ENCAPSULATION OF FLAXSEED OIL WITHIN MODIFIED LENTIL PROTEIN ISOLATE MATRICES

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ABSTRACT

The overarching goal of this research was to formulate an encapsulated powder using a modified lentil protein isolate-maltodextrin mixture to encapsulate flaxseed oil by freeze drying. The primary objectives were: a) to examine the physicochemical and emulsifying properties of lentil protein isolates with different degrees of hydrolysis; b) to design and test the physicochemical properties of encapsulated flaxseed oil using a wall material with native, heat treated and partially hydrolyzed lentil proteins in combination with maltodextrin; and c) test the oxidative stability of encapsulated flaxseed oil with the capsule design with the lowest surface oil and highest encapsulation efficiency versus free oil.

Within Chapter 3 the physicochemical and emulsifying properties of lentil protein isolates (LPI) were investigated as a function of their degree of hydrolysis (DH of 4, 9 and 20%) following exposure to trypsin/heat. Interfacial tension, surface characteristics (charge and hydrophobicity) and intrinsic fluorescence were determined and related to changes in the emulsification activity (EAI) and stability indices (ESI) of unhydrolyzed (u-LPI) and hydrolyzed LPI (h-LPI) in a flaxseed oil-water emulsion. Most importantly surface hydrophobicity declined from ~30 to ~24 for the u-LPI and h-LPI (DH 4-20%), respectively. The changes in physicochemical properties induced by hydrolysis had a detrimental effect on EAI and ESI values, which declined from ~51 to ~47 m^2 g^{-1} and ~12 to ~ 11 min for u-LPI and h-LPI (DH 4-20%), respectively.

Within Chapter 4, the physicochemical properties of encapsulated flaxseed oil within lentil protein-based maltodextrin microcapsules were investigated using native (n-LPI), pre-treated (heated, un-hydrolyzed (u-LPI); and heated, hydrolyzed (h-LPI)) lentil protein isolates and as a function of oil load (10.0, 20.0 and 30.0% of total solids). The moisture, water activity, surface oil and encapsulation efficiency (EE) were assessed, along with droplet size and emulsion morphology. Light microscopy imaging of the emulsions, showed that the h-LPI had larger oil droplets than the n-LPI and u-LPI, which both appeared similar. Microcapsules prepared from h-LPI showed significantly higher surface oil and lower EE than both the n-LPI and u-LPI materials. The microcapsules prepared using n-LPI with 10.0% oil loading were found to have the lowest surface oil content (~3.7%) and highest EE (~62.8%) for all formulations, and were subjected to an oxidative storage stability test over a 30 d period vs. free oil. The encapsulation process however induced autooxidation leading the production of a

greater amount of primary oxidative products than free oil. Findings indicate that future studies are necessary to enhance the stability of the flaxseed oil through the encapsulation process.

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LIST OF SYMBOLS AND ABBREVIATIONS

ALA	α-Linolenic acid
ANOVA	Analysis of variance
ANS	8-Anilino-1-naphthalenesulfonic acid
A_w	Water activity
BHT	Butylated hydroxytoluene
d	Day
DE	Dextrose equivalence
DF	Dilution factor
DH	Degree of hydrolysis
DMSO	Dimethyl sulfoxide
E/S	Enzyme/substrate ratio
EE	Encapsulation efficiency
EAI	Emulsion activity index
ESI	Emulsion stability index
FI	Fluorescent intensity
GI	Human gastrointestinal tract
h-LPI	Hydrolyzed/heated lentil protein isolate
S_o-ANS	Surface hydrophobicity
h	Yield of hydrolysis equivalents (of α-amino groups formed during hydrolysis reaction; or α-NH_2-Gly equivalent)
h_c	mM concentration of α-NH_2-Gly equivalent from non-trypsin treated lentil protein isolate (control)
h_t	mM concentration of α-NH_2-Gly equivalent obtained from the trypsin catalyzed protein hydrolysis reaction
h_{tot}	mM concentration of α-NH_2-Gly equivalent from total lentil protein isolate hydrolysis
LA	Linoleic acid
LPI	Lentil protein isolate

MDA	Malondialdehyde
MM	Molecular mass
MWM	Molecular weight marker
n-LPI	Native lentil protein isolate
pI	Isoelectric point
PUFA	Polyunsaturated fatty acid
PV	Peroxide value
S	Svedberg Unit
SDS	Sodium dodecyl sulfate
TBARS	2-Thiobarbituric acid reactive substances
TNBS	Trinitrobenzene sulfonic acid
U_E	Electrophoretic mobility
u-LPI	Unhydrolyzed/heated lentil protein isolate
$d_{4,3}$	Volume-length mean diameter
γ	Interfacial tension
ζ	Zeta potential
ε	Permittivity
κ	Debye length
η	Dispersion viscosity
σ	Surface charge density
ω	Omega

1. INTRODUCTION

1.1 Overview

Flaxseed oil represents a rich plant source of essential omega-3 (ω-3) and omega-6 (ω-6) polyunsaturated fatty acids (PUFAs) (e.g., α-linolenic acid [ALA] and linoleic acid [LA]) (Lukaszewic et al., 2004; Abuzaytoun & Shahidi, 2006). Over the past decade, there has been increased interest by the food industry to incorporate larger amounts of flaxseed oil into both human and animal diets based on their purported health promoting properties. These include, but are not limited to, reducing the risks for coronary heart disease, some types of cancers, neurological diseases and hormonal disorders (Lukaszewic et al., 2004; Coskuner & Karababa, 2007; El-Beltagi et al., 2007). However, the incorporation of flaxseed oil into products by the food industry has been limited due to its low oxidative stability caused by its high content of PUFAs, off-flavours and off-colours associated with lipid rancidity, and poor miscibility within aqueous-based products. Oxidation is typically induced by processing and storage conditions, resulting in reduced product shelf life (Lee & Ying, 2008). To circumvent these issues, encapsulation technologies can be employed as a means of providing protection to oxygen-sensitive oils from the harsh conditions of the food processing and storage environment, as well as the harsh conditions of the human gastrointestinal tract (GI). Inclusion of the oil within an amphiphilic carrier also improves its miscibility in aqueous-based products.

Encapsulation is defined as the process whereby solids, liquids, or gaseous materials are enclosed in small capsules which can be formulated of proteins, polysaccharides and/or lipid based materials (Desai & Park, 2005; Champagne & Fustier, 2007; Lee & Ying, 2008; Augustin & Hemar, 2009; Kailasapathy, 2009; Fang & Bhandari, 2010). Encapsulation also provides a means to mask the taste and smell of the oils within food formulations to maintain the product's sensory appeal and consumer satisfaction (Champagne & Fustier, 2007). A wide range of wall materials have been used to encapsulate edible oils, including polysaccharides (modified starch, gum Arabic (Tonon et al., 2012), gum Arabic and maltodextrin, (Carneiro et al., 2013) chitosan and maltodextrin (Klaypradit & Huang, 2008), alginate and chitosan (Li & McClements, 2011))

and proteins (whey (Kim et al., 1996; Partanen et al., 2008; Tonon et al., 2012), soy (Kim et al., 1996), sodium caseinate and caesin (Kim et al., 1996; Drusch et al., 2012), pea (Pierucci et al., 2007)) or in-combination (whey protein-gum Arabic (Weinbreck et al., 2004a), sodium caseinate-maltodextrins and corn syrup solids (Hogan et al., 2001), gelatin-gum Arabic (Chang et al., 2006)). Protein-based wall materials are especially attractive due to their amphiphilic nature and ability to stabilize oil-in-water emulsions. Furthermore, a protein's sensitivities to pH and enzymes provide a means for controlled delivery especially during transit through the gastrointestinal tract. Based on the growing consumer demand for plant protein ingredients as an alternative to animal derived products, it is advantageous to develop these delivery systems to capitalize on new market niches (i.e., those that restrict the use of animal proteins based on religious, moral or dietary restrictions) and a wider range of products (e.g., vegan-based foods). Although soy (Kim et al., 1996; Nesterenko et al., 2012) and wheat (Mauguet et al., 2002; Ducel et al., 2004b; Ducel et al., 2005) proteins have been used for encapsulating oils, there are associated allergen concerns with their use. Based on the excellent emulsifying properties of other pulse proteins, particularly lentil protein isolates, could serve as an alternative (Can Karaca et al. 2011a,b). Can Karaca (2012) encapsulated flaxseed oil using mixtures of chickpea protein-maltodextrin and lentil protein-maltodextrin with some success, showing its potential for producing encapsulated powders that had low surface oil and high encapsulation efficiencies, that offered protection against oxidation over a 25 day (d) storage period, and displayed excellent release properties under simulated gastric conditions.

The overall goal of this research was to encapsulate flaxseed oil within a modified lentil protein isolate-maltodextrin mixture followed by freeze drying to produce a functional powder. In contrast to work performed by Can Karaca (2012), native, heat treated and partially hydrolyzed lentil protein isolates were explored as a means of improving the performance of the developed functional powders.

1.2 Hypotheses

To achieve the overall goal of this research the following hypotheses were tested: a) that lentil protein isolate (LPI) emulsifying properties will be improved with limited hydrolysis up to a critical level, and that further hydrolysis will have a deleterious effect on emulsion stability; b)

LPI with a limited degree of hydrolysis in combination with maltodextrin as the wall material will result in the production of powders with lower surface oil content and higher flaxseed oil encapsulation efficiencies than if native or heat treated LPI was used; and c) the wall material will offer better oxidative protection to the flaxseed oil versus free oil over a 30 d room temperature storage trial.

1.3 Objectives

To achieve the overall goal of this research and to test the associated hypotheses, the following objectives were established: a) to examine the physicochemical and emulsifying properties of lentil protein isolates with different degrees of hydrolysis; b) to design and test the physicochemical properties of encapsulated flaxseed oil using the following wall materials: native, heat treated and partially hydrolyzed lentil protein isolates combined with maltodextrin; as a function of oil payload; and c) test the oxidative stability of encapsulated flaxseed oil versus free flaxseed oil using the capsule design with the lowest surface oil content and highest encapsulation efficiency.

2. LITERATURE REVIEW

2.1 Encapsulation technologies in the food industry

Encapsulation is the process by which solids, liquids, or gaseous materials are packaged into small capsules/particles comprised of proteins, polysaccharides and/or lipid-based materials (Desai & Park, 2005; Champagne & Fustier, 2007; Lee & Ying, 2008; Augustin & Hemar, 2009; Kailasapathy, 2009; Fang & Bhandari, 2010). Encapsulation technology offers tremendous advantages for delivering lipid core materials (e.g., polyunsaturated fatty acids (PUFAs)), such as: a) protection against heat, moisture, pH, gases and light that could lead to lipid degradation; b) improved miscibility of lipids within an aqueous product; c) partition of the lipid core to prevent coalescence of lipid droplets and the formation of a surface film; and d) controlled release of the core material to a targeted site (e.g., small intestines) at a specific burst or prolonged release rate (Desai & Park, 2005; Champagne & Fustier, 2007; Lee & Ying, 2008; Augustin & Hemar, 2009; Fang & Bhandari, 2010).

Capsules are comprised of a coating material (also referred to as the wall material, capsule membrane or shell) normally composed of a protein, polysaccharide and/or lipid-based material which encapsulates an active ingredient (e.g., PUFAs, probiotics, flavours/colourants, acidulants), which is also known as the core material or payload (Lee & Ying, 2008; Kailasapathy, 2009; Fang & Bhandari, 2010). Typically, microcapsules range in size between 1 and 1000 μm and are classified based on their morphologies (Thies, 2003; Lee & Ying, 2008). Two of the more common designs are described as either having mononuclear or multinuclear morphologies. The former has one large inner core surrounded by an outer wall material, whereas in the latter, numerous smaller core particles are embedded within a continuous wall matrix (Thies, 2003; Desai & Park, 2005; Augustin & Hemar, 2009; Fang & Bhandari, 2010). The morphology of the wall material can be tailored to achieve its desired functionality (e.g., level of protection, release properties) by altering its formulation and preparation (Desai & Park 2005). Table 2.1 presents a selection of the published techniques and applications which are/have been employed by the food industry for the encapsulation of lipids and other sensitive bioactive

Table 2.1 Summary of encapsulation technologies for the entrapment of bioactive ingredients.

Technique	Process	Wall Material	Applications
A) Mechanical			
Spray drying[3,5]	Core dispersed in the wall material. Dispersion is homogenized and then atomized into hot air.[1, 2, 3, 4, 5]	Water soluble polymers: polysaccharide or proteins (e.g. gum Arabic, modified starches, maltodextrin, whey protein, soy proteins, gelatin).[1, 4, 5]	Flavours and aromas, vitamins, fats, fish or plant oils, antioxidants, essential oils, enzymes, probiotics.[1, 4, 5]
Spray cooling/chilling[3,5]	Core dispersed in the wall material. Dispersion is homogenized and then atomized into cooled or chilled air.[1, 2,3, 4, 5]	Usually high melting fats are used[4] but also various fats, waxes, fatty alcohols, fatty acids or combinations (e.g. vegetable oil and its derivatives).[1, 2, 3, 5]	Volatile water soluble materials: flavour and aroma compounds [3], mineral salts, enzymes, acidulants, protein hydrolysates incorporated into fat particles.[4]
Extrusion[3,5]	Core is dispersed in a molten polymer coating wall material solution, and then is cooled or a core-coating mixture passes through a dehydrating liquid.[1, 2, 4]	Glassy carbohydrate matrices (e.g. sugars, corn syrup solids, maltodextrins, modified starches, and gum Arabic).[1, 3, 4, 5]	Volatile unstable flavors (citrus oils), vitamin C, colors, lactic acid bacteria.[1, 2, 3]
Fluidized bed coating (air suspension)[3,5]	Core is fluidized into solid particles and the wall material is atomized onto the particles to coat the core.[1, 2, 3, 4]	Cellulose derivatives, dextrins, emulsifiers, lipids, protein derivatives, and modified starch.[2, 4, 5]	Acidulants, vitamins, salts, minerals, polyunsaturated oils.[4, 2, 5]
Lyophilization (freeze drying)[3,5]	Core dispersed in the wall material. Dispersion is homogenized and then frozen and freeze dried.[3, 5]	Gum acacia and modified starch.[3]	Water soluble flavours and natural aromas, essential oils, fish oils.[2, 3]

Table 2.1 Summary of encapsulation technologies for the entrapment of bioactive ingredients (continued).

