWPI – Fenugreek gum conjugates (A) Before removal of unreacted proteins, (B) After removal of unreacted proteins, (C) Fenugreek gum.**

The extent of reaction between SWPI-fenugreek gums can be observed from infrared spectra. Figure 3.6 shows an infrared spectrum of SWPI – Fenugreek gum conjugates before and after the removal of unreacted proteins and compared to the spectrum of fenugreek gum. It was
found that an amide band I and II were still observed after the unreacted protein was removed, which showed that some of the protein had been incorporated into fenugreek gum. The amide I band (1700-1600) cm\(^{-1}\) arises almost entirely from the C=O stretching vibration of the peptide group. The amide II band (1480-1575) cm\(^{-1}\) is primarily N–H bending and a contribution from C–N stretching vibrations (Krimm & Bandekar, 1986).

### 3.3.4 Monosaccharide composition

Monosaccharide composition of unhydrolyzed and partially hydrolyzed fenugreek gum is shown in Table 3.4 and Figure 3.7. Hydrolysis reduced both the amount of galactose and mannose of fenugreek gum. As shown in Table 3.3, as the hydrolysis time is increased, the amount of mannose and galactose decreased. The amount of galactose reduced at higher rate compared to mannose as can be seen from the ratio between galactose and mannose before and after hydrolysis. The initial ratio was 1:1.26 of galactose to mannose (Table 3.4). After hydrolysis, the proportion of galactose decreased while the proportion of mannose increased as can be observed from galactose to mannose ratio.

<table>
<thead>
<tr>
<th>Table 3-4 Monosaccharides composition of unhydrolyzed and partially hydrolyzed fenugreek gum</th>
<th>Galactose, G (%)</th>
<th>Mannose, M (%)</th>
<th>Ratio G:M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified fenugreek gum</td>
<td>34.35 ± 2.77</td>
<td>43.38 ± 0.77</td>
<td>1 : 1.26</td>
</tr>
<tr>
<td>Hydrolyzed 10 min</td>
<td>16.67 ± 0.04</td>
<td>30.04 ± 2.44</td>
<td>1 : 1.80</td>
</tr>
<tr>
<td>Hydrolyzed 30 min</td>
<td>14.69 ± 1.37</td>
<td>29.54 ± 1.70</td>
<td>1 : 2.01</td>
</tr>
<tr>
<td>Hydrolyzed 50 min</td>
<td>13.70 ± 0.25</td>
<td>27.81 ± 1.65</td>
<td>1 : 2.03</td>
</tr>
</tbody>
</table>
Figure 3-7 Monosaccharide composition of unhydrolyzed and partially hydrolyzed fenugreek gum.

3.4 Conclusion

Fenugreek gum was successfully conjugated to soy whey protein isolate. SDS-PAGE analysis showed polydispersed bands at the top of the separating gel in the conjugate suggesting the formation of high molecular weight products with a distinct amount. Refractive index spectrum of HPSEC profiles shows the reduction of protein peak of unconjugated mixture and a shift of a peak to a higher molecular weight of the conjugates on conjugation between SWPI and fenugreek gum. UV spectrum of HPSEC profiles shows the protein peak intensity at fenugreek gum region was slightly increased indicating that conjugation had taken place. After removal of unreacted proteins, about 45% of proteins had interacted to polysaccharide. An infrared spectrum of SWPI – Fenugreek gum conjugates before and after removal of unreacted protein and
compared to the spectrum of fenugreek gum showed that an amide band I and II were still observed after the unreacted protein was removed, which showed that some of the protein had been incorporated into fenugreek gum. Partial hydrolysis of fenugreek gum reduced both the amount of mannose and galactose but increased the ratio of mannose to galactose.
4.0 RHEOLOGICAL PROPERTIES AND NMR STRUCTURE DETERMINATION OF SWPI – FENUGREEK GUM CONJUGATES

4.1 Introduction

Polysaccharides and food proteins are two key structural components to control texture, structure, and stability of food materials. It is now extensively studied and well recognized that the solubility and functional properties of proteins can be improved by their conjugation with polysaccharides (Akhtar & Dickinson, 2003, 2007; O’Regan & Mulvihill, 2009; Dunlap & Côté, 2005; Einhorn-Stoll et al., 2005; Jimenez-Castano, Villamiel & Lopez-Fandino, 2007). The protein-polysaccharides conjugates (i.e proteins covalently linked to polysaccharides) are typically formed by dry-heating reaction through condensation between the carbonyl group of a reducing end sugar and unprotonated amino group at controlled humidity via Maillard reaction (Oliver, Melton, & Stanley, 2006). The resulting high molecular weight glycoprotein conjugate is believed to enhance emulsion stability by combining the hydrophobic property of proteins to strongly adsorb to oil-water interfaces and the typical hydrophilic property of polysaccharides to dissolve well in an aqueous medium (Dickinson & Galazka, 1991).

Gum Arabic is a naturally occurring polysaccharide obtained as the exudate of the Acacia tree and is widely used to stabilize food emulsions and dispersions. About 12% (w/w) is required to give stable 20% (w/w) orange oil emulsions of small droplet size (Randall et al., 1988). The great emulsifying property of gum Arabic is associated with the presence of a protein-rich fraction which predominantly adsorbs and hence is responsible for the emulsifying ability of the gum (Randall et al., 1988). Structural investigation shows that this fraction was an arabinogalactan-protein complex (AGP). Acacia (sen) SUPER GUM\textsuperscript{TM}, \textit{A. senegal} gum, in

\footnote{Note: To be published as Kasran, M., Cui, S. W., & Goff, H. D. (2013). \textit{LWT – Food Science and Technology.}}
which the amount of arabinogalactan protein (AGP) was increased up to more than double the amount present originally by agglomeration of the proteinaceous components within the molecularly-dispersed system showed a dramatic increase in the interfacial surface properties and coverage of the oil droplet in oil-in-water emulsion (Al-Assaf, Phillips, Aoki, & Sasaki, 2007). An AGP consists of a hydroxyproline-rich core protein which was identified as O-galactosylhydroxyproline, as the glycopeptide linkage (Qi, Fong, & Lamport, 1991). One of the proposed models for AGP was that of hydrophilic carbohydrate blocks linked to a protein chain with a wattle-blossom-type structure that was readily degradable by proteolytic enzymes (Osman, Menzies, Williams, Phillips, & Baldwin, 1993). Latest studies shows that gum Arabic is a highly branched polysaccharide with the backbone composed of 1,3-linked galactopyranosyl (Galp) residues substituted at O-2, O-6 or O-4 positions. The residues of \( \rightarrow 2,3,6-\beta -D\text{-Galp1} \rightarrow, \rightarrow 3,4\text{-Galp1} \rightarrow, \rightarrow 3,4,6\text{-Galp1} \rightarrow \) and substitutions at O-2 and O-4 position were not identified in previous studies (Nie, Wang, Cui, Wang, Xie, & Phillips, 2013).

Gum ghatti is another naturally occurring polysaccharide that exhibits good emulsifying ability and has been reported to be superior to that of gum Arabic when used at lower concentration (Al-Assaf, Amar, & Phillips, 2008). The superior emulsification property of gum ghatti has been attributed to at least three factors: more gum component adsorbed onto the oil droplets in the emulsions of gum ghatti (30%, w/w) compared to gum Arabic (7–10%) (Katayama et al., 2008). Secondly, the adsorbed components of gum ghatti in the emulsion distributed evenly in the whole molecular weight range: from high molecular to low molecular weight; on the contrary, only the high Mw fraction of gum Arabic was adsorbed on to the oil surface (Katayama et al., 2008). Thirdly, the protein content of gum ghatti is higher than that of gum Arabic (Al-Assaf et al., 2008). Kang et al. (2012) reported that gum ghatti is comprised of a
1,6-linked galactose backbone, to which were attached numerous sugar and peptide side chains. By using 1D and 2D NMR spectra, the linkage site of amino acids and polysaccharides was determined as N-linked (Hex)n-GlcNAc-Asn.

The aim of this work was to analyse the rheological properties and NMR structure determination of fenugreek gum and SWPI – Fenugreek gum conjugates. The rheological properties include steady shear viscosity and oscillatory frequency sweep test to compare the viscoelastic properties of fenugreek gum and the SWPI – Fenugreek mixture and conjugates. NMR studies include both $^1$H and $^{13}$C NMR spectra and 2D NMR (COSY, HMQC, and HMBC).

4.2 Materials and methods

SWPI and purified fenugreek gum were obtained as described in section 3.2.

4.2.1 Rheological properties of SWPI – Unhydrolyzed fenugreek gum mixture and conjugates

Rheological tests were conducted using an AR 1000 Rheometer (TA Instruments Ltd., Leatherhead, Surrey, England). Steady shear and oscillatory tests were performed at 25°C using a 40 mm parallel plate geometry ($4^o$). SWPI – Unhydrolyzed fenugreek gum mixture and conjugate solutions were prepared by dispersing in water for 3-4 h. Steady shear viscosity was determined for 0.5%, 1% and 2%, (w/v). The dispersions were transferred onto the rheometer lower plate. The upper plate was lowered onto the gum dispersion to establish a gap of 0.8 mm, and excess material was trimmed from the periphery; the sides were covered with a thin layer of low-viscosity standard oil (Brookfield Engineering Laboratories, Stoughton, MA, USA) to minimize evaporation losses. After each sample was equilibrated (25°C, 2 min), an oscillatory sweep at a frequency range from 0.01 to 50 rads/s was carried out using 2% samples; oscillatory
strain was 5% which is within the linear viscoelastic region. The elastic modulus $G'$, viscous modulus $G''$, and complex viscosity $\eta^*$ of the samples were determined within the linear viscoelastic region.