Technique	Process	Wall Material	Applications
B) Chemical			
Coacervation[3, 5]	Involves the formation of a 3 phase system; made up of a liquid manufacturing vehicle phase, core material phase, and coating material phase; followed by separation of a liquid phase coating material from a polymeric solution; coating of that phase occurs uniformly around the suspended active core particles; coating is then solidified.[2, 5]	Water soluble polymers of proteins and ionic polysaccharides (e.g. whey, gelatin, gum acacia, gliadin, carrageenan, chitosan, soy protein, polyvinyl alcohol, gelatin, carboxymethyylcellulose, β-lactoglobulin, guar gum, dextran).[1, 2, 3, 5]	Flavour oils, essential oils, fish oils, nutrients, vitamins.[1, 2, 3, 4, 5]
Liposome encapsulation[5]	A phospholipid-based membrane (lipid-bilayers) surrounds an aqueous phase. The bilayer forms spherical core-shell structures through techniques like microfluidization, ultrasonicaiton, and reverse-phase evaporation (solvent evaporation).[2, 4, 5]	Lecithin (from soy or egg yolk), fatty acids[2], liposomes (phospholipids).[5]	Enzymes, oils (ω-3 fatty acids), water soluble vitamins, flavors, food antimicrobials in liquid suspensions.[5]
Co-crystalization[3, 5]	Core material is added to a supersaturated solution and there is a substantial emission of heat once the solution reaches the sucrose crystallization temperature.[2, 3, 5]	Supersaturated sucrose solution.[2, 5]	Fruit juices, essential oils, flavours, brown sugar.[2, 3, 5]
Molecular inclusion[3, 5]	The internal apolar cavity of β-cyclodextrin entraps the apolar core molecules through hydrophobic interactions.[2, 3, 4, 5]	β-cyclodextrins (cyclic carbohydrates, enzymatically modified starch granules).[2, 3, 4, 5]	Essential oils, flavour oils, colourings, micronutrients, vitamins (A, E, K).[2, 3, 5]

References: [1]Thies (2003), [2]Desai & Park (2005), [3]Madene et al. (2006), [4]Augustin & Hemar (2009), [5]Kailasapathy (2009)

ingredients using mechanical or chemical-based encapsulation methods. Spray and freeze drying methods will be discussed in greater detail as they are more common within this research field.

Spray drying as a drying method

One of the most common final steps in microencapsulation technology is drying, and a widely used technique is spray drying. This technique has been used in the food industry for ~60 years, because of its flexibility, relatively low cost (relative to other drying processes) and continuous/batch process capabilities (Gouin, 2004; Desai & Park, 2005; Augustin & Sanguansari, 2008; de Vos et al., 2010, Fang & Bhandari; 2010). Spray drying has been used for capsules containing hydrophobic materials such as PUFAs, flavours, essential oils and other lipid-soluble bioactive compounds (e.g., vitamins) so as to produce dried stable powders (Thies, 2003; Gouin, 2004; Desai & Park 2005; Augustin & Sanguansari, 2008; Fang & Bhandari, 2010). During this process, the core ingredient is dispersed within the wall material, homogenized and then atomized in heated air within the spray dryer (Desai & Park, 2005; Augustin & Sanguansari, 2008; de Vos et al., 2010; Fang & Bhandari, 2010). Dried microcapsules typically range in size from 1 to 50 μm (Nesterenko et al., 2013).

Spray drying is advantageous for capsule formation due to the ability to tailor processing conditions to give the desired powder properties such as, particle size, moisture content, bulk density, flowability, dispersability, appearance, and structural strength (Reineccius, 2001; Augustin & Sanguansri, 2008). Spray drying is a well establish technology, it utilizes easily accessible equipment and can produce large amounts of capsules economically; and production costs are lower relative to other drying methods (Thies, 2003; Desai & Park, 2005). Despite these advantages, some limitations to the technique also arise. For instance, the wall materials (e.g., proteins or polysaccharides) must be soluble in water and form stable oil-in-water emulsions (Gouin, 2004; Desai & Park, 2005; Fang & Bhandari, 2010). Also, poor surface coating and emulsion stability can lead to free or surface oil on the final product, which in turn leads to off-odours, colours or flavours in the final product. Another disadvantage of the technique is a significant loss of material as the result of it adhering to the inner wall of the spray dryer (Nesterenko et al., 2013). Despite these limitations, spray drying is widely applied for

encapsulating flavours, fish and plant oils, and liquid vitamin formulations (Beristain et al., 2001; Thies, 2003; Turchiuli et al., 2005; Augustin et al., 2006; Ahn et al., 2008a).

Freeze drying as a drying method

Freeze drying is typically employed for the drying of heat sensitive ingredients and tends to be more restricted to higher value applications (Augustin & Hemar, 2009; de Vos et al., 2010). Freeze drying is performed at low temperatures under a vacuum; where the material is first frozen, then the surrounding pressure is reduced and heat is added to allow the frozen water in the material to be sublimated, thus avoiding a liquid phase transition and possible core material oxidation (Kailasapathy, 2009; Fang & Bhandari, 2010). Aside from the lengthy dehydration period required (~20 h), freeze drying has proven to be a simple technique to encapsulate aromas and water-soluble flavours (Desai & Park, 2005; Fang & Bhandari, 2010). The resulting dried mixture is ground into fine particles with wide size distributions (Kailasapathy, 2009). Freeze dried products have been shown to exhibit good shelf life stability (Fang & Bhandari, 2010). Previously, freeze drying has been used in the microencapsulation process of fish oil so as to improve the oxidative stability of the final products (Heinzelmann & Franke, 1999; Heinzelmann et al., 2000; Lee & Ying, 2008). Lee and Ying (2008) reported the freezing rate of emulsions prior to drying affects the oxidative stability of encapsulated oil, however stability can be improved through the addition of antioxidants and optimization of the freezing conditions. Heinzelmann and Franke (1999) studied the oxidative stability of fish oil encapsulated with sodium caseinate and/or maltodextrin, and lactose. The authors reported that the sodium caseinate-maltodextrin combined with low initial freeze drying temperature gave a better oxidative shelf life than the sodium-lactose mixture. Those capsules that were created with either slow or fast initial freezing rate followed by freeze drying, and those with the addition of antioxidants provided better oxidative stability than those without antioxidants and medium freezing rates. Heinzelmann et al. (2000) encapsulated fish oil in combination with sodium caseinate-lactose and reported that capsules that were quick frozen using liquid nitrogen and then freeze dried provided the greatest oxidative protection.

2.2 Choice of wall materials

The choice of wall materials in the design of microcapsules for hydrophobic core ingredients is of the utmost importance as it can have a major impact on encapsulation efficiencies, yield, shelf life and level of protection against oxidation (Nesterenko et al., 2013). Proteins and polysaccharides are common wall materials employed alone or in combination for hydrophobic core ingredients. Proteins are especially advantageous since they typically have good water solubility, film forming abilities, water/lipid holding abilities, emulsifying properties and have low viscosities at relatively high concentrations. Proteins have the added benefit of being susceptible to enzymatic, chemical or physical degradation in response to the presence of enzymes (e.g., trypsin), changes in solvent pH or salt levels, or to processing (e.g., shear and heat). Depending on the desired release profiles, wall materials can be designed to have controlled delivery capabilities (Augustin & Hemar, 2009). Quite often, proteins are used in combination with polysaccharides, such as maltodextrin so as to increase the dry solid content without drastically affecting viscosity, which is important for the drying process (Kagami et al., 2003). Proteins may also be mixed with other polysaccharides, such as alginate (Yosha et al., 2008), chitosan (Lee & Hong, 2009), pectin (Zimet & Livney, 2009) and carrageenan (Weinbreck et al., 2004b) of opposing net charge in a process known as complex coacervation (Weinbreck et al., 2004a). The latter typically occurs at pH values less than the isoelectric point of the protein where the protein carries a positive net charge, and opposes the anionic polysaccharide. Complex formation occurs primarily through electrostatic interactions to create a shell around the oil droplet, either as a mixed biopolymer wall or a multi-layer wall depending on the emulsification procedure used. Due to their amphiphilic nature, proteins under the right solvent and biopolymer conditions have excellent emulsifying properties.

Various animal-derived proteins have been employed for the encapsulation of hydrophobic cores, including but not limited to, casein (Drusch et al., 2012), whey (Kim et al., 1996; Partanen et al., 2008; Tonon et al., 2012) and gelatin (Chang et al., 2006) alone or in-combination with polysaccharides. Furthermore, encapsulation using plant proteins, such as soy (Kim et al., 1996; Nesterenko et al., 2012), corn zein (Quispe-condori et al., 2011), barley (Wang et al., 2011) and, wheat gluten (Liao et al., 2012) or gliadins (Mauguet et al., 2002; Ducel et al., 2004b; Ducel et al., 2005) have also been investigated. However, consumer trends are shifting

away from animal-derived products due to consumer perceived fears (e.g., prion disease), and dietary preferences based on religious or moral beliefs (Ducel et al., 2004a; Nesterenko et al., 2013); and away from some plant proteins (e.g., soy and wheat) based on concerns over allergens. Pulse proteins, such as pea, chickpea and lentils represent potential alternatives as wall materials for hydrophobic core ingredient encapsulation. Pea proteins have already been studied as encapsulating materials for triacylglycerols (Ducel et al. 2004a,b) and α-tocopherol (Pierucci et al., 2007). Previously, Can Karaca (2012) microencapsulated flaxseed oil employing a wall material matrix of lentil protein isolate (4.0%) and maltodextrin, followed by freeze-drying. Effects of oil concentration (5.3-21.0%), and maltodextrin type (DE 9 and 18) and concentration (25.0-40.7%) on both the physicochemical characteristics and microstructure of the microcapsules were investigated. The researcher found that an increase in emulsion oil concentration resulted in a concomitant increase in oil droplet diameter and microcapsule surface oil content, and a decrease in oil encapsulation efficiency. Optimum flaxseed oil encapsulation efficiency (~83.5%), minimum surface oil content (~2.8%) and acceptable mean droplet diameter (3.0 μm) was afforded with 35.5% maltodextrin-DE 9 and 10.5% oil. Microcapsules formed employing these experimental conditions showed a protective effect against oxidation versus free oil over a storage period of 25 d at room temperature.

Lentil proteins

Protein contents within lentils seeds (*Lens culinaris*) range between ~24.2 and 31.3% depending on the cultivar, environment and growing conditions (Bhatty et al., 1976; Bhatty, 1986). These proteins are dominated by globulin (salt-soluble) and albumin (water-soluble) proteins, with minor amounts of prolamin (alcohol-soluble) and glutelin (dilute acid-soluble) proteins (Bhatty et al., 1976; Swanson, 1990; Boye et al., 2010). The globulin fraction represents ~47% of the total seed proteins in lentils and can be sub-classed into legumin, vicilin and convicilin proteins. Legumin is an 11 S (S, Svedberg unit) hexameric protein with a molecular mass (MM) of 300-400 kDa. Each monomer has as acidic (MM ~40 kDa) and basic (MM ~20 kDa) chain linked together by disulfide bridges between cysteine residues. Hexamers are held together primarily through hydrophobic interactions, hydrogen bonding and van der Waals forces. In contrast, vicilin is a 7S trimeric protein with a MM of 175-180 kDa (Swanson, 1990;

Boye et al., 2010). Each monomer has a MM of ~50-60 kDa and lacks stabilizing disulfide bridges due to a limiting amount of sulfur containing amino acids (cysteine). Vicilin's trimeric structure is maintained by hydrophobic interactions, hydrogen bonding and van der Waals forces. The third globulin, convicilin has subunits with a MM of 71 kDa and has a MM of 290 kDa in its native form (Swanson, 1990; Boye et al., 2010). The pI of a native globulin isolate was found to occur at pH 4.5 (Bora, 2002).

Can Karaca et al. (2011a) studied the emulsifying and physicochemical properties of chickpea, faba bean, lentil, pea and soy protein isolates produced by isoelectric precipitation and salt extraction. The authors reported that the source of legume proteins and the method of isolate production impacted their emulsifying and physicochemical properties. All legume protein isolates carried a net negative charge at neutral pH, and had surface hydrophobicity values ranging between 53.0 and 84.8 (S_o-ANS). Isolates formed by isoelectric precipitation showed higher surface charge and water solubility compared to those produced via salt extraction. Emulsion capacity values for all legume protein isolates were found to range between 476-542 g oil/g protein with LPI showing the highest capacity. Isoelectric-precipitated chickpea and lentil protein isolates had relatively high surface charges and formed emulsions with smaller droplet sizes (~1.6 μm) when compared to the other protein isolates. The authors also reported similar emulsification activity and stability indices, creaming stability and emulsification capacity for chickpea, lentil and soy proteins produced by isoelectric precipitation. The findings from this extensive emulsification study involving legume proteins, combined with an initial encapsulation study with flaxseed oil (Can Karaca, 2012), suggested that lentil protein isolates have potential as an encapsulating agent.

Maltodextrin

Maltodextrin is produced from starch hydrolysis, and is classified based on its dextrose equivalence (DE), which is directly related to the level of hydrolysis (Hogan et al., 2001). Typically, maltodextrins have DE values <20, whereas at levels >20 the starch hydrolysis products are typically referred to as syrups or liquid glucose (Hogan et al., 2001; Madene et al., 2006). Maltodextrins are commonly used in the capsule drying process as it raises the total dry solids content within the formulation without substantially increasing the viscosity. Since they

have little emulsifying properties on their own, they are typically mixed with a surface active polymer, such as a protein, to form the outer shell upon drying (Kagami et al., 2003; Augustin et al., 2006). The mixed wall formulations, such as sodium caseinate-maltodextrin (Hogan et al., 2001; Kagami et al., 2003) and whey protein-maltodextrin (Carneiro et al., 2013) have been shown to increase the encapsulation efficiencies and oxidative stability of encapsulated oils. Grattard and co-workers (2002) studied the influence of the physical state and molecular mobility of freeze dried maltodextrin matrices on the oxidation of encapsulated oils. The authors reported that sufficient protection was afforded by the maltodextrin matrices against lipid oxidation provided the moisture content corresponded to the monolayer value.

2.3 Emulsion stability and how it affects microencapsulation

The first stage in the formation of microcapsules under emulsification conditions is to create a stable emulsion. Broadly defined, an emulsion is composed of at least two immiscible liquids, where one of the liquids becomes dispersed as small droplets (dispersed phase) in the other (continuous phase) (Damodaran, 2005; McClements, 2005a; Fang & Bhandari, 2010). To encapsulate a core material (e.g. bioactive lipid) that must be added in the first liquid (wall material) by homogenization (de Vos et al., 2010). The spatial organization of the oil and water phases allows for emulsion classification. When the oil droplets are dispersed in the aqueous phase it is classified as an oil-in-water emulsion (O/W) and when the aqueous phase droplets are dispersed in the oil phase it is classified as a water-in-oil emulsion (W/O) (Damodaran, 2005; Fang & Bhandari, 2010). The properties of O/W emulsions can be manipulated by varying the emulsifiers used as well as the components that are present in the aqueous phase (Dalgleish, 2004). Due to the fact that emulsions are thermodynamically unstable systems they begin to lose their structure and breakdown over time (Dalgleish, 2004; McClements, 2005a; McClements et al., 2007b).

Emulsion stability and functional behavior is controlled by the three components of the system: the oil that makes up the interior of the droplets, the interfacial material between the lipid and the continuous aqueous phase (e.g. the protein), and the aqueous phase (Dalgleish, 2004; McClements, 2005a). To create more stable emulsions, emulsifiers, which are surface-active molecules, adsorb to the surface of formed droplets during homogenization and form a

layer to prevent droplet aggregation (Dickinson, 2009). Emulsifiers are amphiphilic molecules, they can interact with both the water (hydrophilic/polar) and the oil (hydrophobic/nonpolar) phases (Dickinson, 2003; Dalgleish, 2004; McClements, 2005a). A good emulsifier is one of the crucial factors in the stabilization of an emulsion and helps define the physicochemical properties of an emulsion-based delivery system (McClements, 2007a; Lee & Ying, 2008). The factors which affect an emulsion's stability include: the particle size and distribution of emulsified oil droplets; viscosity and total solid content of the emulsion; type and concentration of the carrier; ratio of core to the carrier (wall material); the type of emulsifier, which allows for control of interfacial properties like charge, thickness, rheology, and response to environmental stresses such as pH, ionic strength, temperature and enzyme activity; and the homogenization temperature, pressure, and time (Dickinson, 2003; Tcholakova, 2003; Lee & Ying, 2008; McClements & Li, 2010). Emulsion stability is a necessary prerequisite for the preparation of stable microcapsules containing the desired properties and performance, especially in achieving oxidative stability of an encapsulated unsaturated oil (Lee & Ying, 2008).

For the creation of a stable emulsion for oil encapsulation, its properties should encompass: a narrow distribution and small size of the oil droplets; a high solid content to afford formation of a continuous matrix resulting in a protective barrier; and a low viscosity to allow for easy flow and drying (Tcholakova, 2003; Dalgleish, 2004; McClements, 2005c; Lee & Ying, 2008). These properties will be discussed in some detail in the following sections.

Droplet size

The ideal droplet size to enhance emulsion stabilization is small with a narrow distribution as these factors prevent agglomeration (creaming) and coalescence (Dalgleish, 2004; McClements, 2005c; Lee & Ying, 2008; Dickinson, 2009). Typically within food emulsions, the droplet diameters range from 0.1-100 μm (McClements, 2005a; Bouyer et al., 2012). When droplet radii are less than 10 nm creaming should be retarded almost completely due to Brownian motion (Damodaran, 2005; McClements, 2005c; Dickinson, 2010). Droplets that are larger (radius > 1 μm), and with broad distribution tend to agglomerate and coalesce more rapidly and are responsible for the negative result of high capsule surface oil content (McClements, 2005d; Lee & Ying, 2008). Not only are encapsulation efficiency and capsule

physicochemical properties adversely affected by the resulting surface oil, the rate of lipid oxidation is also adversely affected (Lee & Ying, 2008; Drusch & Mannino, 2009). Droplet size is controlled by the pressure of the homogenizer, viscosity of the suspension, number of passes through the homogenizer, and the amount of emulsifier present (Dickinson, 2003; Dalgleish, 2004; McClements, 2005c; Dickinson, 2009).

To create small droplets, higher homogenization pressures can be used (Dalgleish, 2004; Lee & Ying, 2008). Intensity and duration of the energy input of the homogenizer are two parameters that can be manipulated to modify droplet size and distribution (Krog & Sparso, 2004; Pegg & Shahidi, 2007). By using homogenization to convert immiscible liquids into an emulsion, there is the primary homogenization, which is the creation of an emulsion from two separate liquids, and a secondary homogenization, which is the reduction in size of droplets already in existence (McClements, 2005d). When multiple passes of the emulsion are put through the homogenizer this allows for the creation of small oil droplets with a narrow range distribution (Lee & Ying, 2008).

The choice of emulsifier and its concentration can also be used to modify droplet size (Dickinson, 2003; Tcholakova, 2003). When the concentration of the emulsifier is increased, droplet size decreases (Tcholakova, 2003; Pegg & Shahidi, 2007; Dickinson, 2009). This can be achieved by increasing the protein concentration (i.e. emulsifier) in an emulsion whereby a thicker emulsifier layer on the droplets occurs, which in turn prevents the close approach of the emulsifier coated oil droplets due to charge repulsion between similarly charged droplets (Tcholakova, 2003; Dalgleish, 2004; Dickinson, 2009). The emulsifier plays a role in droplet size by decreasing the interfacial tension present between the aqueous and oil phases, and this aids in droplet distribution (McClements, 2005d). Hence, the more quickly an emulsifier can adsorb at the interface, the more the interfacial tension will be reduced and the smaller the droplets that can be created at specific homogenizer energy outputs (McClements, 2005d). Therefore, emulsifier choice and concentration can be used to control the kinetics of destabilization of the emulsion droplets so as to create emulsions with longer shelf lives (Dalgleish, 2004).

Use of emulsifiers

Emulsifiers can be added to emulsions to create systems that are kinetically stable over time (Krog & Sparso, 2004; Dickinson, 2009; Fang & Bhandari, 2010). Emulsifiers are defined as surface active molecules, which form protective layers by adsorbing to the surface of newly formed droplets produced by homogenization (Krog & Sparso, 2004; Dickinson, 2009). When proteins are used as emulsifiers, due to their amphiphilic nature, they form a layer at the oil-water interface thus allowing them to interact with, and form a viscoelastic layer around the oil droplet (Dalgleish, 2004; Augustin & Hemar, 2009). Depending on the nature of the protein and the emulsion pH, oil droplets can exert either stabilizing repulsive forces or destabilizing attractive forces between neighboring droplets; or contribute to steric interactions (Dalgleish, 2004; McClements, 2004; McClements et al., 2007b). Sufficient emulsifier concentration must be present in the system for oil droplet coverage to avoid droplet aggregation (Dickinson, 2003; Tcholakova, 2003).

Proteins are subject to conformational changes and denaturation due to environmental factors such as pH, temperature, and ionic strength; making the emulsion system subject to the effects of these parameters (Dickinson, 2003; Dalgleish, 2004; van Aken, 2004; Damodaran, 2005). Numerous studies have investigated ways to increase the emulsifying capacity of proteins through physical, chemical and enzymatic modifications (proteolysis/hydrolysis with trypsin, and cross-linking with transglutaminase) (Dalgleish, 2004; Augustin & Hemar, 2009). These techniques have been found to increase oil-water emulsion stability employing whey and soy proteins as emulsifiers by creating strong and rigid interfacial layers (Dalgleish, 2004). The use of biopolymers as stabilizers produced by mixing proteins and polysaccharides to enhance emulsifying capacities have also been studied (Dalgleish, 2004; Augustin & Hemar, 2009). Commonly, food proteins used as emulsifiers for core ingredient encapsulation include those from soy, milk (caseins and whey), egg, corn (zein) or their hydrolysates (Augustin & Hemar, 2009). As mentioned previously, pH, ionic strength and interfacial tension are also factors which affect how the protein can function as a stabilizer within the emulsion as these factors impart conformational changes to the protein and impact its overall charge.

pH and ionic strength

To manipulate the electrostatic interactions between droplets in emulsions, modification of pH and/or ionic strength of the aqueous solution are employed (McClements et al., 2009 Dickinson, 2010). Droplet charge properties are determined by the surface electrical potential, which is a measure of the free energy required to increase the surface charge density (σ) from zero to a specific value (McClements, 2007a; Bouyer et al., 2012). Surface electrical potential is determined by the ionic composition of the surrounding medium, which is dependent on electrostatic screening effects, which usually decreases with an increase in the ionic strength of the aqueous phase (McClements, 2007a; Shaw et al., 2008; Bouyer et al., 2012). Protein stabilized emulsions are especially susceptible to pH and ionic strength changes due to the fact that the interfacial membranes that are formed by proteins are thin and electrically charged, so the major mechanism that will prevent/delay droplet aggregation is electrostatic repulsion (McClements, 2004; Shaw et al., 2008; Dickinson, 2010).

Protein stabilized emulsions tend to flocculate at pH values that are close to their pI and when the ionic strength of the medium exceeds a certain level. This is because the electrostatic repulsion between the droplets become insufficient to overcome droplet attractive forces (McClements, 2004; van Aken, 2004; Dickinson, 2010). Therefore, if the pH of the aqueous phase is altered such that the overall protein charge is lost, or if salt is added to screen the electrostatic interactions among droplets, the repulsive forces will be insufficient to prevent droplet aggregation (van Aken, 2004; McClements, 2005a). On the droplet surface, the build-up of charged species is dependent on the sign of the charge relative to the surface, the strength of electrostatic interactions, their concentration, and the presence of other charged species that may compete for the droplet surface (McClements, 2005a). Electrostatic repulsion between droplets decreases as the ionic strength of the aqueous solution increases due to electrostatic screening, which is the accumulation of counterions around droplet surfaces (Dalgleish, 2004; McClements, 2005b; Dickinson, 2010). When the concentration and valency of counterions in the aqueous solution increases, the effect of electrostatic screening increases, which would further result in destabilizing emulsion stability and can result in flocculation; therefore, ionic strength of the

surrounding aqueous phase must be low so as to enhance emulsion stability (van Aken, 2004; McClements, 2005b).

Interfacial tension

Interfacial tension is the measure of the free energy that is stored in the interface (McClements, 2005e; Bouyer et al., 2012). Interfacial tension is created by the imbalance of molecular interactions between molecules located at the interface, however the introduction of a surface active agent (e.g., protein) can reduce the interfacial tension because the agent/emulsifier minimizes the thermodynamically unfavorable interactions between the various molecules at the interface (Dickinson, 2003; Damodaran, 2005; McClements, 2005e; Bouyer et al., 2012). This interfacial energy is important in the formation of emulsions because it plays a role in determining the amount of mechanical energy needed via homogenization to break up system droplets (Damodaran, 2005; McClements, 2005e). The resistance of droplets towards coalescence and Ostwald's ripening, and the packing of large droplets in concentrated emulsions are also affected by interfacial tension (Damodaran, 2005; McClements, 2005e; Bouyer et al., 2012). Therefore, the value of the interfacial tension of a system can provide valuable information about the emulsifier and the interface including: excess surface concentration; surface activity; adsorption rates; and interfacial rheology (McClements, 2005e). In order to reduce the droplet size, the pressure required by the homogenizer must increase with increasing interfacial tension (Damodaran, 2005; McClements, 2005d). Therefore, in order to decrease interfacial tension so as to enable small droplet formation, a surface active agent (i.e., emulsifier) such as a protein can be added (Krog & Sparso, 2004; Damodaran, 2005; McClements, 2005e). The presence of the emulsifier would decrease the thermodynamically unfavourable contact between the oil and the water phase. Therefore, a high concentration of the emulsifier at the interface would result in a lowering of the interfacial tension of the system (Dickinson, 2003; Tcholakova, 2003; McClements, 2005e).

2.4 Protein modification

Complete or limited hydrolysis is utilized by the food industry to manipulate the functional properties of proteins. Both chemical and enzymatic methods can be employed to

create protein hydrolysates; however this research project will only focus on the use of enzymatic hydrolysis with trypsin. The advantages for using enzymatic methods for protein modification over chemical means include: improved consumer acceptance with respect to ingredient labels (consumers tend to view enzymes to be more 'natural' than chemicals); milder processing conditions; high specificity; and are less likely to create possible toxic by-products (Panyam & Kilara, 1996; Guan et al., 2007).

The food industry has employed protein hydrolysis (proteolysis) to improve the functional properties of proteins including: solubility; emulsification; gelation; water and fat holding capacities; and foaming ability (Panyam & Kilara, 1996; Henning et al., 1997; Guan et al., 2007). The degree of protein degradation, also referred to as the degree of hydrolysis (DH), significantly impacts the functional properties of the resulting hydrolysate (Guan et al., 2007). An endopeptidase enzyme functions to cleave the peptide linkage between adjacent amino acid residues in the primary sequence of the protein, to create two peptides (Panyam & Kilara, 1996). The specificity of the enzyme is a key factor in hydrolysis as it affects the number and location of the peptide linkages that are being hydrolyzed (Panyam & Kilara, 1996). Other factors which affect the effectiveness of enzymatic hydrolysis are: extent of protein denaturation; substrate and enzyme concentrations; pH; ionic strength; temperature; and either the presence or absence of inhibitory substances (Panyam & Kilara, 1996).

Based on published literature, limited proteolysis appears to be more beneficial in improving protein functionality than extended proteolysis, which can greatly impair protein functionality (Guan et al., 2007; Karayannidou et al., 2007; Pinterits & Arntfield, 2007). Hydrolysis alters the proteins' functionality by causing changes to the proteins' molecular size, conformation, and strength of protein-protein interactions (Chabanon et al., 2007; Guan et al., 2007). Hydrolysis decreases the molecular weight of the protein, increases the ionizable group number, and can expose previously covered hydrophobic groups (Panyam & Kilara, 1996; Lamsal et al., 2007; Pinterits & Arntfield, 2007). In the present research, limited hydrolysis of a lentil protein isolate was studied as a means for improving its emulsifying properties so as to obtain more stable emulsions for flaxseed oil encapsulation purposes.