4.2.2 Hydrolysis of fenugreek gum by dilute HCl aqueous solution

Purified fenugreek gum (0.4 g) was dissolved in water at 80°C in water bath for 6 h, followed by stirring at room temperature overnight. HCl was added to the solution to make a concentration of HCl in solution of 0.05 N and stirred for 30 min at 90°C. After cooling in water bath, the reaction mixture was neutralized with 0.2 M NaOH, and then dialyzed (6 – 8 kDa MWCO) against deionized water overnight. The dialysate was filtered to remove the water insoluble precipitate and then freeze dried for further use.

4.2.3 Preparations of SWPI – Partially hydrolyzed fenugreek gum conjugates for NMR analysis

Protein and polysaccharide were brought into contact by dissolving the SWPI and partially hydrolyzed fenugreek gum in distilled water at a weight ratio of 1:3 (SWPI to fenugreek gum). The solution was then freeze dried, milled to make a powder and sieved (80 mesh). A desiccator containing saturated NaCl was placed in the oven at 60°C for 30 min to achieve equilibrium temperature and relative humidity. The dried polymer admixture was incubated at 60°C for 3 days after which the conjugates were frozen at -20°C overnight, freeze dried and stored at 4°C until further use. About 0.4 g conjugates were dispersed in 100 mL distilled water and stirred for 2 h. The dispersion was adjusted to pH 4.5 with 2 N HCl enabling the precipitation of unreacted protein. The precipitate was separated by centrifugation at 17,210 g for 30 min at 4°C and the supernatant was collected. The supernatant was adjusted to pH 7.0 with 2 N NaOH. The solution was dialyzed overnight at 4°C and freeze dried. The unreacted protein
from SWPI – Fenugreek gum conjugates was further removed by precipitating conjugate solution in 60% ethanol. Conjugates (0.1 g) were dissolved in 100 mL distilled water. Ethanol was added to make a final concentration of ethanol in solution of 60%. The solution was stirred for 2 h and stored at 4°C overnight to precipitate the high molecular polymer and centrifuged at 17,210 g for 30 min. The supernatant was discarded and the precipitate was re-dissolved in ultrapure water, stored at -20°C overnight and freeze dried.

4.2.4 $^1$H, $^{13}$C, and 2D NMR spectroscopy

Fenugreek gum and SWPI – Fenugreek gum conjugates (90 mg precipitated at 60% ethanol) were dissolved in 5 mL D$_2$O with stirring for 2 h, and then freeze dried. This procedure was repeated three times to completely replace H$_2$O with D$_2$O. Samples were finally dissolved in 2 mL D$_2$O for 3 h before NMR analysis. Both $^1$H and $^{13}$C NMR spectra were recorded at 500.13 and 125.78 MHz, respectively, on a Bruker ARX500 NMR spectrometer operating at 30°C. A 5 mm inverse geometry $^1$H/$^{13}$C/$^{15}$N probe was used. Chemical shifts are reported relative to trimethylsilyl propionate (TSP) in D$_2$O for 1H (0.0 ppm, external standard) and 1,4-dioxane in D$_2$O for $^{13}$C (66.5 ppm, external standard). Homonuclear $^1$H/$^1$H correlation spectroscopy (COSY) and heteronuclear $^1$H/$^{13}$C correlation experiments (HMQC/HMBC) were run using the standard Bruker pulse sequence.

4.3 Results and discussion

4.3.1 Rheological properties of SWPI – Unhydrolyzed fenugreek gum mixture and conjugates

The effect of shear rate on viscosity for SWPI – Unhydrolyzed fenugreek gum mixture and conjugate at 0.5, 1 and 2% concentration is shown in Figure 4.1. The viscosity of SWPI –
Unhydrolyzed fenugreek gum mixture and conjugate were shear rate dependent at high concentration. However, for SWPI – Fenugreek gum conjugate Newtonian behavior was observed at 0.5 and 1% concentration (Figure 4.1B). SWPI – Unhydrolyzed fenugreek gum conjugate showed less viscosity as compared to SWPI – Unhydrolyzed fenugreek gum mixture. As shown in Table 3.3 (chapter 3), the molecular weight of SWPI – Unhydrolyzed fenugreek gum conjugates was lower compared SWPI – Unhydrolyzed fenugreek mixture. It might be due to the degradation of fenugreek gum during Maillard reaction.

Figure 4.2(A) shows the mechanical spectra of a 2% fenugreek gum solution. Oscillatory measurement results were characteristic of a random coil polysaccharide (Mitchell, 1979; Morris, 1995; Rees, Morris, Thom, & Maden, 1982). At low frequencies, $G''$ is above $G'$; as frequency increases the value of $G'$ approach the $G''$. At certain frequency, $G'$ surpasses $G''$. The mechanical spectra of SWPI – Unhydrolyzed Fenugreek gum mixture and conjugate showed that $G''$ is above $G'$ at all frequency (Figure 4.2 B and Figure 4.2 C). It is also noted that the mechanical spectra of fenugreek gum has a greater magnitude as compared to SWPI – Unhydrolyzed Fenugreek gum mixture and conjugate while SWPI – Unhydrolyzed Fenugreek gum conjugate showed the least magnitude of mechanical spectra. The weaker rheological properties of SWPI – Fenugreek conjugate are in agreement with the intrinsic viscosity measurements as described Table 3 - 3.
Figure 4-1 Steady shear rate dependence of viscosity for SWPI – Fenugreek gum mixture (A), SWPI – Fenugreek gum conjugate (B) at different concentration.
Figure 4-2 Mechanical spectra at 5% strain for Fenugreek gum (A), SWPI – Fenugreek gum mixture (B), and SWPI – Fenugreek gum conjugate (C) at 2% concentration.
4.3.2 Structural analysis of fenugreek gum and SWPI – Fenugreek gum conjugates

4.3.2.1 NMR studies of Fenugreek gum

Fenugreek gum is a high molecular weight galactomannan and hence was difficult to dissolve in water. Therefore, in this work, the fenugreek gum was hydrolyzed using 0.05 M HCl at 90°C for 30 min to give low molecular weights and to easily dissolve in water. In the $^{13}$C spectra, the original fenugreek gum had low peak resolutions and gave noisy spectrum in the highly viscous solution due to high molecular weight. The peak resolutions were found to increase with decreasing molecular weight.

Due to simplicity of the fenugreek gum structure, complete structural characterization could be accomplished exclusively based on NMR methods without methylation analysis. Results of both 1D and 2D $^1$H and $^{13}$C NMR studies gave a complete and reliable assignment of $^1$H and $^{13}$C signal. In 2D NMR, COSY (Homonuclear shift correlation spectrum) was used in the determination of $^1$H assignments. $^1$H assignments were verified by TOCSY (Total correlated spectroscopy). HMQC (Heteronuclear correlated spectroscopy) allows one to match the protons with the corresponding carbon in a molecule. In such spectrum, cross peaks arise from connectivity between a $^{13}$C nucleus and its corresponding directly linked proton. NOESY (Nuclear overhauser effect spectroscopy) provides information through space rather than through bond couplings. NOE connectivities are often observed between the anomeric protons of particular sugar residue. HMBC (Heteronuclear multiple bond connectivity) detects long range coupling between proton and carbon (two or three bonds away) with great sensitivity (Cui, 2005).
Figure 4.3 shows the $^1$H and $^{13}$C NMR spectrum of fenugreek gum. As shown in Figure 4.3A, the resonance of the anomeric protons in the $^1$H NMR spectra are well separated. The assignment of the $^1$H resonances of mannose and galactose as being either due to α or β was compared with available data (Bock & Thogersen, 1982; Agrawal, 1992). The spectrum gives a signal at 4.61 ppm of the anomeric proton (H-1) of the D-mannopyranosyl units, which, accordingly, have the β-D configuration. Another signal at 4.88 ppm is assigned to H-1 of the galactopyranosyl units, which therefore have α-D configuration. The integrated ratio of the H-1 signals of the $^1$H NMR spectra showed that fenugreek gum consisted of galactose and mannose in the proportions of 1.0 and 0.83; that is, a backbone of 1,4-β-D-mannan with substitution degree of C-6-carbon atoms by a single α-D galactopyranosyl residue equal to 0.83 (Jiang, Zhu, Zhang, & Sun, 2007).