2.5 Omgea-3 fatty acids from flaxseed oil as the bioactive core material

Flaxseed (*Linum usitatissimum* L.*)* is a plant source rich in the ω-3 polyunsaturated fatty acid, α-linolenic acid (ALA) (Lukaszewic et al., 2004; Abuzaytoun & Shahidi, 2006). Polyunsaturated fatty acids (PUFAs) play a key role in human health, and because ALA is an essential PUFA and cannot be synthesized in the body, good food sources for this compound are of interest to consumers and industry (Lukaszewic et al., 2004). Flaxseed contains about 30-40% oil with more than 50% being ALA (Babu & Wiesenfeld, 2003; Coskuner & Karababa, 2007). Flaxseed oil continues to be the subject of increasing interest in human and animal diets due to its potential beneficial health effects as related to coronary heart disease, some types of cancers, neurological disorders and hormonal disorders (Lukaszewic et al., 2004; Coskuner & Karababa, 2007; El-Beltagi et al., 2007). PUFAs are highly susceptible to oxidation, which can lead to the formation of free radicals, which can have adverse effects on your health (Kubow, 1992; Lee & Ying, 2008), and lead to lipid rancidity and the production of off flavours/odours (Lukaszewic et al., 2004; Lee & Ying, 2008).

The stability of oil against lipid oxidation is dependent upon its fatty acid composition, content of natural or added antioxidants, the presence of oxygen, processing parameters, and storage and packaging conditions (Abuzaytoun & Shahidi, 2006). Flaxseed oil can be protected through different mechanisms so as to increase its shelf life and protect against lipid oxidation. One of these mechanisms is through the use of antioxidants. Flaxseed contains naturally occurring antioxidants present in the form of lignans, and commercial products contain added vitamin E (Lukaszewic et al., 2004; El-Beltagi et al., 2007). Another method to protect unsaturated lipids from oxidation is through encapsulation. This process has been used in numerous studies to encapsulate bioactive lipids to protect against lipid oxidation as it provides a barrier to oxygen and other environmental factors (Beristain et al., 2001; Thies, 2003; Turchiuli et al., 2005; Augustin et al., 2006; Ahn et al, 2008a; Lee & Ying, 2008). In addition, encapsulation also provides a means to mask the off flavours/aromas from lipid oxidation so that foods maintain their sensory appeal and consumer satisfaction (Champagne & Fustier, 2007).

3. THE EFFECTS OF LIMITED ENZYMATIC HYDROLYSIS ON THE PHYSICOCHEMICAL AND EMULSIFYING PROPERTIES OF A LENTIL PROTEIN ISOLATE[1]

3.1 Abstract

The physicochemical and emulsifying properties of lentil protein isolates (LPI) were investigated as a function of their degree of hydrolysis (DH of 4, 9 and 20%) following exposure to trypsin/heat. Specifically, interfacial tension, surface characteristics (charge and hydrophobicity) and intrinsic fluorescence were determined. These parameters were then related to changes in the emulsification activity (EAI) and stability indices (ESI) of unhydrolyzed (u-LPI) and hydrolyzed LPI (h-LPI) in a flaxseed oil-water emulsion. Interfacial tension was found to decrease from ~6.5 to ~6.1 mN m^{-1} for u-LPI and h-LPI (DH 4-20%), respectively. A similar trend was observed for surface hydrophobicity, which declined from ~30 to ~24 for the u-LPI and h-LPI (DH 4-20%), respectively. In contrast, surface charge values were similar for all materials (~-37 mV). Intrinsic fluorescence as a function of emission wavelengths (300-400 nm) indicated a slight change in the tertiary conformation of LPI upon hydrolysis, where the magnitude of fluorescence intensity declined relative to that of u-LPI. Changes in physicochemical properties upon hydrolysis had a detrimental effect on EAI and ESI values, which declined from ~51 to ~47 m^2 g^{-1} and ~12 to ~ 11 min for u-LPI and h-LPI (DH 4-20%), respectively.

[1]Partially reproduced with permission. Avramenko, N.A., Low, N.H. and Nickerson M.T. (2013). The effects of limited enzymatic hydrolysis on the physicochemical and emulsifying properties of a lentil protein isolate. Food Research International, 51, 162-169.

3.2 Introduction

In general, food proteins are effective at stabilizing oil-in-water emulsions due to their amphiphilic nature (i.e., possessing both hydrophilic and hydrophobic reactive sites on their surface) and interfacial film-forming abilities. Emulsions are defined as dispersions of two (or more) immiscible liquids which are inherently thermodynamically unstable and tend to phase separate overtime via creaming, flocculation and/or coalescence. Emulsion stability is highly dependent upon liquid droplet size and distribution, emulsion processing conditions (e.g., homogenization rates), protein characteristics (e.g., size, conformation, surface reactivity, concentration and solubility), solvent conditions (e.g., pH, salts and temperature), phase volume ratio and continuous phase viscosity (McClements, 2007a; Can Karaca et al., 2011a,b). During emulsion formation, proteins diffuse from the bulk solution to the oil-water interface, where they unravel and re-orient to form a viscoelastic interfacial film around the dispersed oil droplets which acts as a physical and/or electrostatic barrier towards destabilization (Damodaran, 2005). Emulsion stability can arise from electrostatic repulsive forces between neighboring droplets (depending on the salt and pH conditions) or steric hindrance induced by hydrophilic protein segments extending into the continuous phase that physically restricts coalescence (Can Karaca et al., 2011a).

In contrast to animal-derived proteins (e.g., whey, casein or ovalbumin), the emulsifying properties of plant proteins including legumes, have not been as extensively studied and their mechanism(s) of action are less well understood. Plant protein-based emulsifiers are attractive to the food industry for both product development and re-formulation, based on their low cost, nutritional benefits and greater consumer/market acceptability (Duranti, 2006; Can Karaca et al., 2011a). The emulsifying properties of a group of legume proteins have been studied and include those extracted from pea (Ducel et al., 2004a), cowpea, (Kimura et al., 2008), faba bean (Galazka, et al., 1999), soy (Martinez et al., 2009) and lentil (Bora, 2002). Research arising from our laboratory compared the emulsifying properties of protein isolates derived from chickpea, lentil, faba bean, pea and soy produced by both isoelectric precipitation and salt extraction (Can Karaca et al., 2011a). Findings indicated that lentil protein isolates, produced by isoelectric precipitation gave the best emulsifying properties, having the highest emulsion capacity of the legumes tested, and comparable emulsifying activity/stability indices and creaming behaviour to

soy. Emulsion capacity (phase inversion) refers to the maximum amount of oil in which can be dispersed within a solution of emulsifiers (e.g., proteins) without the emulsion phase separating or undergoing an inversion from an oil-in-water emulsion to a water-in-oil emulsion (McClements, 2007a). The emulsifying activity index relates to the emulsion forming properties of the protein (Hill, 1996), and provides an estimate of the interfacial area stabilized by a given weight of protein based on turbidimetric analysis of a diluted emulsion (Pearce & Kinsella, 1978). The emulsifying stability index provides a measure of stability over a defined period of time for the same diluted emulsion (Pearce & Kinsella, 1978).

In order to further enhance the emulsifying properties of proteins, some researchers have explored limited enzymatic hydrolysis by proteases (e.g., trypsin) as a means to enhance functionality. Limited hydrolysis can lead to a partial unraveling of the protein structure (altering its conformational stability) to expose more ionizable and hydrophobic groups, decrease protein mass, and release polypeptides/peptides into solution (Panyam & Kilara, 1996). A limited degree of protein hydrolysis (DH) (<10%) has been found by some to enhance protein functionality, however above a critical point a detrimental effect can be seen (Panyam & Kilara, 1996; Govindaraju & Srinivas, 2006; Guan et al., 2007; Karayannidou et al., 2007). Karayannidou et al. (2007) reported that trypsin-treated sunflower protein isolate (DH ~10%) showed improved emulsifying and foaming properties relative to the non-hydrolyzed material. Guan et al. (2007) reported enhanced solubility, water holding, emulsifying and foaming properties for trypsin-treated oat bran protein (DH ~4-8%) relative to the native protein. Also, Ventureira et al. (2010) reported that trypsin-treated amaranth protein (DH of 2.2%) showed enhanced oil-water emulsion stability relative to the native form. In contrast, Govindaraju & Srinivas (2006) reported that the hydrolysis of arachin protein (DH ~19%) using papain, alcalase and fungal protease resulted in a significant decrease in emulsification, foaming capacity and stability in an oil-water emulsion. Chabanon et al. (2007) reported that a 15% hydrolysis of canola protein with alcalase resulted in lower foaming capacity, stability, emulsion activity and stability in an oil-water emulsion in comparison to that of the unhydrolyzed protein.

The overall goal of this research was to investigate structure-function relationships associated with the limited enzymatic hydrolysis of a lentil protein isolate (LPI), as it related to its surface characteristics, protein conformation and emulsifying properties. Lentil proteins are

primarily comprised of globulins (~49%) and albumins (~16.8%) (Boye et al., 2010). The salt-soluble globulin fraction consists mainly of legumin (11S, S – Svedberg Unit; ~340-360 kDa) and vicilin (7S; ~175-180 kDa) (Swanson, 1990). Legumin is a hexameric protein comprised of ~60 kDa subunits of α (~40 kDa) and β (~20 kDa) chains, whereas vicilin is a trimeric protein comprised of ~50-60 kDa subunits (Swanson, 1990). In contrast, the water-soluble albumins range in size from 5 to 80 kDa, and include protease and amylase inhibitors, and lectins (Boye et al., 2010).

3.3. Materials and Methods

3.3.1 Materials

Whole green lentil seeds (CDC Grandora) and flaxseed oil were provided by the Crop Development Centre (Saskatoon, SK, Canada) and Bioriginal Food and Science Corp. (Saskatoon, SK, Canada), respectively. The following materials were purchased from Bio-Rad (Mississauga, ON, Canada): Bio-Rad Broad Range Marker, Bio-Rad Tris-HCl gel (15%), Coomassie blue R-350 and Laemmli Sample Buffer. Hexane was purchased from Fischer Scientific (Ottawa, ON, Canada). Picrylsulfonic acid (trinitrobenzene sulfonic acid (TNBS)) and trypsin (10,600 units/mg) were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). The following chemicals were purchased from VWR (Edmonton, AB, Canada): hydrochloric acid, sodium dihydrogen phosphate, sodium dodecyl sulfate, sodium hydroxide, and sodium monohydrogen phosphate. All chemicals used in this study were of reagent grade except for sodium dodecyl sulfate which was ultrapure. The water used in this research was produced from a Millipore Milli-Q^{TM} water purification system (Millipore Corp., Milford, MA, USA).

3.3.2 LPI preparation

Whole green lentil seeds were ground into a fine flour using a food processor (Cuisinart mini-prep plus grinder) (~1 min), followed by an IKA A11 analytical mill treatment (IKA Works Inc., Wilmington, NC, USA) (~1 min). The flour was then defatted by stirring in hexane (1:3 [w/v], flour: hexane) for 40 min; this procedure was repeated two additional times. Protein isolates were prepared from the defatted flour based on methods of Papalamprou et al. (2010) and Can Karaca et al. (2011a). In brief, 100 g of defatted flour was mixed with water at a 1:10

(w/v) ratio. The pH of the resulting suspension was adjusted to 9.00 using 1.0 N NaOH followed by mechanical stirring at 500 rpm for 1 h at room temperature (21-23^{o}C). The mixture was then centrifuged at 5,000 x *g* at 4^{o}C for 20 min using a Sorvall RC-6 Plus centrifuge (Thermo Scientific, Asheville, NC, USA) to collect the supernatant. The resulting pellet was re-suspended in water at a ratio of 1:5 (w/v), adjusted to pH 9.00 using 1.0 N NaOH, stirred for 1 h at room temperature, followed by centrifugation (5,000 x *g*, 20 min, 4^{o}C). Supernatants were pooled and adjusted to pH 4.50 with 0.1 M HCl to precipitate the protein (Bora, 2002). The LPI was washed with water, frozen at -30^{o}C and then freeze dried using a Labconco FreeZone 6 freeze drier (Labconco Corp., Kansas City, MO, USA). Proximate composition for the resulting LPI were conducted according to AOAC Official Methods 925.10 (moisture), 923.03 (ash), 920.85 (lipid), and 920.87 (crude protein by using %N × 6.25) (AOAC, 2003). Carbohydrate content was determined on the basis of percent differential from 100%. Proximate analyses were performed on three separate protein isolate preparations with each preparation analyzed in triplicate (n=3).

3.3.3 LPI hydrolysis as a function of enzyme/substrate ratio

This study included the preparation of a LPI control, hydrolyzed LPI products, and determination of a total LPI hydrolysis value.

Preparation of the control sample (unhydrolyzed and heat treated): One hundred milliliters of a 1.0% (w/v) lentil protein isolate in 35 mM sodium phosphate (pH 7.80) was stirred overnight at 4^{o}C. The resulting solution was transferred to a shaking (90 rpm) water bath (PolyScience, Niles, IL, USA) at 37^{o}C for 1 h. A 250 µL aliquot of the mixture was removed and added to 2.00 mL of 1.0% SDS in 35 mM sodium phosphate (pH 7.80) and heated at 85^{o}C in a water bath for 10 min and then a 250 µL aliquot was taken and added to 2.00 mL of 35 mM sodium phosphate buffer (pH 7.80). This reaction was repeated in triplicate and analyzed employing the TNBS reaction. The LPI control sample is identified as h_c in equation 3.1.

Preparation of trypsin catalyzed protein hydrolysates: One hundred milliliters of a 1.0% (w/v) LPI in 35 mM sodium phosphate (pH 7.80) was stirred overnight at 4^{o}C. The resulting solution was transferred to a shaking (90 rpm) water bath at 37^{o}C for 1 h. Trypsin was then added to lentil protein solutions at the following enzyme/substrate (E/S [w/w]) ratios: 1/100, 1/250, 1/500 and 1/1000. A 250 µL aliquot of each E/S ratio experiment was removed at time

intervals of 5, 10, 20, 30, 40, 60, 80, 100, 120 min, and were individually added to 2.00 mL of 1.0% SDS in 35 mM sodium phosphate (pH 7.80) and heated at 85^{o}C in a water bath for 10 min to quench the hydrolysis reaction and then a 250 µL aliquot was taken and added to 2.00 mL of 35 mM sodium phosphate buffer (pH 7.80). A sample blank consisting of 250 µL of 1.0% SDS in 35 mM sodium phosphate (pH 7.80) and 2.00 mL of 35 mM sodium phosphate buffer (pH 7.80) was run with each batch of hydrolysis experiments. All partial hydrolysis reactions were performed in triplicate and all samples and blanks were analyzed employing the TNBS reaction. Partially hydrolyzed LPI samples are referred to as h_t in equation 1.

Preparation of total protein hydrolysates: Total LPI hydrolysis was performed based on the methods of Adler-Nissen (1979), Jung et al. (2005) and Barbana & Boye (2011). In brief, ~24 mg of LPI was weighed into a 20 x 150 mm screw cap Pyrex tube, followed by the addition of 15 mL of 6.0 N HCl. The tube was then flushed with nitrogen gas and sealed. Acid digestion was afforded by heating the sample in a forced-air oven (Yamoto Mechanical Convection DKN600, Yamoto Scientific America, Inc., CA, USA) at 110^{o}C for 20 h. The pH of the sample solution was adjusted to pH 7.00 ± 0.20 with 2 M NaOH, and was filtered through Whatman Grade 3 filter paper (Whatman International Ltd., Maidstone, UK). Sample aliquots of 250 µL were added to 2.00 mL of 1.0% SDS in 35 mM sodium phosphate (pH 7.80) and then a 250 µL aliquot was taken and added to 2.00 mL of 35 mM sodium phosphate buffer (pH 7.80). A sample blank was prepared by mixing volumes of 6.0 N NaOH and 6.0 N HCl to obtain a solution with a final pH of 7.00 ± 0.20. All total LPI hydrolysis reactions were performed in triplicate and all samples and blanks were analyzed employing the TNBS reaction. Total LPI hydrolyzed samples are referred to as h_{tot} in equation 2.

Trinitrobenzene sulfonic acid (TNBS) reaction: The TNBS reaction was conducted in accordance with the combined methods of Adler-Nissen (1979) and del Toro & Garcia-Carreno (2005) in order to determine the degree of hydrolysis. Sample/blank solutions were transferred to a covered (i.e. protected from light) water bath maintained at 50^{o}C for 1 h. To each sample/blank 4.00 mL of 0.1 N HCl was added so as to quench the derivatization reaction. Samples were then cooled to room temperature (~5 min), and their absorbance read at 340 nm (Genesys 10UV Scanning Thermo Scientific, USA). A standard curve was prepared based on the methods of Adler-Nissen (1979) and del Toro & Garcia-Carreno (2005). Briefly, a 35 mM sodium phosphate

solution at pH 7.80 containing 1.5 mM glycine was prepared. A range of glycine standards (0.03, 0.04, 0.06, 0.08, 0.10, and 0.30 mM) were prepared from the stock solution by dilution to a final volume of 2.25 mL with 35 mM sodium phosphate solution (pH 7.80). As an example, the 0.03 mM glycine standard consisted of 45 µL of the 1.5 mM glycine stock solution and 2.205 mL of 35 mM sodium phosphate buffer. The sample blank consisted of 0.25 mL of 1.0% SDS in 35 mM sodium phosphate (pH 7.80) and 2.00 mL of 35 mM sodium phosphate (pH 7.80). All standards and blanks were analyzed in triplicate employing the TNBS reaction.

Degree of hydrolysis calculation: The degree of LPI hydrolysis (% DH) was calculated based on the protocols of Adler-Nissen (1979) and del Toro & Garcia-Carreno (2005) employing the following equations:

$$h = (h_t - h_c)\, x\, DF \qquad \text{(eq. 3.1)}$$

$$\%DH = \frac{h}{h_{tot}}\, x\, 100 \qquad \text{(eq. 3.2)}$$

where, h is the yield of hydrolysis equivalents (of α-amino groups formed during the hydrolysis reaction; or α-NH_2-Gly equivalent), h_t is the mM concentration of α-NH_2-Gly equivalent (obtained using the glycine standard curve) obtained from the trypsin catalyzed protein hydrolysis reactions, h_c is the mM concentration of α-NH_2-Gly equivalent from the non-trypsin treated LPI (control), h_{tot} is the mM concentration of α-NH_2-Gly equivalent from the total LPI hydrolysis, and DF is a dilution factor. The value used for total hydrolysis of the LPI had a mean value of 43.98 ± 3.65 mM (n=3).

3.3.4 Preparation of lentil protein hydrolysates for physicochemical and emulsion stability testing

Partial protein hydrolysis was carried out using an E/S ratio of 1:250 (w/w) at times 0 (control), 5, 15 and 30 min, which corresponded to % DH of 0, 4.38, 8.78 and 19.56 (% DH 0, 4, 9 and 20), respectively, as determined by the TNBS method. Unhydrolyzed (u-LPI) and hydrolyzed LPI (h-LPI) materials were prepared as previously described (under *Preparation of the control sample* and *Preparation of trypsin catalyzed protein hydrolysates*), except with a total volume of 150 mL, and an increased enzyme inactivation time that was based on sample volume. Aliquots of 30 mL were removed from the hydrolysis reaction at the aforementioned designated

times, and placed directly in a hot water bath (85°C) for 20 min to inactivate the enzyme (based on preliminary studies, not shown). Samples were then cooled to room temperature, separated into multiple sample tubes (~3-4 mL), and frozen for later use. Prior to conducting each experiment, sample tubes were removed and left to thaw overnight at 4°C, and then allowed to warm to room temperature (21-23°C) prior to analysis. Sample tubes were vortexed for 10 s at speed 4 prior to use. The hydrolysis reaction was conducted three separate times, to enable triplicate measurements to be performed for all treatments and analyses.

3.3.5 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The polypeptide profiles of u-LPI and h-LPI samples were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using the Laemmli (1970) method. Sample concentrations of 0.5% (v/v) (e. g. 75 µL of 1.0% sample in 75 µL water) both the u-LPI and h-LPI were employed for SDS-PAGE analysis. Briefly, 150 µL of each sample was pipetted into separate Eppendorf tubes followed by the addition of 150 µL of Laemmli sample buffer. Samples were heated to ~95^{o} C for 10 min and were centrifuged (Eppendorf Centrifuge 5424, Hamburg, Germany) for 5 min at 12,000 x *g*. Molecular weight standards (Bio-Rad Broad Range Marker) were run in conjunction with samples on a Bio-Rad Tris-HCl gel (15%) at 100-110V for ~1.5 h. Protein bands were stained using Coomassie blue R-350.

3.3.6 Physicochemical properties of protein hydrolysates

Surface charge (zeta potential or ZP): Surface charge for u-LPI and h-LPI (% DH 4, 9 and 20) materials were measured at pH 7.80, at a concentration of 0.0625% (w/w) and at room temperature (21-23^{o}C) using a Zetasizer Nano-ZS90 Instrument (Malvern Instruments, Westborough, MA). The electrophoretic mobility (U_E) of the protein solutions were used to calculate the zeta potential (ζ; units: mV) by applying the Henry equation:

$$U_E = \frac{2\varepsilon \cdot \zeta \cdot f(\kappa\alpha)}{3\eta} \qquad \text{(eq. 3.3)}$$

where ε is the permittivity (units: F (Farad)/m), $f(\kappa\alpha)$ is a function related to the ratio of particle radius (α; units: nm) and the Debye length (κ; units: nm^{-1}), and η is the dispersion viscosity (units: mPa·s). The Smoluchowski approximation $f(\kappa\alpha)$ was set as 1.5, as is accustom for folded

capillary cells and, with particles larger than 0.2 µm dispersed in moderate electrolyte concentration (> 1 mM). The Smoluchowski approximation assumes that a) concentration of particles (proteins) is sufficiently high such that such thickness of the electric double layer (Debye length) is small relative to the particle size ($\kappa\alpha>>1$); and b) ζ is linearly related to U_E. All measurements are reported as the mean ± one standard deviation (n=3).

Surface hydrophobicity: Surface hydrophobicity (S_o-ANS) for u-LPI and h-LPI (% DH 4, 9 and 20) samples was measured at pH 7.80 using a modified method of Kato & Nakai (1980). In brief, u-LPI and h-LPI (1.0% w/w) solutions were diluted with 35 mM sodium phosphate buffer to give a concentration range of 0.005 to 0.025% (w/w). For instance, 50 µL of the 1.0% (w/w) h-LPI stock underwent a 40-fold dilution by adding 1.95 mL of buffer to give a final concentration of 0.025% (w/w) (total volume of 2 mL). To 800 µL of these solutions, 8 µL of the fluorescent probe, 8-anilino-1-naphthalenesulfonic acid (ANS; 8mM ANS solution in 35 mM sodium phosphate buffer (pH 7.80)) was added, followed by vortexing for 10 s. A second set of protein solutions were also prepared without the ANS probe. An ANS blank was also prepared which consisted of 800 µL of buffer and 8 µL of the ANS probe. Samples were kept in the dark for 5 min. Sample net fluorescent intensity (FI) was determined by subtracting the intensity measured for the protein sample without ANS and an ANS blank from that of the sample containing both the protein and ANS. All fluorescence measurements were made using a FluoroMax-4 spectrophotometer (Horiba Jobin Yvon Inc., Edison, NJ, USA) with excitation and emission wavelengths at 390 and 470 nm, respectively. Both emission and excitation slit widths were set to 1 nm. The initial slope of the plot of net FI versus % protein concentration was calculated by linear regression analysis and is used as an index of protein surface hydrophobicity. All measurements are reported as the mean ± one standard deviation (n=3).

Intrinsic fluorescence: Changes to the protein's tertiary conformation as a function of % DH was determined for u-LPI and h-LPI samples as a function of emission wavelength (300-400 nm; at 0.5 nm increments) at a constant protein concentration (0.05%, w/w) and excitation wavelength (280 nm) using a FluoroMax 4 spectrofluorometer (Aluko & Yada, 1995). Both excitation and emission slit widths were set at 5 nm. Fluorescent intensity (FI) was reported as a function of emission wavelength and maximum FI value (Klassen & Nickerson, 2012). All measurements are reported as the mean ± one standard deviation (n=3).

Interfacial tension: Interfacial tension between u-LPI and h-LPI solutions (0.25%; w/w, in the 35 mM sodium phosphate buffer (pH 7.80)) and flaxseed oil was measured using a semi-automatic tensiometer (Lauda TD2, Lauda Dr. R. Wobser GmbH & Co., Lauda-Königshofen, Germany) and a Du Noüy ring (Can Karaca et al., 2011a). Interfacial tension for a sample consisting of 35 mM sodium phosphate buffer-flaxseed oil (without protein) served as a control. Interfacial tension was calculated from the maximum force (F_{max}; units: milli-Newtons; instrument measures mg x gravity) using the following equation:

$$\gamma = \frac{F_{max}}{4\pi R\beta} \qquad \text{(eq. 3.4)}$$

where, γ is the interfacial tension (mN/m), R is the radius of the ring (20 mm), β is a correction factor that depends on the dimensions of the ring and the density of the liquid involved (McClements, 2005e). All measurements are reported as the mean ± one standard deviation (n=3).

3.3.7 Emulsifying properties

Emulsion activity (EAI) and stability (ESI) indices: The emulsifying properties (*EAI*, *ESI*) for u-LPI and h-LPI were determined using the modified methods of Pearce & Kinsella (1978). In brief, 5.0 g of 0.25% (w/w) protein solution and 4.0 g of flaxseed oil were homogenized using an Omni Macro Homogenizer (Omni International, Marietta, GA, USA) with a 20 mm saw tooth generating probe at speed 4 (~7,200 rpm) for 5 min. A 50 µL emulsion sample was immediately removed from the bottom of the tube (0 min) and added to 7.5 mL of 35 mM sodium phosphate buffer (pH 7.80) containing 0.1% sodium dodecyl sulfate (SDS). A second 50 µL aliquot of the emulsion (from the bottom of the tube) was also taken at 10 min of static storage at room temperature. Sample absorbance was measured at 500 nm using a Genesys 10 UV-visible spectrophotometer (Thermo Scientific, Madison, WI, USA) using plastic cuvettes (1 cm path length). *EAI* and *ESI* were calculated using the following equations:

$$EAI\ (m^2/g) = \frac{2 \cdot 2.303 \cdot A_0 \cdot N}{c \cdot \varphi \cdot 10000} \qquad \text{(eq. 3.5)}$$

$$ESI(\text{min}) = \frac{A_0}{\Delta A} \cdot t \qquad \text{(eq. 3.6)}$$

where, A_0 is the absorbance of the diluted emulsion immediately after homogenization, N is the dilution factor, c is the weight of protein per volume (g/mL), φ is the oil volume fraction of the emulsion, ΔA is the change in absorbance between 0 and 10 min $(A_0 - A_{10})$ and t is the time interval, 10 min (Gu et al., 2005; Guzey & McClements, 2007; Gu et al., 2009). All measurements are reported as the mean ± one standard deviation (n=3).

3.3.8 Statistical analyses

A one-way analysis of variance (ANOVA) with a Scheffe post-hoc test was used to determine statistical differences among the physicochemical and emulsifying tests for the u-LPI and h-LPI materials. All statistical analyses were performed with SPSS version 17.0 software (SPSS Inc., 2008, Chicago, IL, USA).

3.4 Results and Discussion

3.4.1 Proximate composition of LPI

The mean and standard deviation values for the proximate composition of the LPI were, 87.27 ± 1.66% protein, 2.20 ± 0.20% moisture, 3.25 ± 0.03% ash, 1.12 ± 0.23% lipid and 6.16% carbohydrate; with the three separate preparations yielding a similar composition. Levels were also comparable to what others have reported for LPI produced by isoelectric precipitation (Alsohaimy et al., 2007; Can Karaca et al., 2011a; Joshi et al., 2011). Based on literature reports, the protein concentration of the prepared LPI of 87.27 or 89.23% on a wet or dry weight basis, respectively, supports its identification as an isolate. Currently, there is no universal classification scheme to separate protein concentrates from isolates for all legume proteins (Can Karaca et al., 2011a). However, Pearson (1983) reported that a minimum protein content of 85% on a dry weight basis (6.25 nitrogen conversion factor) was required for a soy isolate designation.