The $^{13}$C NMR spectra of fenugreek gum is shown in Figure 4.3B. The structural units are the same as reported previously (Jiang et al., 2007; Muschin & Yoshida, 2012). As can be seen, three types of structural units can be clearly differentiated and identified. These units are (i) the α-D-galactopyranosyl non-reducing end-units, (ii) the un-substituted (1 → 4)-linked β-D-mannopyranosyl units of the mannan backbone, and (iii) the O-6-substituted (1 → 4)-linked β-D-mannopyranosyl units of the mannan backbone. The signal at 67 and 61 ppm were assigned to C6 mannose by COSY and HMQC spectra. The signal at 61 ppm was assigned to C6 without an attached galactose side chain while the signal at 67 ppm was due to the C6 with an attached galactose side chain (Muschin & Yoshida, 2012).
Figure 4–3. 1D NMR spectrum of fenugreek gum, (A) $^1$H NMR, (B) $^{13}$C NMR.
Fenugreek gum was measured by the COSY and HMQC spectra in D$_2$O to assign the signals. The chemical shifts (ppm) are recorded in Table 4.1. Figure 4.4 exhibits the 2D spectra of fenugreek gum. In the COSY spectrum (Figure 4.4A), the H-1 signals due to galactose (GH1) and mannose (MH1) residues appeared at 4.88 and 4.61 ppm, respectively. The correlation signal of the GH1 – GH2 was observed to assign the GH2 signal to 3.70 ppm. The GH3 signal was exhibited at 3.79 ppm assigned by correlation signals of GH2 – GH3. GH4 and GH5 signals as well as GH5 and GH6 were determined in a similar manner. The GH6 was also assigned by correlation of the GH6 and GC6 signals in the HMQC spectrum (Figure 4.5B). The correlation signal of the MH2 and MH3 signals were found to appear at 4.02 and 3.63 ppm respectively, by correlation signals of MH1 – MH2 and MH2 – MH3. However, other mannose proton signals were difficult to assign by only the COSY spectrum because of the complex overlapping. In the HMQC spectrum shown in Figure 4.4B, the carbon signals of mannose were assigned from the corresponding proton signals. The MC6 at 61 and 67 ppm were separated into two correlation signals, respectively of their corresponding protons, due to the presence and absence of attached galactose side chains.
Figure 4-4. COSY (A) and $^1$H – $^{13}$C HMQC (B) spectrum of fenugreek gum, G – Galactose; M – Mannose; * – mannose is substituted with galactose at C6.
Table 4-1 $^1$H and $^{13}$C chemical shifts (ppm) of Fenugreek gum

<table>
<thead>
<tr>
<th>Position</th>
<th>$^1$H</th>
<th>$^{13}$C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4.88</td>
<td>99.58</td>
</tr>
<tr>
<td>2</td>
<td>3.70</td>
<td>69.42</td>
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<tr>
<td>3</td>
<td>3.79</td>
<td>70.27</td>
</tr>
<tr>
<td>4</td>
<td>3.88</td>
<td>70.04</td>
</tr>
<tr>
<td>5</td>
<td>3.78</td>
<td>72.08</td>
</tr>
<tr>
<td>6</td>
<td>3.63$^a$</td>
<td>62.12</td>
</tr>
<tr>
<td>Mannose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4.61</td>
<td>100.8</td>
</tr>
<tr>
<td>2</td>
<td>4.02</td>
<td>70.94</td>
</tr>
<tr>
<td>3</td>
<td>4.61</td>
<td>72.46</td>
</tr>
<tr>
<td>4</td>
<td>3.73</td>
<td>77.77</td>
</tr>
<tr>
<td>5</td>
<td>3.60</td>
<td>74.07</td>
</tr>
<tr>
<td>6</td>
<td>3.86</td>
<td>67.33 – substituted</td>
</tr>
<tr>
<td></td>
<td>3.68</td>
<td>67.33 – substituted</td>
</tr>
<tr>
<td></td>
<td>3.63</td>
<td>61.21 – unsubstituted</td>
</tr>
</tbody>
</table>

$^a$ Two signals overlapped

HMBC measurement is suitable for the determination of multiple bond connectivity and used for sequencing. Figure 4.5 shows the HMBC spectrum of fenugreek gum. The correlation signals of relevant long range C – H connectivities of the constituent glycosyl residues are clear. The long range correlation signals between GH1 and MC6 through the G1-O-M6 oxygen appeared on the GH1 track, suggesting that galactose branches were attached at the C-6 of the main chain mannose. The H-1 (gal) at δ 4.88 is coupled to the $^{13}$C resonance at δ 67.33 (C-6 of β-man). The correlation signals between MH1–MC4 and MC1–MH4 on the MH1 and MC1 tracks appeared clearly, respectively, due to the long-ranged correlation signals through the M4-O-M1 oxygen between two neighboring mannopyranose residues, indicating that mannose has a
1→4-linked residue. The H-4 (man) at δ 3.73 is coupled to the $^{13}$C resonance at δ 100.8 (C-1 of β-man) and H-1 (man) at δ 4.61 is coupled to the $^{13}$C resonance at δ 77.77 (C-4 of β-man). The anomeric inter residue linkage, β-man-(1→4)-β-man and α-gal-(1→6)-β-man is in agreement with previously reported structure (Ramesh, Yamaki, & Tsushida, 2001) is shown in Figure 4.6.

Figure 4-5 $^1$H – $^{13}$C HMBC spectrum of Fenugreek gum, G – Galactose; M - Mannose.

Figure 4-6 Repeating unit of fenugreek gum (Ramesh et al., 2001).
4.3.2.2 NMR studies of SWPI – Fenugreek gum conjugates

In principle, Maillard reaction involves the condensation of the carbonyl group of the reducing sugar with the available ε-amino group of the protein, resulting in an Amadori product being produced via the formation of a Schiff base with the release of water and the Amadori rearrangement (Ames, 1992).

In this study, Amadori product was subjected to 1D NMR investigation. The $^1$H spectrum of SWPI – Partially hydrolyzed fenugreek gum conjugate is shown in Figure 4.7. Most of the NMR signals of protein and polysaccharide were located in different regions of 1D spectra, except the H$^\alpha$ signals of amino acids overlapped with the signals from polysaccharides. The signals from 7.1 to 9.0 ppm were derived from the backbone signals of protein, while 6.0 to 7.5 ppm originated from the side chains of protein; the resonances from 0 to 3 ppm belonged to the aliphatic group of protein and polysaccharide. The resonance of aliphatic group of protein was virtually identical to the spectrum of lysine (Chazin, Rance, & Wright, 1987), in which lysine contained C$^a$, C$^b$, C$^\gamma$, C$^\delta$, and C$^\varepsilon$ protons. The chemical shifts at 1.98 to 2.02 ppm showed a strong resonance and represents the signals of C$^\beta^r$ and C$^\beta^v$ protons and the resonance at 1.75 ppm is that of C$^\delta$H$_2$. The resonance at 1.42 and 1.57 ppm could be tentatively assigned to C$^\gamma^r$ and C$^\gamma^v$ protons while C$^\varepsilon$H$_2$ resonates near 2.80 and 3.05 ppm. Figure 4.8 also exhibited the occurrence of new peak at δ 3.59 and 3.60 ppm. These peaks correspond to the H-1 of the Amadori products. According to Yaylayan, Huyghues-Despointes, & Feather (1994), the H-1 protons of Amadori products resonate at δ 3.25 to 3.60 ppm in D$_2$O and the chemical shifts were not markedly affected by the pH of the solutions (Röper, Röper, & Heyns, 1983). The schematic illustration of the reaction mode of SWPI and partially hydrolyzed fenugreek gum is shown in Figure 4.8.
Figure 4-7 1D NMR spectrum of SWPI – Partially hydrolyzed fenugreek gum conjugate.

Figure 4-8 Schematic illustration of the reaction mode of SWPI and partially hydrolyzed fenugreek gum through Maillard reaction.
Conclusion

SWPI – Unhydrolyzed fenugreek gum conjugate showed less viscosity as compared to SWPI – Unhydrolyzed fenugreek gum mixture. The mechanical spectra of fenugreek gum has a greater magnitude as compared to SWPI – Unhydrolyzed Fenugreek gum mixture and conjugate while SWPI – Unhydrolyzed Fenugreek gum conjugate showed the least magnitude of mechanical spectra. HMBC of NMR spectra shows that fenugreek gum has anomeric inter residue linkage, β-man-(1→4)-β-man and α-gal-(1→6)-β-man. In 1D NMR investigation, the $^1$H spectrum of SWPI – Partially hydrolyzed fenugreek gum conjugate showed that most of the NMR signals of protein and polysaccharide were located in different regions except the $\text{H}^\alpha$ signals of amino acids overlapped with the signals from polysaccharides. The resonance of aliphatic group of protein was virtually identical to the spectrum of lysine. In addition, a new peak appeared that resonates at δ 3.59 and 3.60 ppm. These peaks correspond to the H-1 of the Amadori products. Hence, the Amadori product being produced was due to the condensation of the carbonyl group of the reducing end mannose residue with the available ε-amino group of lysine.
5.0 FUNCTIONAL PROPERTIES OF SOY WHEY PROTEIN ISOLATE – FENUGREEK GUM CONJUGATES IN OIL-IN-WATER EMULSION MODEL SYSTEMS

5.1 Introduction

Many proteins are highly effective emulsifiers because they contain both hydrophobic regions and charged hydrophilic regions, which lower the surface tension and interact at the emulsion interface. However, this emulsification activity may be lost close to the isoelectric point of protein, where charge and solubility are minimal, and in the presence of high concentration of sodium chloride due to charge shielding effect (Shepherd et al., 2000). Since most foods are acidic this is a significant limitation on the utility of these emulsifiers in the food industry (Damodaran, 1996).

Protein-polysaccharide conjugates could potentially combine the excellent emulsification properties of the protein with the stabilizing effect of the polysaccharide (Shepherd et al., 2000). The high molecular weight glyco-conjugate is supposed to combine the properties of a hydrophobic protein, being firmly attached to the oil droplet surface, with the property of a hydrophilic polysaccharide, being highly solvated by the aqueous phase medium (Dickinson & Galazka, 1991). The complexation between proteins and polysaccharides at the emulsion droplet surface can improve steric stabilization.

In general, two different interaction mechanisms may be distinguished: either covalent coupling to form protein-polysaccharide hybrid molecules or non-covalent interaction through electrostatic interactions (Neirynck et al., 2004). In the former case, the biopolymers are

Note: Published, Kasran, M., Cui, S. W., & Goff, H. D. (2013). Food Hydrocolloids. 30, 552-558; Kasran, M., Cui, S. W., & Goff, H. D. (2013). Food Hydrocolloids. 30, 691-697.
permanently linked together, whereas in the latter case the association may be relatively weak and hence readily reversible. As a consequence, non-covalent complexes may dissociate on changing temperature or pH.