3.4.2 Effect of enzyme/substrate ratio on protein hydrolysis

The degree of lentil protein hydrolysis over a 2 h time period was investigated for different E/S ratios using trypsin. The E/S ratios tested ranged from 1/100 to 1/1000 and were based on those reported in literature for other plant protein-protease reactions (Henning et al.,

1997, Ventureira et al., 2010; Yust et al., 2010). Enzyme catalyzed hydrolysis of LPI results showing the concentration of free amino groups (*h*) and corresponding degree of hydrolysis (% DH) as a function of E/S ratio and reaction time are shown in Figures 3.1A and 3.1B, respectively. At the highest E/S ratio (1/100) studied, LPI hydrolysis occurred rapidly reaching a DH of ~42% within the first 40 min, which increased at a much slower rate to ~57% over the 120 min reaction period (Figure 3.1B). At an E/S ratio of 1/250, the initial increase in % DH was slower reaching ~15% DH after 40 min and plateaued at ~31% DH after 60 min (i.e., 60-120 min) (Figure 3.1B). At E/S ratios of 1/500 and 1/1000, protein hydrolysis proceeded very slowly over the 120 min reaction time period and reached maximums of ~17 and ~4%, respectively (Figure 3.1B). Based on these results, an E/S ratio of 1/250 was selected for LPI hydrolysis as the reaction conditions could be readily controlled to achieve the desired 4-20% DH products. The % DH range selected was hypothesized to produce products that would impact functionality, specifically emulsion formation and stability, in both a positive (% DH < 10) and negative (% DH >10) manner.

3.4.3 Characterization of LPI hydrolysates

The application of the selected E/S (1/250) ratio on a higher LPI volume (150 mL) gave % DH values for hydrolysis times of 5, 15 and 30 min of, 4.39 ± 1.21, 8.78 ± 1.66, and 19.56 ± 0.71, respectively. These % DH values were higher than those shown in Figure 3.1B and may be explained by the longer time (10 vs. 20 min) associated with trypsin inactivation due to the extra volume of the scaled up reaction.

SDS-PAGE analysis of the u-LPI and h-LPI samples is shown in Figure 3.2. Unhydrolyzed LPI (lane 2) showed multiple major bands between ~80-100 kDa, ~50 kDa, ~31 kDa and ~28 kDa. As the % DH of the h-LPI samples increased from ~4 to ~20% (lanes 3-5) a significant reduction in the ~80-100 kDa molecular weight bands with a concomitant increase in the intensity and/or appearance of protein bands in the ~30-50 kDa and <30 kDa range was evident. Identification of major bands to known molecular weight protein sub-units (e.g., 11S or 7S) could not reliably be matched, since protein structure was already presumed altered during the deactivation of trypsin (i.e., heating to 85^{o}C for 20 min). These results would support the

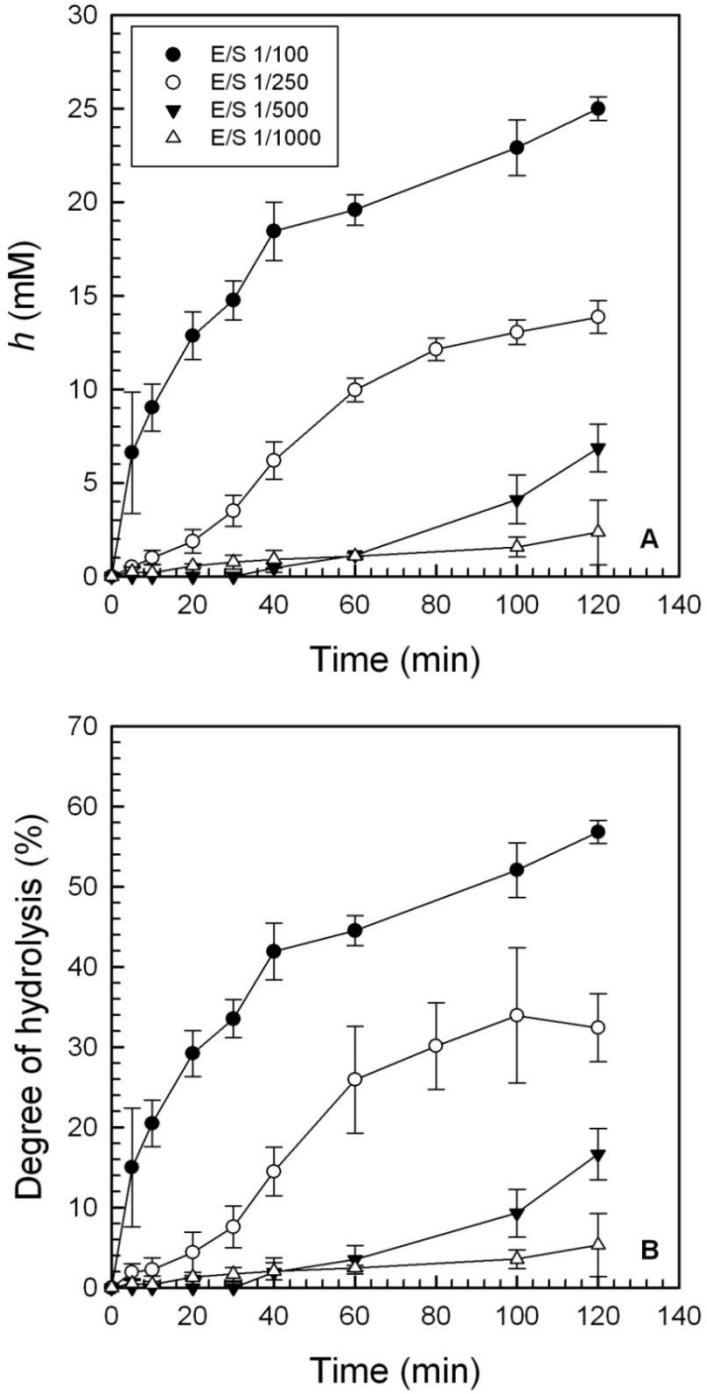

Figure 3.1 TNBS reaction value associated with the number of free amino groups (*h*; glycine mmoles of free amino acids) (A) and degree of hydrolysis (%) (B) as a function of time (min) and E/S ratio (1:100, 1:250, 1:500 and 1:1000). Data represents the mean ± one standard deviation (n=3)

conclusion that significant changes to the protein structure of LPI occurred upon treatment with trypsin to produced h-LPI. Trypsin catalyzed reactions could influence the quaternary and tertiary conformations of proteins by cleaving peptide bonds within individual or aggregated proteins to give smaller protein sub-units and/or smaller peptides. In addition, only minor differences were evident by SDS-PAGE amongst the three hydrolyzed products (% DH 4-20).

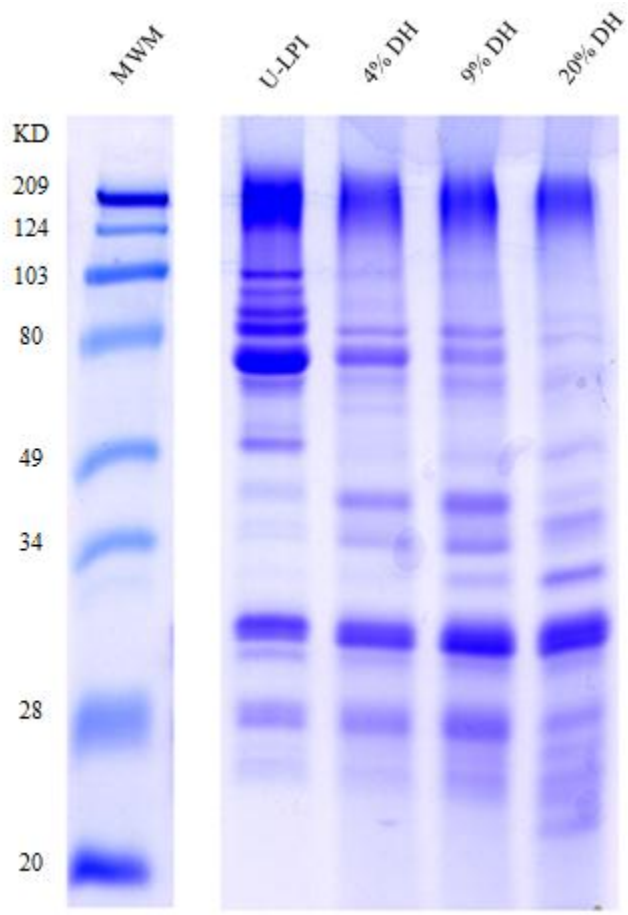

Figure 3.2 SDS PAGE (non-reducing) of LPI as a function of degree of hydrolysis (%) (Lanes: (1) molecular weight marker (MWM); (2) u-LPI, (3) 4% DH, (4) 9% DH and (5) 20% DH).

3.4.4 Physicochemical properties of u-LPI and h-LPI

Surface characteristics

As a prerequisite for appreciable surface activity, a protein should possess both a charge and patches of hydrophobicity on its surface. A plot of both surface hydrophobicity and charge (zeta potential) of u-LPI and the h-LPI samples as a function of % DH is shown in Figure 3.3. The surface charge for all samples, as indicated by their zeta potentials, carried a net negative charge at pH 7.80, which is readily explained by the fact that this pH value was well above the isoelectric point of LPI (pI = 4.5; Bora, 2002). Sample surface charge was found to change only

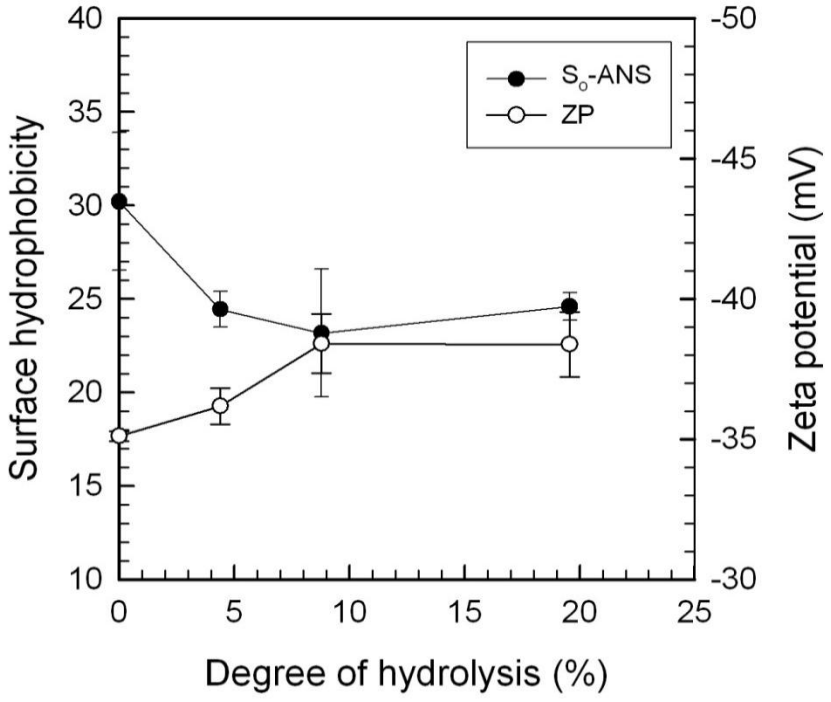

Figure 3.3 Surface hydrophobicity (S_o-ANS) and zeta potential (ZP) for trypsin-treated LPI as a function of degree of hydrolysis (%) at pH 7.80. Data represents the mean ± one standard deviation (n=3).

slightly from ~-35 mV for the u-LPI to ~-37 mV for h-LPI, but was found to be insignificant ($p>0.05$). Can Karaca et al. (2011a) reported a zeta potential of -22.6 mV for an unheated and unhydrolyzed LPI at pH 7.00. In this study, the LPI was heated to 37^{o}C for 1-1.5 h during the hydrolysis process, followed by an additional heating at 85^{o}C for 20 min during the trypsin

inactivation process. It is postulated that these heating steps resulted in protein unraveling, which could account for the increased surface charge values obtained in this study when compared to those of Can Karaca et al (2011a). It has been reported that it is difficult to distinguish the effects of enzymatic hydrolysis, heating and the combination of these processes on the surface properties of proteins (Panyam & Kilara, 1996).

An analysis of variance revealed that the surface hydrophobicity of u-LPI (~30 S_o-ANS) was significantly higher than that of h-LPI (~24 S_o-ANS) ($p<0.05$), however no differences were observed as a function of % DH (4-20%) ($p>0.05$) (Figure 3.3). It has been reported that protein surface hydrophobicity is dependent on experimental (i.e. heat/hydrolysis) conditions, protein characteristics (source, amino acid profile) and enzyme specificity; and can either increase or decrease with hydrolysis (Calderon de la Barca et al., 2000). For example, the limited hydrolysis of faba bean legumin with trypsin (Henning et al., 1997) and soy protein isolate with pancreatin (Hettiarachchy & Kalapathy, 1997) lead to a rise in S_o-ANS, which was associated with the partial unraveling of the protein's conformation and the cleaving off/or release of small polypeptides/peptides into solution; both of which exposes a greater number of hydrophobic amino groups previously buried within the interior of the protein. In contrast, others have reported a decline in S_o-ANS with limited hydrolysis, as was the case in the present study. As examples, a decline in hydrophobicity has been reported for trypsin-treated soy flour (Jung et al., 2005), bromelain-treated soy protein isolate (Ortiz & Wagner, 2002), extruded/unextruded soy protein concentrate with alcalase and esperase (Surowka et al., 2004) and, an alcalase-treated brewers' spent grain protein concentrate (Celus et al., 2007). Jung et al. (2005) proposed that this decline in S_o-ANS is associated with the exposure of buried hydrophobic groups (through partial unraveling or release of polypeptides/peptides), followed by their aggregation via hydrophobic interactions; effectively re-burying them within the interior of a larger aggregated structure. Conversely, Surowka et al. (2004) suggested that the partial unraveling and/or cleaving of polypeptides/peptides upon limited protein hydrolysis could lead to modifications with higher conformational entropy (or flexibility), enabling it to re-fold so that hydrophobic groups are re-buried within the interior of the new structure.

Intrinsic fluorescence

Changes in intrinsic fluorescence for u-LPI and h-LPI samples as a function of their degree of hydrolysis were studied over an emission wavelength range of 300-400 nm (Figure 3.4). The fluorescent intensities for h-LPI samples (DH 4-20%) were found to be slightly lower than that observed for u-LPI at peak maximums of 322 and 332 nm (Figure 3.4). Although this decrease was not significant ($p>0.05$), the trend supported the S_o-ANS data for these samples. A similar trend was found between 340 nm and 400 nm, where the u-LPI material was higher than the h-LPI samples (which were similar). Fluorescence intensity is indicative of the level of exposure of aromatic amino acids (i.e. tryptophan, tyrosine and phenylalanine) to the reaction solvent, a phenomenon which is highly sensitive to a protein's tertiary conformation in solution and the neighboring amino acids to the aromatic groups (Kronman & Holmes, 1971; Pain, 2005). The findings in the present study differ from what would typically be expected, where enzymatic hydrolysis would lead to increased exposure of buried aromatic groups to the solvent and a higher fluorescent intensity (Pain, 2005). However, a decrease in intensity would be expected if hydrolysis lead to first the exposure of aromatic groups, followed by protein aggregation (via hydrophobic interactions) which then re-buried the groups within the interior of the protein cluster; or if hydrolysis led to increased protein flexibility, which upon re-folding influenced aromatic site exposure. A similar decline in fluorescence intensity with limited enzymatic hydrolysis has been previously reported for broad bean legumin-trypsin (Braudo et al., 2006) and peanut protein-alcalase-trypsin (Zhao et al., 2011).

Interfacial properties

Changes to the interfacial tension of LPI as a function of % DH in a flaxseed oil-water mixture is shown in Figure 3.5. An analysis of variance revealed that the interfacial tension of u-LPI (~6.5 mN m^{-1}) was significantly greater than that of h-LPI (~6.1 mN m^{-1}) ($p<0.05$), however no differences were observed in samples as a function of % DH ($p>0.05$) (Figure 3.5). In the absence of LPI, the measured interfacial tension between the oil and water phases was 7.65 mN m^{-1}. The reduced interfacial tension in the presence of h-LPI relative to u-LPI may be due to the ability of the modified proteins to align with, and integrate into the oil-water interface. Based on

hydrophobicity data, it was proposed that hydrolysis of LPI lead to an initial exposure of hydrophobic groups, followed by their aggregation (via hydrophobic interactions) to form larger

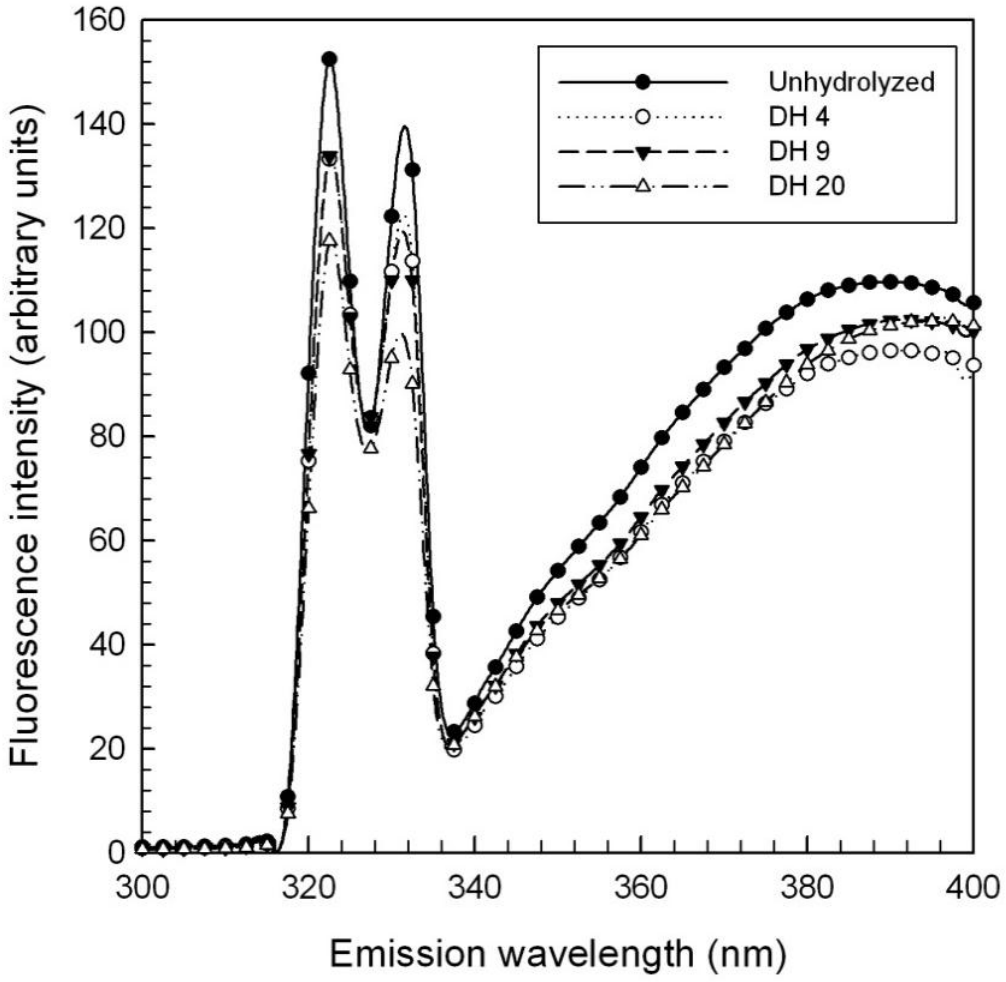

Figure 3.4 Mean intrinsic fluorescence intensity (arbitrary units) emission scans for trypsin-treated LPI as a function of degree of hydrolysis (%) (at an excitation wavelength of 280 nm) (n=3).

aggregates. Consequently, surface hydrophobicity was less for h-LPI than u-LPI materials. Interfacial tension was lower for h-LPI material possibly because of greater solubility (less hydrophobic) in the aqueous phase allowing for greater rates of diffusion to the interface. Surface charge was similar between u-LPI and h-LPI materials, therefore was not considered to be a major determinant in interfacial tension.

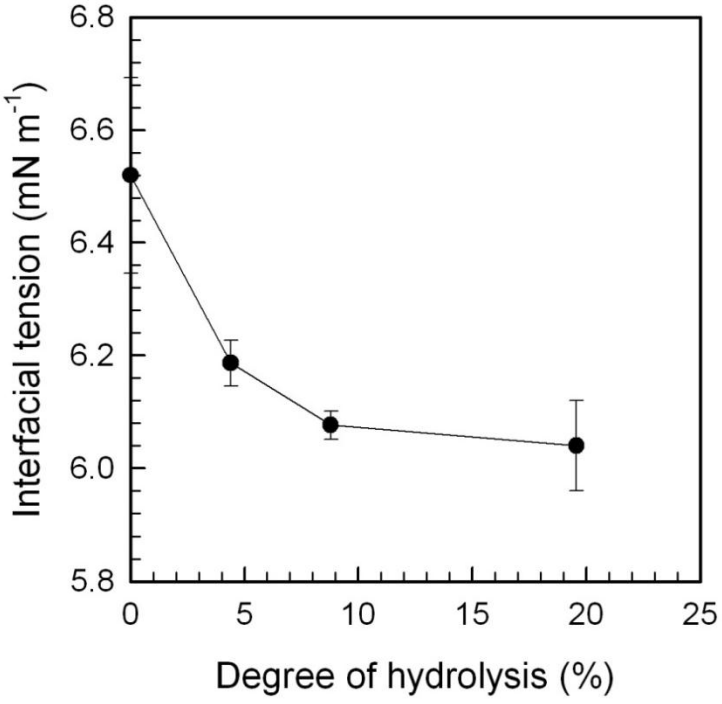

Figure 3.5 Interfacial tension (mN m^{-1}) for trypsin-treated LPI as a function of degree of hydrolysis (%) at pH 7.80. Data represents the mean ± one standard deviation (n=3).

3.4.5 Emulsifying properties

The emulsification activity and stability indices for LPI as a function of the % DH are shown in Figure 3.6. Protein hydrolysis was found to have a negative effect on its emulsifying properties in a flaxseed oil-water system, where the EAI and ESI values declined from ~51 to ~47 m^2 g^{-1} ($p<0.05$) and ~12 to ~11 min ($p<0.001$), respectively, for u-LPI relative to h-LPI. No significant differences were observed for either EAI or ESI as a function of % DH over the range studied ($p>0.05$) (Figure 3.6). Similar results were reported by Zhao et al. (2011), where EAI values for native peanut proteins were greater than those that had undergone limited hydrolysis by alcalase. In literature, the effect of limited hydrolysis on the emulsifying properties of a protein seems to be related to both the system and extent of hydrolysis. Govindaraju & Srinivas

(2006) and, Severin & Xia (2006) both reported improved emulsifying properties (e.g., capacity) for enzymatically hydrolyzed arachin protein (major oilseed protein of groundnuts) and whey

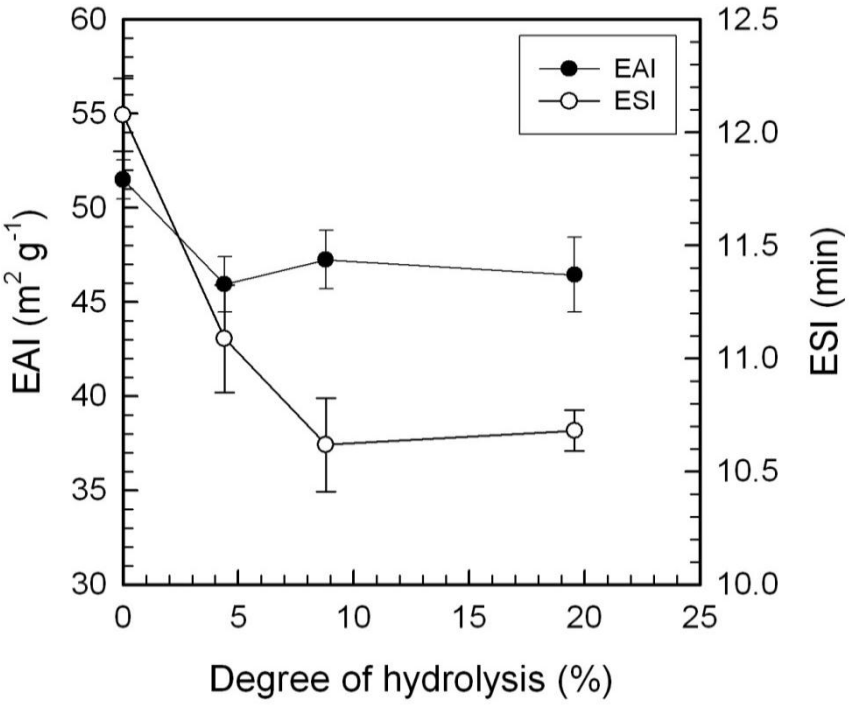

Figure 3.6 Emulsion activity (EAI, $m^2 g^{-1}$) and stability (ESI, min) indices for trypsin-treated LPI as a function of degree of hydrolysis (%) at pH 7.80. Data represents the mean ± one standard deviation (n=3).

proteins, respectively at low % DH, after which continued hydrolysis had a negative effect on emulsification. Severin & Xia (2006) proposed this phenomenon was related the molecular weight distribution of peptides produced during the hydrolysis process. The initially improved emulsifying properties with increased levels of DH was presumed to be related to a steady decrease the in molecular weight of the peptides generated, enabling greater alignment at the oil-water interface. This continued until an optimal molecular weight distribution was reached, afterwards continued hydrolysis led to reduced emulsifying properties. The authors proposed the decline could be attributed to the formation of more hydrophilic peptides that only weakly associated with the oil-water interface, or the viscoelastic film formed at the interface with the smaller peptides was insufficient to resist coalescence of neighboring droplets. In the present

study, h-LPI showed reduced hydrophobicity and increased surface charge relative to the u-LPI material (Figure 3.1); resulting in a material with greater difficulty aligning at the oil-water interface.

3.5 Conclusions

Overall, trypsin catalyzed hydrolysis of LPI yielding % DH of 4-20 produced materials with reduced surface hydrophobicity (S_o-ANS) and intrinsic fluorescence relative to u-LPI. These surface characteristic changes are postulated to be due to the initial exposure of reactive aromatic amino acids, which is followed by protein aggregation to re-bury these hydrophobic sites. The observed reduction in surface hydrophobicity for h-LPI materials was thought to play a key role in reducing their emulsifying properties relative to u-LPI. No differences in physicochemical and emulsifying properties were observed among the three h-LPI (4, 9 and 20%) materials studied. Findings from this study suggest that an unhydrolyzed LPI could be effectively used as plant-based food emulsifier.

3.6 Linkage to Chapter 4

The findings from this study provided foundational knowledge associated with structure-function relationships of lentil protein isolates modified by limited degrees of hydrolysis. This information will aid in a better understanding of the role of lentil protein isolates alone or as modified by controlled hydrolysis as wall materials in stabilizing emulsions used in encapsulation processes, and the impact they have on surface oil content and encapsulation efficiencies in the resulting freeze dried powders.

4. ENCAPSULATION OF FLAXSEED OIL WITHIN LENTIL PROTEIN-BASED MICROCAPSULES

4.1 Abstract

The physicochemical properties of microcapsules comprised of lentil protein and maltodextrin containing encapsulated flaxseed oil was investigated using native (n-LPI), pre-treated (heated, unhydrolyzed (u-LPI) and heated, hydrolyzed with trypsin (h-LPI)) lentil protein isolates and as a function of oil load (10.0, 20.0 and 30.0% of total solids). Specifically, the moisture, water activity, surface oil and flaxseed oil encapsulation efficiency (EE) were assessed, along with droplet size and emulsion morphology for all formulations. Moisture content (<6.0%) and water activity (<0.2) of all microcapsules were characteristics of dried powder ingredients. Light microscopy imaging of the emulsions prior to freeze drying, revealed that the h-LPI had larger oil droplets than the n-LPI and u-LPI, which both appeared similar. Findings were confirmed by light scattering, where droplet sizes were 6.7, 4.2 and 4.2 µm for the h-LPI, u-LPI and n-LPI stabilized emulsions, respectively. Overall, the microcapsules prepared from h-LPI showed significantly higher surface oil and lower EE than both the n-LPI and u-LPI formulations. Furthermore, as the flaxseed oil formulation content increased, surface oil increased with a concomitant decrease in EE. Experimental results showed that microcapsules prepared using n-LPI with 10.0% oil loading was found to have the lowest surface oil content (~3.7%) and highest EE (~62.8%). Product from this microcapsule formulation was subjected to an oxidative storage stability test over a 30 d room temperature (21-23°C) period vs. free oil. Unexpectedly, the encapsulation process induced autooxidation leading the production of a greater amount of primary oxidative products than free oil, as evident by higher peroxide values for the entrapped (15.6 meq. active 0_2/kg) vs. free oil (5.7 meq. active 0_2/kg) at day zero. During the 30 d storage stability test, PV values for the entrapped oil increased to 37.3 meq. active 0_2/kg, whereas values in the free oil remained constant.