The main advantage of covalent protein-polysaccharide conjugates over non-covalent complexes is the retention of molecular integrity and solubility over a wide range of experimental conditions (Dickinson & Galazka, 1991). The conjugates have been shown to be very stable to changes in pH, ionic strength and temperature (Dickinson & Euston, 1991; Schmitt et al., 1998). These conjugates also possess improved functional properties, including enhanced emulsifying properties (Akhtar & Dickinson, 2007; Shepherd et al., 2000), increased solubility particularly around the isoelectric pH of the protein (O’Regan & Mulvihill, 2009; Chevalier et al., 2001) and increased heat stability compared to protein itself (Aoki et al., 1999; Chevalier et al., 2001).

The aim of this work was to assess the suitability of SWPI – Fenugreek gum conjugates prepared by controlled dry heating of soy whey protein isolate-fenugreek gum mixtures in stabilizing o/w emulsions. The effects of protein-polysaccharide ratios, incubation time and hydrolysis of fenugreek gum before conjugation on the emulsion stability were investigated. SWPI-fenugreek gum conjugates were tested in o/w emulsion model systems. The protein polysaccharide complexes were tested in emulsions under acidic condition, high salt concentration and applying heat treatment on the conjugates before emulsification.

5.2 Materials and methods

SWPI – Fenugreek gum conjugates were obtained by the method described in section 3.2.
5.2.1 Protein solubility

Protein solubility of SWPI, SWPI – Fenugreek gum non-conjugated mixtures and SWPI – Fenugreek gum conjugates as a function of pH was determined by the method of Babiker et al. (1998). Samples solution (1 mg/mL protein) at various pH values (pH 3-5, 0.05 M acetate buffer; pH 6-8, 0.05 M phosphate buffer and pH 9, 0.05 M Tris buffer) were centrifuged at 10,000 g for 20 min. After centrifugation, each supernatant was decanted and filtered (Whatman No. 1 filter paper) and its protein content was determined by BIO-RAD DC protein assay, Lowry method (Lowry, Rosenbrough, Farr, & Randall, 1951). Protein solubility at various pH was indicated by the ratio of the protein concentration in the supernatant to that in the original protein solution at pH 9.

5.2.2 Emulsion preparation

SWPI – Unhydrolyzed fenugreek gum (1:3) mixture and conjugates, or SWPI – Partially hydrolyzed fenugreek gum (1:3) mixture and corresponding conjugates were dispersed in 0.1 M acetate buffer, pH 4 at 0.8% (w/v) in the presence of 0.01% sodium azide as an antimicrobial agent. The solutions were stored at 4°C overnight to fully hydrate. The solutions were then centrifuged at 17,210 g for 30 min. The emulsions were prepared by slowly mixing canola oil into the dispersions (10% vol. of canola oil) and pre-homogenizing the mixture with a Polytron (Kinematica GmbH, Brinkman Instruments, Rexdale, ON) at power level 5 for 5 min at room temperature. The pre-homogenized mixture was homogenized with laboratory scale jet homogenizer (Nano DeBee, B.E.E. International Inc., Easton, MA) for 3 passes with overall pressure of 35 MPa. In addition to 1:3 ratio, emulsions were also prepared with conjugates of 1:1 and 1:5 ratio. The emulsions were also prepared at pH 7 in comparison to pH 4.
In order to get the optimum incubation time of conjugation for stabilization of emulsion, the mixture of SWPI – Partially hydrolyzed fenugreek gum (HD30) was prepared at 1:3 ratio. The dried polymer admixture was incubated at 60°C for 12 h, 1 day, 2 days or 3 days in a desiccator, in the presence of saturated NaCl (relative humidity, 75%). The efficiency of the conjugates to stabilize 10 vol.% o/w emulsions was then tested.

5.2.2.1 Heat treatment of SWPI – Fenugreek gum conjugates

Heating was carried out at two different temperatures, 75°C or 85°C for 15 min. These temperatures were chosen as the SPI solutions show two denaturation peaks, the first at 68°C and the second at 85°C, attributable to β-conglycinin and glycinin denaturation (Keerati-u-rai & Corredig, 2009b). Samples were then removed and cooled in ice bath. Emulsifications were carried out immediately after heat treatment.

5.2.2.2 Salt addition on SWPI – Fenugreek gum conjugates solution

Soy whey protein isolate (SWPI) powder and SWPI-fenugreek gum conjugates were dispersed in ultrapure water, pH 7 at 0.8% (w/v) in the presence of 0.01% sodium azide as an antimicrobial agent. Sodium chloride (0.5 M) was added and the solutions were stored at 4°C overnight to fully hydrate after which the emulsification was carried out. Samples without addition of sodium chloride were also prepared as a control.

5.2.3 Emulsion droplets distribution and size measurements

Static light scattering (Mastersizer 2000S, Malvern Instruments Inc. Westborough, MA) was employed to measure the average particle size and particle size distribution. The optical parameters selected were a dispersed phase refractive index of 1.466, a droplet absorbance of 0 and a continuous phase refractive index of 1.333. The average droplet size was characterized by
two mean diameters, surface weighted mean diameter, $d_{32}$ and volume weighted mean diameter, $d_{43}$. The $d_{32}$ value was used to estimate the specific surface area of freshly made emulsions, and the $d_{43}$ value was used to monitor changes in droplet-size distribution on storage.

5.2.4 Statistical analysis

Each treatment was performed in at least triplicate. The statistical analysis was completed using SAS 9.1.3, SAS Institute Inc. Cary, NC, USA. Analysis of variance (ANOVA) was performed on the data, and a least significant difference (LSD) test with a confidence interval of 95% was used to compare the means.

5.3 Results and discussion

5.3.1 Protein solubility

Solubility of protein for SWPI, SWPI – Fenugreek gum mixture and SWPI – Fenugreek gum conjugates as a function of pH from 3.0 to 8.0 is shown in Figure 5.1. The solubility of SWPI in solution is influenced by pH. As the pH of SWPI solution is adjusted to near the isoelectric of protein (pH 4.5), the net charge of proteins is reduced, which results in the precipitation of SWPI. SWPI – Fenugreek mixture also showed a reduction in protein solubility as the pH approached the isoelectric point of protein. However, SWPI – Fenugreek gum conjugate was almost completely soluble (>90%) over the entire pH range measured, including around the isoelectric point of protein. However, SWPI – Fenugreek gum mixture showed slightly better protein solubility at isoelectric point of protein as compared to SWPI alone. An improvement of protein solubility of SWPI – Fenugreek gum mixture at isoelectric point of protein might be due to the presence of polysaccharide in the solution mixture. The presence of polysaccharide increases the viscosity of the solutions and hence prevents aggregation of some
proteins at isoelectric pH of protein. After centrifugation some proteins remained in the solution mixtures.

The results indicate that the protein solubility of SWPI – Fenugreek gum conjugate was independent of pH suggesting that conjugation confers a protecting effect against precipitation in the isoelectric point of protein (Chevalier et al., 2001). The improvement in solubility of the SWPI – Fenugreek gum conjugates when compared to SWPI alone near the isoelectric point of protein corroborates other findings in sodium caseinate-maltodextrin system (Shepherd et al., 2000; O’Regan & Mulvihill, 2009), soy protein isolate-dextran system (Diftis & Kiosseoglou, 2006) and whey protein isolate-pectins system (Neirynck et al., 2004).

![Figure 5-1](image)

**Figure 5-1** Protein solubility of SWPI, SWPI – Fenugreek gum mixture and SWPI – Fenugreek conjugates as a function of pH from 3.0 to 8.0 at 22°C.

5.3.2 Emulsifying properties of SWPI – Fenugreek gum conjugates

5.3.2.1 Optimization of conjugation for stabilization of emulsion
5.3.2.1.1 Effect of the ratio of SWPI to fenugreek gum

In order to determine the approximate protein-polysaccharide ratio giving optimum emulsion stability, experiments were carried out with various conjugates as a function of the conjugate composition. O/w emulsions (0.8% emulsifier, 10 vol% canola oil) were prepared at pH 4.0. Figure 5.2 compares the average droplet sizes of emulsions stabilized by SWPI – Fenugreek gum conjugates at 1:1, 1:3 and 1:5 ratios immediately after emulsion preparation and after storage at 25°C for 14 days. The results showed that at 1:1 ratio, after 14 days of storage the droplet size was significantly increased compared to 1:3 and 1:5 ratio. The amount of fenugreek gum may not have been sufficient to react with all of the protein molecules that could provide steric stabilization at emulsion droplet interfaces. There was no significant difference of average droplet size at 1:3 and 1:5 ratio even after 14 days of storage ($d_{43} \sim 1.6 \mu m$).

Figure 5-2 Comparison of average droplet size ($d_{43}$) of canola oil (10%) emulsified with SWPI – Fenugreek gum at 1:1, 1:3 and 1:5 ratio incubated at 60°C for 3 days freshly after emulsion preparation and after 14 days of storage at 25°C at pH 4.0.
However, the optimum ratio for better emulsion stability depends on the types of protein and polysaccharide employed. Akhtar & Dickinson (2007) reported that the optimum ratio of whey protein-maltodextrin conjugates was 1:1 or 1:2. In other emulsification work with Maillard-type complexes of bovine serum albumin (BSA) + dextran (Dickinson & Semenova, 1992) and whey protein + dextran (Akhtar & Dickinson, 2003), the optimum protein/polysaccharide ratio was found to be around 1:3. In these studies the polysaccharide moiety was of substantially higher molecular weight compared to maltodextrin. Based on the above results and supported by the previous work, 1:3 ratio was therefore chosen for the rest of the experiment.

5.3.2.1.2 Effect of incubation time

The effect of incubation time on the emulsifying properties of SWPI – Fenugreek conjugates was investigated. Samples were prepared by dry-heating of SWPI – HD30 in the weight ratio of 1:3 at 60°C for 12 h, 1 day, 2 days or 3 days. The results were compared to the non-conjugated SWPI – HD30. Figure 5.3 shows the effect of incubation conditions on the average droplet size of o/w emulsions (0.8% SWPI – HD30 conjugate, 10% oil) at pH 4.0 on storage at 25°C for 28 days. It can be seen that the conjugates showed much better emulsifying properties in terms of droplet size compared to non-conjugated SWPI – HD30 mixture irrespective of incubation time.

Comparing the average droplet size at different incubation time as a function of storage time, it was observed that there was no significant difference on average droplet size in the first 3 days of storage at 25°C irrespective of incubation time. However, a sharp increase in droplet size was observed on incubation at 60°C for 12 h on storage for up to 28 days. Overall, as the incubation time increased, the average droplet size decreased. As shown in Figure 5.3,
incubation of SWPI – HD30 at 60°C for 3 days showed the lowest average droplet size. The average droplet sizes (d_{43}) only increased slightly after 7 days of storage and remained constant on storage for up to 28 days. After 28 days of storage at 25°C the average droplet sizes (d_{43}) were 23.46 ± 0.17, 10.84 ± 0.20, 6.39 ± 0.41 and 5.14 ± 0.26 µm respectively after 12 h, 1 day, 2 days and 3 days of incubation. Emulsification with the conjugates at incubation time less than 3 days shows the destabilization of emulsion as storage time is increased. The destabilization of emulsion could be due to less amount of protein reacted to polysaccharide. The unreacted proteins aggregate during preparation of the conjugate solution at pH 4 before emulsification.

Figure 5-3 Comparison of average droplet size (d_{43}) for emulsions of canola oil (10 vol% oil, 0.8% emulsifier) stabilized by SWPI – HD30 (1:3) mixture and conjugates incubated at 60°C for 12 h, 1 day, 2 days and 3 days as a function of storage time from 0 – 28 days at pH 4.0 and stored at 25°C.
5.3.2.1.3 Effect of hydrolysis of fenugreek gum on emulsifying properties

To determine the effect of hydrolysis of fenugreek gum on emulsion stability, experiments were carried out with SWPI – Unhydrolyzed fenugreek gum as well as SWPI – HD10, SWPI – HD30 and SWPI – HD50. The conjugates were produced by incubating SWPI - Fenugreek gum (partially hydrolyzed and unhydrolyzed) mixture at 60°C for 3 days. O/w emulsions (0.8% emulsifier, 10 vol% canola oil) were prepared at pH 4.0. Figure 5.4 compares the average droplet sizes of emulsions stabilized by SWPI – Fenugreek gum (partially hydrolyzed and unhydrolyzed) conjugates at 1:3 ratios immediately after emulsion preparation and on storage at 25°C for up to 28 days. The results showed that SWPI – Unhydrolyzed fenugreek gum conjugate produced much smaller emulsion droplets compared to the SWPI – Partially hydrolyzed fenugreek gum conjugates. The average droplet size \(d_{43}\) was constant over a storage period of 28 days at 25°C. As the hydrolysis time is increased, a larger droplet size was produced especially after 50 min of hydrolysis.

Cumulative particle size distributions of the emulsions on storage for up to 28 days are shown in Figure 5.5. The resulting plots appear as double S-shaped curves. The particle size where the cumulative distribution is 50% is known as the median droplet diameter \(d_{v,0.5}\). The cumulative distribution showed SWPI – Unhydrolyzed fenugreek gum conjugate (Figure 5.5A) had a small \(d_{v,0.5}\) on storage for up to 28 days at 25°C and produced an emulsion with monomodal distribution throughout the storage period indicating a stable emulsion. Emulsions prepared with SWPI – HD10 and SWPI – HD30 showed a small \(d_{v,0.5}\) value and a monomodal distribution in fresh emulsion (Figure 5.5B & 5.5C). However, after 3 days of storage and onwards a bimodal distribution and an increase of \(d_{v,0.5}\) value was observed indicating that oil droplets have shifted to a larger size. Cumulative particle size distribution of an emulsion
prepared with SWPI – HD50 showed an increase in $d_{v,0.5}$ value on storage for up to 28 days (Figure 5.5D). Bimodal distribution was observed as soon as the emulsion was prepared and the average particle size increased gradually on storage for up to 28 days, which indicates that the emulsion was less stable.

Figure 5-4 Comparison of average droplet size ($d_{43}$) for emulsions of canola oil (10 vol% oil, 0.8% emulsifier) stabilized by SWPI – Unhydrolyzed fenugreek gum (1:3) conjugate, SWPI – HD10 (1:3) conjugate, SWPI – HD30 (1:3) conjugate and SWPI – HD50 (1:3) conjugate (60°C, 3 days) as a function of storage time from 0 – 28 days at pH 4.0 and stored at 25°C.
Figure 5-5 Comparison of cumulative particle size distribution for emulsions of canola oil (10 vol% oil, 0.8% emulsifier) stabilized by SWPI: unhydrolyzed fenugreek gum (A), SWPI – HD10 (B), SWPI – HD30 (C) and SWPI – HD50 (D) conjugates (60°C, 3 days) as a function of storage time from 0 – 28 days at pH 4.0 and stored at 25°C (●, 0 day; □, 28 days).

Our hypothesis is that hydrolysis could produce more reactive sites by producing more reducing ends of polysaccharides to interact with protein during Maillard reaction. Indeed, as can be seen in SDS-PAGE electropherogram (Figure 3.2, chapter 3), it was noted that as the hydrolysis time is increased the characteristics of 7S fraction as well as 11S fractions disappeared on conjugation with a distinct shift to a higher molecular weight. It was also observed that the intensity of polydispersed band at the top of the separating gel was more pronounced in the partially hydrolyzed fenugreek gum particularly after 50 min of hydrolysis. However, other factors contribute to the stability of emulsion. Hydrolysis reduced the molecular weight and hydrodynamic radius of fenugreek gum (Table 3.3, chapter 3) and viscosity of the solution. As the viscosity of the solution could reduce the rate of gravitational separation
(McClements, 2005a; 2007), a reduced viscosity could reduce the efficiency of SWPI – Partially fenugreek gum conjugate to stabilize the emulsion. The viscosity of the continuous phase slows down the movement of droplets due to gravity or Brownian motion, as well as provides the product with characteristic textural attributes (Cui, 2005; Doublier & Cuvelier, 2006). The stabilizing effect against creaming is achieved at high polysaccharide concentration, which is believed to be due to formation of a weak gel-like network of the emulsion particles (Parker, Gunning, Ng, & Robins, 1995). The viscoelastic properties of such gels in emulsions were due to the high yield stress of the particle network that retards the creaming rate (Tadros, 1994; Meller, Gisler, Weitz, & Stavans, 1999). In addition, a reduced hydrodynamic radius of partially fenugreek gum would reduce the ability of the conjugates to provide steric stabilizing effect towards flocculation and coalescence.

5.3.2.2 Application of SWPI – Fenugreek gum conjugates in oil-in-water emulsion model systems

5.3.2.2.1 Effect of pH on stability of emulsion

To determine the efficiency of the conjugates at pH close to isoelectric point in stabilizing emulsions, experiments were carried out at pH 4.0 and compared to fenugreek gum, SWPI and SWPI – Fenugreek gum non-conjugated mixture. Preparation of emulsions with SWPI – Fenugreek gum conjugates at pH 7.0 was also carried out.

Figure 5.6 shows average droplet sizes of emulsions (10% oil, 0.8% emulsifier) stabilized by fenugreek gum, SWPI, SWPI – Fenugreek gum non-conjugated mixture and SWPI – Fenugreek gum conjugate. The results showed that under pH close to isoelectric point of protein, SWPI was unable to stabilize the emulsion as the average droplet size was around 100 µm. Emulsification with fenugreek gum also produced large initial droplet size with an average
droplet size was around 40 µm. Since the emulsions has separated into two layers after 28 days of storage for SWPI and fenugreek gum, the data for particle size measurements was no longer reliable and the data was not shown in Figure 5.6. Furthermore, emulsification with SWPI - Fenugreek gum non-conjugated mixture also produced large droplet sizes. However, a small average droplet size was produced for emulsion prepared with SWPI – Fenugreek gum conjugates. The results indicate that the conjugate is a highly effective stabilizer of o/w emulsion under this condition. In comparison, preparation of emulsion with SWPI – Fenugreek gum conjugate at pH 7.0 also produced small initial emulsion droplets ($d_{43} \sim 1.01\pm0.03$ µm).

![Figure 5-6](image.png)

**Figure 5-6** Comparison of average droplet size ($d_{43}$) for emulsions of canola oil (10 vol% oil, 0.8% emulsifier) at pH 4.0 as a function of storage time from 0 – 28 days and stored at 25°C stabilized by Fenugreek gum, SWPI, SWPI – Fenugreek gum (1:3) mixture and SWPI – Fenugreek gum (1:3) conjugates. The conjugates were prepared at 60°C for 3 days.
5.3.2.2.2 Effect of heating of SWPI – Fenugreek gum conjugates

Heat treatment has a great impact on the structure and functionality of proteins. To determine the effect of heating, emulsions were prepared at pH 4.0 with heated SWPI – Unhydrolyzed fenugreek gum conjugates solution at 75°C and 85°C before emulsification was carried out. The average droplet sizes of the emulsions were compared to the unheated SWPI – Unhydrolyzed fenugreek gum conjugate. Static light scattering was employed to determine the changes in droplet size distribution of the emulsions as a function of storage time.

Figure 5-7 Comparison of particle size distribution for fresh emulsions of canola oil at pH 4.0 (10 vol% oil, 0.8% emulsifier) stabilized by unheated SWPI - Fenugreek gum (1:3) conjugate solution and heated SWPI – Fenugreek gum (1:3) conjugate solutions at 75°C and 85°C for 15 min.
Figure 5.7 shows the particle size distribution of emulsions prepared by unheated SWPI – Unhydrolyzed fenugreek gum conjugates solutions as well as heated solutions at 75°C and 85°C. Particle size distribution showed that in fresh emulsion, the distribution shifted to a smaller size for emulsions prepared by heated SWPI – Fenugreek gum conjugates. The results in Figure 5.8 showed that heating before homogenization changes the emulsifying behaviour of the soy protein fractions. Emulsions prepared with unheated 0.8% SWPI – Unhydrolyzed fenugreek gum conjugates showed a monomodal distribution of particle sizes with an average diameter of about 1.6 µm. After 28 days of storage at 25°C, the particle size distribution still showed a monomodal distribution with an average diameter of particle size of about 1.8 µm. Emulsions prepared with heated SWPI – Unhydrolyzed fenugreek gum conjugates solution at 75°C and 85°C showed a significant decrease of average particle sizes compared to unheated conjugates with an average diameter of about 1.4 µm. Kato, Minaki & Kobayashi (1993) reported that the emulsifying properties of dried egg white-galactomannan conjugate were greatly increased by heating the conjugate at 100°C for 3 min. After 14 days of storage, the average diameter of particle sizes was still constant at 1.4 µm. There was no significant difference of average droplet size either heated at 75°C or 85°C and particle size distributions showed a monomodal distribution. However, after 21 days of storage, the average particle size increased to 2 µm and further increased after 28 days of storage to an average diameter of about 2.2 µm. There was no significant difference of average particle size of emulsion prepared by heated SWPI – Fenugreek gum conjugates either at 75°C or 85°C after 28 days of storage.
The emulsifying properties of proteins were dramatically increased by conjugation with polysaccharide. The attachment of polysaccharide causes protein to form stable structures. Upon heating in aqueous solution, the protein molecule partially unfolds and results in aggregates due to the heat-induced disruption of the delicate balance of various noncovalent interactions. This process may be reversible in the protein-polysaccharide conjugates, because of the inhibition of the unfolded protein-protein interaction due to the attached polysaccharide (Kato, 1996). The resulting unfolded form of protein was maintained by the attached polysaccharide and better amphiphilic balance was formed to result in better emulsifying properties (Kato et al., 1993).
It has been known that surface hydrophobicity of glycinin was increased by heating (Matsudomi et al., 1985). Glycinin shows a change in the secondary structure and increase in the surface hydrophobicity with heat (Lakemond, de Jongh, Hessing, Gruppen, & Voragen, 2000; Tezuka, Yagasaki, & Ono, 2004). Heating β-conglycinin above 70°C leads to dissociation of protein subunits, which causes the formation of soluble complexes (Iwabuchi, Watanabe & Yamaguchi, 1991; Utsumi, Damodaran, & Kinsella, 1984).

Previous studies demonstrated that heating 1% SPI protein solutions at 75°C results in emulsions with a bimodal particle size distribution, with one population of sizes smaller than 1 µm and significantly smaller than the control emulsions, as well as second population of aggregated material between 1 and 10 µm (Keerati-u-rai & Corredig, 2009b). The shift to a smaller size distribution for oil droplets prepared with solutions heated at 75°C may suggest the dissociation of β-conglycinin together with the limited denaturation of glycinin, causing an improvement of the emulsifying properties of SPI (Keerati-u-rai & Corredig, 2009b). The second population of particles appeared immediately after emulsion preparation. By conjugating protein with polysaccharide, the same phenomena were observed where particle sizes of emulsions decreased when heated conjugates solution at 75°C or 85°C were used to stabilize the emulsion. However, the particle size distribution showed monomodal distribution until 21 days of storage at 25°C. Bimodal distribution was only observed after 3 weeks of storage.

Emulsions prepared with dry-heated protein-polysaccharide mixture, steric repulsion effects should operate as a result of the presence of the adsorbed conjugate. Since, the particle size increased when heated conjugate solutions at 75°C or 85°C were used to stabilize the emulsion after 21 days of storage, one has to consider the possibility of droplet interaction through ‘bridging’ effects. SDS-PAGE data showed a significant unreacted protein band exist
after conjugation, which shows that not all proteins involved in the conjugation process. It has been previously reported that soluble aggregates formed between the basic subunit of glycinin and the β-subunit of β-conglycinin during heating (Damodaran & Kinsella, 1982; German, Damodaran, & Kinsella, 1982; Utsumi et al., 1984). Thus, protein aggregates may ‘bridge’ the gap between neighbouring droplets by adsorbing on both sides of thin liquid films, forming between the droplets (Diftis, Biliaderis, & Kiosseoglou, 2005). This can be the reason for the bridging and coalescence as shown in the cumulative particle size distribution in Figure 5.9A & 5.9B. Velev et al. (1993) reported the existence of protein aggregates and ‘hard’ particles in plain-parallel vegetable protein-stabilized aqueous films of sizes large enough to become adsorbed on both sides of the thin films separating the oil droplets. However, by employing SWPI – Fenugreek conjugates the stability of emulsions was further improved compared to SPI alone. Moreover, the emulsification was carried out at pH 4.

Figure 5-9 Cumulative particle size distribution for emulsions of canola oil (10 vol% oil, 0.8% emulsifier) as a function of storage time from 0 – 28 days at pH 4.0 and stored at 25°C stabilized by heated SWPI – Fenugreek gum (1:3) conjugates (60°C, 3 days) at 75°C (A) and 85°C (B) for 15 min.
5.3.2.2.3 Effect of salt addition

Tests were carried out to determine the effect of high concentration of sodium chloride on the stability of emulsions. Fig. 5.10 compares average droplet sizes of emulsions stabilized by SWPI and SWPI – Fenugreek gum conjugates in the presence or absence of 0.5 M NaCl. It was noted that addition of sodium chloride increased the average droplet size of emulsions stabilized by both SWPI and SWPI-fenugreek conjugates compared to controls. The average droplet size significantly increased after 21 days of storage at 25°C for emulsions stabilized by SWPI in the presence of 0.5 M NaCl. However, for emulsions stabilized by SWPI – Fenugreek gum conjugates in the presence of 0.5 M NaCl, there was no significant difference of average droplet size after 7 days of storage and onwards. In terms of high salt concentration tolerance, SWPI – Fenugreek gum conjugate was effective in stabilizing o/w emulsions over extended storage period compared to SWPI alone. Significantly, a large increase in average droplet size of emulsions stabilized by SWPI indicates that SWPI has poor emulsifying properties in the presence of sodium chloride. The improvements of emulsion stability at high salt concentration were previously reported in other protein-polysaccharide conjugate systems (Akhtar & Dickinson, 2003; Kato et al., 1993; Nakamura, Kato, & Kobayashi, 1992).
Figure 5-10 Comparison of average droplet size ($d_{43}$) for emulsions of canola oil (10 vol% oil, 0.8% emulsifier, pH 7.0) stabilized by SWPI (☐); SWPI + 0.5M NaCl (□); SWPI – Fenugreek gum (1:3) conjugate (60°C, 3 days) (▲) and SWPI – Fenugreek gum (1:3) conjugate (60°C, 3 days) + 0.5 M NaCl (×) as a function of storage time from 0 – 21 days and stored at 25°C.

5.4 Conclusion

Soy whey protein isolate (SWPI) was conjugated to hydrolyzed and unhydrolyzed purified fenugreek gum via natural Maillard reaction. The protein solubility of SWPI – Fenugreek gum conjugates improved as compared to SWPI and SWPI – Fenugreek gum mixture especially at isoelectric point of protein when assessed in the pH range 3 to 8 at 22°C. A 1:3 and 1:5 ratio of SWPI – Fenugreek gum gave rise to better emulsion stabilization as compared to 1:1 ratio. Particle size analysis revealed that conjugation of SWPI – Fenugreek gum at 60°C for 3 days was enough to produce relatively small droplet sizes in o/w emulsions. SWPI – Unhydrolyzed fenugreek gum conjugates had better emulsifying properties compared to SWPI – Hydrolyzed fenugreek gum conjugates. In comparison to SWPI, fenugreek gum and SWPI –
Fenugreek gum mixture, SWPI – Fenugreek gum conjugates had improved emulsifying properties, particularly around the isoelectric pH of protein. The emulsifying properties were greatly increased by heating the SWPI – Fenugreek gum conjugates before emulsification. Application of SWPI – Fenugreek gum conjugates also showed an improvement in emulsion stability at high salt concentration compared to SWPI alone.
6.0 GENERAL DISCUSSION AND CONCLUSIONS

Protein-polysaccharide interactions play a significant role in the structure and stability of many processed foods. Polysaccharides are known to modify liquid properties by increasing viscosity and formation of a gel, which could be used to stabilize an emulsion. Fenugreek gum has been shown to exert better emulsifying properties compared to other gums. Proteins are known to exert surface activity that can stabilize emulsions. Soy proteins are important ingredients in many food formulations where they extend or replace the functionality of animal proteins. The tofu whey has the advantage of being low cost since it has little market value (Nguyen et al., 2003) and its disposal actually constitutes an environmental and industrial problem (Peñas et al., 2006). Our hypothesis is by conjugating fenugreek gum and soy whey protein isolate, the new material will exert better functional properties such as emulsion properties. If the new product is successful, it could be very useful in the food industry. Based on the unique characteristics of fenugreek gum and the functional and nutritional value of soy protein, this study focussed on developing fenugreek gum-soy whey protein isolate conjugates through Maillard reaction for stabilizing emulsions, characterizing and elucidating the structure of the protein-polysaccharide conjugates, and applying the protein-polysaccharide conjugates in oil-in-water emulsion model systems.

Soy whey protein powders was extracted from the whey of tofu processing through dialysis and either precipitated at pH 4.5 using HCl (soy whey protein isolate, SWPI) or freeze dried directly. Acid precipitated SWPI powder contained the highest amount of protein, followed by dialyzed soy whey protein powder. The protein band of SDS-PAGE showed that the amount of \(\alpha', \alpha\) and \(\beta\) subunits of \(\beta\)-conglycinin of soy whey protein powders was much lower compared to SPI while glycinin fractions only contained acidic subunit. However, soy whey protein
powders exhibited a pronounced protein band at molecular weight between 20 – 30 kDa. At 0.8% concentration of SWPI, a very fine and stable droplet size was observed on storage for up to 14 days at 25°C. SWPI had better emulsifying properties compared to undialyzed and dialyzed soy whey protein powders.

Since SWPI exhibited better emulsifying properties as compared to dialyzed and undialyzed soy whey protein powders, SWPI was reacted to fenugreek gum (partially hydrolyzed or unhydrolyzed) to form SWPI – Fenugreek gum conjugates through Maillard reaction. Our hypothesis was by hydrolyzing the fenugreek gum it would give more reactive sites of the polysaccharide to be reacted to proteins. The extent of reaction was verified by means of SDS-PAGE, HPSEC and FTIR. The SDS-PAGE of SWPI – Fenugreek gum mixture and conjugate, stained for protein with Coomassie brilliant blue, showed polydispersed bands at the top of the separating gel in the conjugate suggesting the formation of high molecular weight products. A portion of the conjugate also remained immobile in the beginning of the stacking gel, which suggested the presence of high molecular weight conjugated protein that was too large to penetrate the gel. The characteristics of 7S fraction disappeared on conjugation with fenugreek gum, with a distinct shift to a higher molecular weight. The acidic subunits of the 11S fractions also disappeared. The amount of protein at molecular weight 30 kDa of the SWPI – Fenugreek gum conjugate remained unchanged as compared to SWPI – Fenugreek gum mixture. However, in parallel with the disappearance of protein band at molecular weight 21 and 24 kDa, the amount of protein at these molecular weights was much less compared to SWPI – Fenugreek mixture.

Comparing unhydrolyzed and partially hydrolyzed fenugreek gum, it was observed that the intensity of polydispersed band at the top of the separating gel was more pronounced in the
partially hydrolyzed fenugreek gum particularly after 50 min of hydrolysis. The band at molecular weight 30 kDa of the unhydrolyzed fenugreek gum gradually disappeared as the hydrolysis time was increased although it did not fully disappear. Some unreacted proteins were still observed.

To further confirm that conjugation had occurred, HPSEC of SWPI – Fenugreek gum conjugates was performed. The RI and UV spectrum were used to confirm if there were any differences before and after conjugation. RI spectrum of HPSEC profiles for SWPI – Partially hydrolyzed fenugreek gum (90°C, 30 min) showed a reduction of protein peak of unconjugated mixture and a shifted peak to a higher molecular weight of the conjugates on conjugation between SWPI and fenugreek gum. UV spectrum of HPSEC profiles showed an increase of protein peak intensity at polysaccharide region. The intensity of protein peak was more pronounced especially with SWPI – HD50 conjugates. The results indicate that as the hydrolysis time is increased more reaction between SWPI and fenugreek gum is observed. Hydrolysis reduces the chain length of fenugreek gum and produces more reducing end, which then reacts with protein.

To determine the amount of protein reacted to polysaccharide, the unreacted proteins were removed from the conjugates by dispersing the conjugates in ethanol at different ethanol concentration. At 40 and 60% ethanol concentrations the unreacted proteins were totally removed. However, at 80% ethanol concentration, the unreacted proteins were not removed and remained in the conjugates. Protein analysis showed SWPI – Fenugreek gum unconjugated mixture contained about 23% proteins. After precipitation at pH 4.5, the SWPI – Fenugreek conjugates contained 12.93% protein. After treatment of the conjugates with 40 and 60% ethanol, the proteins were further reduced to 10.37 and 10.42% respectively. These values
equivalent to 45% of the proteins interacted to polysaccharide. However, there was no significant difference of protein content precipitated at 80% ethanol as compared to the conjugates without ethanol precipitation.

The molecular weight of SWPI – Unhydrolyzed fenugreek gum conjugate showed that the molecular weight decreased after conjugation. The decrease of molecular weight might be due to the degradation of fenugreek gum during heating. Although there was evidence that protein had conjugated to fenugreek gum, the amount of protein that had conjugated to fenugreek gum was too small compared to the degraded fenugreek gum. However, by incubating SWPI with partially hydrolyzed fenugreek gum, the molecular weight of the conjugates increased as compared to their corresponding mixtures.

The extent of reaction between SWPI and fenugreek gums can be observed from infrared spectra. An infrared spectrum of SWPI – Fenugreek gum conjugates before and after removal of unreacted protein and compared to the spectrum of fenugreek gum showed that an amide band I and II were still observed after the unreacted protein was removed, which showed that some of the protein had been incorporated into fenugreek gum. The amide I band (1700-1600) cm⁻¹ arises almost entirely from the C=O stretching vibration of the peptide group. The amide II band (1480-1575) cm⁻¹ is primarily N–H bending and a contribution from C–N stretching vibrations (Krimm and Bandekar 1986).

In 1D NMR investigation, the ¹H spectrum of SWPI – Partially hydrolyzed fenugreek gum conjugate showed that most of the NMR signals of protein and polysaccharide were located in different regions except the Hα signals of amino acids overlapped with the signals from polysaccharides. The signals from 7.1 to 9.0 ppm were derived from the backbone signals of protein, while 6.0 to 7.5 ppm originated from the side chains of protein; the resonances from 0 to
3 ppm belonged to the aliphatic group of protein and polysaccharide. The resonance of aliphatic group of protein was virtually identical to the spectrum of lysine (Chazin, Rance, & Wright, 1987), in which lysine contained C\(^\alpha\), C\(^\beta\), C\(^\gamma\), C\(^\delta\) and C\(^\varepsilon\) protons. The chemical shifts at 1.98 to 2.02 ppm showed a strong resonance and represents the signals of C\(^\beta\) and C\(^\gamma\) protons and the resonance at 1.75 ppm is that of C\(^\delta\)H\(_2\). The resonance at 1.42 and 1.57 ppm could be tentatively assigned to C\(^\gamma\) and C\(^\varepsilon\) protons while C\(^\delta\)H\(_2\) resonates near 2.80 and 3.05 ppm. A new peak that resonates at \(\delta\) 3.59 and 3.60 ppm correspond to the H-1 of the Amadori products. According to Yaylayan, Huyghues-Despointes, & Feather (1994), the H-1 protons of Amadori products resonate at \(\delta\) 3.25 to 3.60 ppm in D\(_2\)O and the chemical shifts were not markedly affected by the pH of the solutions (Röper, Röper, & Heyns, 1983).

The suitability of SWPI – Fenugreek gum conjugates prepared by controlled dry heating of soy whey protein isolate-fenugreek gum mixtures in stabilizing o/w emulsions and the influence of conjugation on protein solubility as a function of pH were assessed. The effects of protein-polysaccharide ratios, incubation time and hydrolysis of fenugreek gum before conjugation on the emulsion stability were investigated. SWPI – Fenugreek gum conjugates were tested in o/w emulsion model systems under acidic condition, high salt concentration and applying heat treatment on the conjugates before emulsification.

Solubility of protein for SWPI, SWPI – Fenugreek gum mixture and SWPI – Fenugreek gum conjugates was tested as a function of pH from 3.0 to 8.0. The solubility of SWPI in solution was influenced by pH. As the pH of SWPI solution is adjusted to near the isoelectric of protein (pH 4.5), the net charge of proteins is reduced, which results in the precipitation of SWPI. SWPI – Fenugreek mixture also showed a reduction in protein solubility as the pH approached the isoelectric point of protein. However, SWPI – Fenugreek gum conjugate was
almost completely soluble (>90%) over the entire pH range measured, including around the isoelectric point of protein. The results indicate that the protein solubility of SWPI – Fenugreek gum conjugate was independent of pH suggesting that conjugation confers a protecting effect against precipitation at the isoelectric point of protein (Chevalier et al., 2001).

To determine the approximate protein-polysaccharide ratio giving optimum emulsion stability, experiments were carried out at different conjugate composition. O/w emulsions (0.8% emulsifier, 10 vol% canola oil) were prepared at pH 4.0 and the average droplet sizes of emulsions stabilized by SWPI – Fenugreek gum conjugates at 1:1, 1:3 and 1:5 ratios were observed on storage at 25°C for up to 14 days. The results showed that at 1:1 ratio, after 14 days of storage the droplet size was significantly increased compared to 1:3 and 1:5 ratio. The amount of fenugreek gum may not have been sufficient to react with all of the protein molecules that could provide steric stabilization at emulsion droplet interfaces. There was no significant difference of average droplet size at 1:3 and 1:5 ratio even after 14 days of storage (d_{43} ~ 1.6 μm).

The effect of incubation time on the emulsifying properties of SWPI – Fenugreek conjugates was investigated. Samples were prepared by dry-heating of SWPI – HD30 in the weight ratio of 1:3 at 60°C for 12 h, 1 day, 2 days or 3 days. The results were compared to the non-conjugated SWPI – HD30. The results showed that the conjugates exhibited much better emulsifying properties in terms of droplet size compared to non-conjugated SWPI – HD30 mixture irrespective of incubation time.

Comparing the average droplet size at different incubation time as a function of storage time, it was observed that there was no significant difference on average droplet size in the first 3 days of storage at 25°C irrespective of incubation time. However, a sharp increase in droplet size
was observed on incubation at 60°C for 12 h on storage for up to 28 days. Overall, as the incubation time increased, the average droplet size decreased. Incubation of SWPI – HD30 at 60°C for 3 days showed the lowest average droplet size as compared to other incubation times as a function of storage time for up to 28 days at 25°C.

To determine the effect of hydrolysis of fenugreek gum on emulsion stability, experiments were carried out with SWPI – Unhydrolyzed fenugreek gum as well as SWPI – HD10, SWPI – HD30 and SWPI – HD50. The conjugates were produced by incubating SWPI – Fenugreek gum (partially hydrolyzed and unhydrolyzed) mixture at 60°C for 3 days at 1:3 ratios. O/w emulsions (0.8% emulsifier, 10 vol% canola oil) were prepared at pH 4.0 and the emulsions were stored at 25°C for up to 28 days. The results showed that SWPI – Unhydrolyzed fenugreek gum conjugate produced much smaller emulsion droplets compared to the SWPI – Partially hydrolyzed fenugreek gum conjugates. The average droplet size (d_{43}) was constant over a storage period of 28 days at 25°C. As the hydrolysis time increased, a larger droplet size was produced especially after 50 min of hydrolysis.

SWPI – Fenugreek gum conjugates were applied in o/w emulsion model systems under acidic condition, high salt concentration and applying heat treatment on the conjugates before emulsification. To determine the efficiency of the conjugates at pH close to isoelectric point in stabilizing emulsions, experiments were carried out at pH 4.0 and compared to fenugreek gum, SWPI and SWPI-fenugreek gum non-conjugated mixture. Preparation of emulsions with SWPI – Fenugreek gum conjugates at pH 7.0 was also carried out. The results showed that under pH close to isoelectric point of protein, SWPI was unable to stabilize the emulsion as the average droplet size was around 100 µm. Emulsification with fenugreek gum also produced large initial droplet size with an average droplet size was around 40 µm. Furthermore, emulsification with
SWPI – Fenugreek gum non-conjugated mixture also produced large droplet sizes. However, a small average droplet size was produced for emulsion prepared with SWPI – Fenugreek gum conjugates. The results indicate that the conjugate is a highly effective stabilizer of o/w emulsion under this condition.

To determine the effect of heating, emulsions were prepared at pH 4.0 with heated SWPI – Unhydrolyzed fenugreek gum conjugates solution at 75°C and 85°C before emulsification was carried out. The average droplet sizes of the emulsions were compared to the unheated SWPI – Unhydrolyzed fenugreek gum conjugate. The results showed that heating before homogenization changes the emulsifying behaviour of the soy protein fractions. Emulsions prepared with unheated 0.8% SWPI – Unhydrolyzed fenugreek gum conjugates showed a monomodal distribution of particle sizes with an average diameter of about 1.6 µm. After 28 days of storage at 25°C, the particle size distribution still showed a monomodal distribution with an average diameter of particle size of about 1.8 µm. Emulsions prepared with heated SWPI – Unhydrolyzed fenugreek gum conjugates solution at 75°C and 85°C showed a significant decrease of average particle sizes compared to unheated conjugates with an average diameter of about 1.4 µm. However, after 21 days of storage, the average particle size increased to 2 µm and further increased after 28 days of storage to an average diameter of about 2.2 µm.

The emulsifying properties of proteins were dramatically increased by conjugation with polysaccharide. The attachment of polysaccharide causes protein to form stable structures. Upon heating in aqueous solution, the protein molecule partially unfolds and results in aggregates due to the heat-induced disruption of the delicate balance of various noncovalent interactions. This process may be reversible in the protein-polysaccharide conjugates, because of the inhibition of the unfolded protein-protein interaction due to the attached polysaccharide
Kato, 1996). The resulting unfolded form of protein was maintained by the attached polysaccharide and better amphiphilic balance was formed to result in better emulsifying properties (Kato, Minaki, & Kobayashi, 1993).

Since the particle size increased when heated conjugate solutions at 75°C or 85°C were used to stabilize the emulsion after 21 days of storage, one has to consider the possibility of droplet interaction through ‘bridging’ effects. SDS-PAGE data showed a significant unreacted protein band exist after conjugation, which shows that not all proteins involved in the conjugation process. It has been previously reported that soluble aggregates formed between the basic subunit of glycinin and the β-subunit of β-conglycinin during heating (Damodaran & Kinsella, 1982; German, Damodaran, & Kinsella, 1982; Utsumi et al., 1984). Thus, protein aggregates may ‘bridge’ the gap between neighbouring droplets by adsorbing on both sides of thin liquid films, forming between the droplets (Diftis, Biliaderis, & Kiosseoglou, 2005).

Tests were carried out to determine the effect of high concentration of sodium chloride on the stability of emulsions. It was noted that addition of sodium chloride increased the average droplet size of emulsions stabilized by both SWPI and SWPI – Fenugreek conjugates compared to controls. The average droplet size significantly increased after 21 days of storage at 25°C for emulsions stabilized by SWPI in the presence of 0.5 M NaCl. However, for emulsions stabilized by SWPI – Fenugreek gum conjugates in the presence of 0.5 M NaCl, there was no significant difference of average droplet size after 7 days of storage and onwards. In terms of high salt concentration tolerance, SWPI – Fenugreek gum conjugate was effective in stabilizing o/w emulsions over extended storage period compared to SWPI alone. Significantly, a large increase in average droplet size of emulsions stabilized by SWPI indicates that SWPI has poor emulsifying properties in the presence of sodium chloride.
In conclusion, SWPI exhibited better emulsifying properties compared to undialyzed and dialyzed soy whey powder. At 0.8% concentration of SWPI, a very fine and stable droplet size was observed on storage for up to 14 days at 25°C.

Fenugreek gum was successfully conjugated to soy whey protein isolate. SDS-PAGE analysis showed polydisperse bands at the top of the separating gel in the conjugate suggesting the formation of high molecular weight products with a distinct amount. Refractive index spectrum of HPSEC profiles shows the reduction of protein peak of unconjugated mixture and a shift of a peak to a higher molecular weight of the conjugates on conjugation between SWPI and fenugreek gum. UV spectrum of HPSEC profiles shows the protein peak intensity at fenugreek gum region was slightly increased indicating that conjugation had taken place. After removal of unreacted proteins, about 45% of proteins had interacted to polysaccharide. An infrared spectrum of SWPI – Fenugreek gum conjugates before and after removal of unreacted protein and compared to the spectrum of fenugreek gum showed that an amide band I and II were still observed after the unreacted protein was removed, which showed that some of the protein had been incorporated into fenugreek gum. 1D NMR spectrum showed that fenugreek gum was covalently bound to proteins, in which the interaction was between the reducing end of mannose residue and lysine.

The protein solubility of SWP-fenugreek gum conjugates improved as compared to SWPI and SWPI – Fenugreek gum mixture especially at isoelectric point of protein when assessed in the pH range 3 to 8 at 22°C. A 1:3 and 1:5 ratio of SWPI – Fenugreek gum give rise to better emulsion stability as compared to 1:1 ratio. Particle size analysis revealed that conjugation of SWPI-fenugreek gum at 60°C for 3 days was enough to produce relatively small droplet sizes in o/w emulsions. SWPI – Unhydrolyzed fenugreek gum conjugates had better emulsifying
properties compared to SWPI – Hydrolyzed fenugreek gum conjugates. In comparison to SWPI, fenugreek gum and SWPI – Fenugreek gum mixture, SWPI – Fenugreek gum conjugates had improved emulsifying properties, particularly around the isoelectric pH of protein. The emulsifying properties were greatly increased by heating the SWPI – Fenugreek gum conjugates before emulsification. Application of SWPI – Fenugreek gum conjugates also showed an improvement in emulsion stability at high salt concentration compared to SWPI alone.

The current studies showed that the proteins had been incorporated to fenugreek gum by conjugating between SWPI and fenugreek gum through Maillard reaction. The superior emulsification properties of the conjugates have been attributed to the gum component adsorbing onto the oil droplets and providing steric stabilization that prevents flocculation and coalescence. The conjugates could be applied in a wide range of products, especially in acidic foods. However, conformational studies and molecular modelling is needed to further understand the structure-function relationship of SWPI – Fenugreek gum conjugates and their superior emulsifying properties.
### References