4.2 Introduction

Flaxseed oil is a rich plant source of essential ω-3 fatty acids (e. g. α-linolenic acid [ALA]); these unsaturated lipids provide numerous reported beneficial health effects included but not limited to reducing the incidence of: coronary heart disease; some types of cancer; and neurological and hormonal disorders (Lukaszewic et al., 2004; Abuzaytoun & Shahidi, 2006). The limited incorporation of flaxseed oil into products by the food industry is due to flaxseed oil's low oxidative stability due to: its high content of polyunsaturated fatty acids; and off flavours caused by lipid rancidity. The latter typically being induced by processing and storage conditions, which results in reduced product shelf life (Lee & Ying, 2008). Flaxseed oil's sensitivity to oxygen means that product protection must be introduced so that humans can obtain the beneficial effects of the oil (Lukaszewic et al., 2004; Lee & Ying, 2008).

Encapsulation technologies have been employed to provide protection to oxygen sensitive materials from detrimental food processing and storage conditions as well as the harsh conditions of the human gastrointestinal tract (GI). Encapsulation is defined as the process whereby solids, liquids, or gaseous materials are enclosed in small capsules which can be formulated employing proteins, polysaccharides, lipids and their complexes (Desai & Park, 2005; Champagne & Fustier, 2007; Lee & Ying, 2008; Augustin & Hemar, 2009; Kailasapathy, 2009; Fang & Bhandari, 2010). Encapsulation also provides a means to mask the taste and smell of oils within food formulations to maintain final product sensory appeal and consumer satisfaction (Champagne & Fustier, 2007).

Due to their amphiphilic nature (i.e., having both hydrophilic and hydrophobic groups), proteins are ideal wall materials for oil encapsulation due to their emulsifying potential. Proteins migrate to the oil-water interface, re-orient themselves such that hydrophobic moieties are oriented inwards to the oil phase and hydrophilic groups are oriented outwards to the aqueous phase, and then form a viscoelastic film around the enclosed oil droplets (Dalgleish, 2004; Can Karaca et al., 2011a). The protein film provides stabilizing electrostatic repulsive (depending on pH and salts) and steric forces that resist phase separation (Dalgleish, 2004, Can Karaca et al., 2011a). For encapsulation, stable oil-in-water emulsions are dried through various processes to yield a free flowing powder. The use of plant proteins as encapsulating agents for edible oils has been recently reviewed by Nesterenko et al. (2013). The use of plant proteins in food

formulations such as bioactive compound encapsulation (e.g. flaxseed oil), provides a competitive advantage to ingredient suppliers and food manufactures due to significant growth towards specialty markets, such as those that restrict the use of animal-derived proteins based on religious beliefs or dietary preferences.

The emulsifying properties of various protein isolates derived from chickpea, lentil, faba bean, pea and soy produced by both isoelectric precipitation and salt extraction was recently explored by Can Karaca and co-workers (2011a). The authors findings indicated that isolates produced by isoelectric precipitation displayed better emulsifying properties for the legume proteins than those extracted using salts, and that both lentil and chickpea had comparable properties to soy, and better emulsifying potential than pea and faba bean. The authors further optimized lentil and chickpea protein-stabilized emulsions based on pH, protein concentration and oil content (Can Karaca et al., 2011b). Formation of stable emulsions is a prerequisite to appreciable surface coating of the oil droplets and high oil encapsulation efficiencies (Nesterenko et al., 2013). Partial enzymatic hydrolysis represents another means of potentially improving the emulsifying properties of plant proteins. However, this effect is highly dependent on the protein source and the processing conditions used (Panyam & Kilara, 1996). For instance, Karayannidou et al. (2007) and Guan et al. (2007) reported that trypsin-treated sunflower protein isolate (DH ~10%) and oat bran protein (DH ~4-8%), respectively, showed improved emulsifying properties relative to the non-hydrolyzed material. In contrast, Govindaraju & Srinivas (2006) reported that the hydrolysis of arachin protein (DH ~19%) using papain, alcalase and fungal protease resulted in a significant decrease in emulsifying potential. Experimental work presented in Chapter 3 of this thesis investigated the emulsifying properties of lentil protein isolates as a function of their degree of hydrolysis (DH; 4, 9 and 20%) following exposure to trypsin/heat, relative to an unhydrolyzed material. Results showed that protein isolate emulsifying properties were reduced when treated with trypsin relative to its native form, and that no statistical differences in these properties were observed for the DH values studied. However limited information exists relating to the potential use of partially hydrolyzed proteins from plant sources as encapsulating agents.

Lentils (*Lens culinaris*) are primarily comprised of the storage proteins albumins and globulins, along with minor amounts of prolamins and glutelins (Bhatty et al., 1976; Swanson,

1990; Boye et al., 2010). The globulins, which make up the majority of the protein content in lentils (~47% of total seed proteins), are comprised of two main proteins, legumin (11S) and vicilin (7S) (Bhatty et al., 1976; Swanson, 1990; Boye et al., 2010). Legumin is a hexameric quaternary structure with acidic (molecular mass (MM) of ~40 kDa) and basic (MM of ~20 kDa) subunits. Whereas the 7S vicilin has a trimeric structure with a MM of 175-180 kDa (Swanson, 1990; Boye et al., 2010). A third globulin protein, known as convicilin, has a subunit MM of 71 kDa and a MM of 290 kDa in its native form (Swanson, 1990; Boye et al., 2010).

The overall goal of this research study was to investigate the potential use of native and pre-treated (heated/unhydrolyzed and heated/hydrolyzed) lentil protein isolates, in the formulation of wall materials for the encapsulation and oxidative protection of flaxseed oil.

4.3 Materials and Methods

4.3.1 Materials

Whole green lentil seeds (CDC Greenland) and flaxseed oil were provided by the Crop Development Centre (Saskatoon, SK, Canada) and Bioriginal Food and Science Corp. (Saskatoon, SK, Canada), respectively. The following materials were purchased from Bio-Rad (Mississauga, ON, Canada): Bio-Rad Broad Range Marker, Bio-Rad Tris-HCl gel (15%), Coomassie blue R-350 and Laemmli Sample Buffer. Hexane was purchased from Fischer Scientific (Ottawa, ON, Canada). Butylated hydroxytoluene (BHT), malondialdehyde (MDA) (1,1,3,3-tertraethoxy-propane), picrylsulfonic acid (trinitrobenzene sulfonic acid (TNBS)), pyridine, soluble potato starch, 2-thiobarbituric acid (TBA) and trypsin (10,600 units/mg) were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). The following chemicals were purchased from VWR (Edmonton, AB, Canada): acetic acid, chloroform, dimethyl sulfoxide (DMSO), hydrochloric acid, isopropanol, n-butanol, potassium iodide, sodium dihydrogen phosphate, sodium dodecyl sulfate, sodium hydroxide, sodium monohydrogen phosphate, sodium thiosulfate, and sulfuric acid. All chemicals used in this study were of reagent grade except for sodium dodecyl sulfate which was ultrapure. The water used in this research was produced from a Millipore Milli-Q^{TM} water purification system (Millipore Corp., Milford, MA, USA). Maltodextrin (DE 11, M100^{TM} Maltrin) was donated by Cargill Inc. (Cargill Texturizing Solutions, Cedar Rapids, IA, USA).

4.3.2 LPI preparation

Whole green lentil seeds were ground into a fine flour using a food processor (Cuisinart mini-prep plus grinder) (~1 min), followed by an IKA A11 analytical mill treatment (IKA Works Inc., Wilmington, NC, USA) (~1 min). The flour was then defatted by stirring in hexane (1:3 [w/v], flour: hexane) for 40 min; this procedure was repeated two additional times. Protein isolates were prepared from the defatted flour based on methods of Papalamprou et al. (2010) and Can Karaca et al. (2011a). In brief, 100 g of defatted flour was mixed with water at a 1:10 (w/v) ratio. The pH of the resulting suspension was adjusted to 9.00 using 1.0 N NaOH followed by mechanical stirring at 500 rpm for 1 h at room temperature (21-23^{o}C). The mixture was then centrifuged at 5,000 x *g* at 4°C for 20 min using a Sorvall RC-6 Plus centrifuge (Thermo Scientific, Asheville, NC, USA) to collect the supernatant. The resulting pellet was re-suspended in water at a ratio of 1:5 (w/v), adjusted to pH 9.00 using 1.0 N NaOH, stirred for 1 h at room temperature, followed by centrifugation (5,000 x *g*, 20 min, 4°C). Supernatants were pooled and adjusted to pH 4.50 with 0.1 M HCl to precipitate the protein (Bora, 2002). The LPI was washed with water, frozen at -30°C and then freeze dried using a Labconco FreeZone 6 freeze drier (Labconco Corp., Kansas City, MO, USA). This material is referred to as native LPI (n-LPI). Proximate composition for the resulting n-LPI were conducted according to AOAC Official Methods 925.10 (moisture), 923.03 (ash), 920.85 (lipid), and 920.87 (crude protein by using %N × 6.25) (AOAC, 2003). Carbohydrate content was determined on the basis of percent differential from 100%. Proximate analyses were performed on three separate protein isolate preparations with each preparation analyzed in duplicate (n=2).

4.3.3 Preparation of the protein wall material for encapsulation

Three different LPI solutions were produced for use as wall materials: (a) native LPI (n-LPI); (b) unhydrolyzed, heated LPI (u-LPI); and (c) hydrolyzed, heated LPI (h-LPI). All LPI wall material solutions were prepared in triplicate.

The *n-LPI wall material* was prepared by dissolving the freeze dried n-LPI powder in 35 mM sodium phosphate buffer (pH 7.8) to achieve a final protein concentration of 4.0% (w/v), and was then allowed to stir overnight at 4°C to promote protein solubility.

The *u-LPI wall material* (unhydrolyzed, heated) was initially prepared in a similar manner as the n-LPI solution (as above) 4.0% (w/v). However was then heated in a similar manner as the hydrolyzed material, except no enzymes were present. In brief, the n-LPI solution was heated at 37^{o}C for 1 h within a shaking (90 rpm) water bath (PolyScience, Niles, IL, USA), followed by the transfer of nine individual 10 mL aliquots into separate test tubes. These tubes were then heated at 85^{o}C for 20 min in the water bath, cooled to room temperature and then frozen (-30^{o}C) for later use.

The *h-LPI wall material* was prepared as per the u-LPI solution with the exception that after the 1 h heating period at 37^{o}C, trypsin was added at an enzyme/substrate ratio of 1:250 (w/w), followed by a hydrolysis period of 40 min at 37^{o}C. The hydrolysis solution was then heated at 85^{o}C (deactivation of the enzyme) for 20 min, cooled to room temperature and then frozen (-30^{o}C) for later use. Hydrolysis followed a similar method of Ventureira et al. (2010). The degree of hydrolysis was determined to be 18.37 ± 0.20% using the 2, 4, 6-trinitrobenzene sulfonic acid (TNBS) method of Adler-Nissen (1979) and, del Toro & Garcia-Carreno (2005), based on a glycine standard curve (with concentrations of 0.03, 0.04, 0.06, 0.08, 0.10, and 0.30 mM) and a total hydrolysis value of 32.82 mM of α-NH_2-Gly equivalent (based on acid hydrolysis and the standard curve use).

Prior to all experiments, frozen LPI wall materials were thawed overnight at room temperature (21-23^{o}C), transferred to a beaker and mechanically stirred for 20 min to ensure the material was a well distributed solution.

4.3.4 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The polypeptide profiles of n-LPI, u-LPI and h-LPI materials were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions using the Laemmli (1970) method. Sample concentrations of 0.5% (v/v) (e. g. 125 µL of a 4.0% sample in 875 µL water) for n-LPI, u-LPI and h-LPI initially were prepared and then 100 µL of each sample was pipetted into separate Eppendorf tubes followed by the addition of 100 µL of Laemmli sample buffer creating a sample concentration of 0.25% (v/v) for gel loading. Samples were heated to ~95^{o}C for 5 min, and then centrifuged (Eppendorf Centrifuge 5424, Hamburg, Germany) for 5 min at 12,000 x *g*. Molecular weight standards (Bio-Rad Broad Range Marker)

containing myosin 200 kDa, β-galactosidase 116 kDa, phosporylase b 97 kDa, bovine albumin serum 66 kDa, ovalbumin 45 kDa, carbonic anhydrase 31 kDa, soybean trypsin inhibitor 22 kDa, lysozyme 14 kDa, and aprotinin 7 kDa were run in conjunction with samples on a Bio-Rad Tris-HCl gel (15%) at 100-110V for ~1.5 h. Protein bands were stained using Coomassie blue R-350.

4.3.5 Preparation of LPI microcapsules by emulsification

The three wall materials (n-LPI, u-LPI and h-LPI) plus maltodextrin (as a drying aid and a capsule filling material) were used to encapsulate flaxseed oil at three different oil concentrations (10.0, 20.0 and 30.0%) so as to test the effect of oil concentration on encapsulation efficiency at core-to-wall ratios of 10.0, 20.0, and 30.0%. The wall materials used for capsule design were held constant at 4.0% (w/w) protein (n-LPI, u-LPI or h-LPI) and 36.0% (w/w) maltodextrin (dextrose equivalent of 11) to give a total of 40.0% dry solids. In brief, to 16 g of the unfrozen protein material (4.0%), 5.76 g of maltodextrin was added and then stirred for 30 min at room temperature (21-23^{o}C). The material was then transferred to a 50 mL centrifuge tube, to which flaxseed oil was added to give the desired core-to-wall ratio in the final dried product (10.0% oil level, 0.71 g of flaxseed oil; 20.0% oil level, 1.60 g of flaxseed oil; 30.0% oil level, 2.74 g of flaxseed oil). Samples were then homogenized using a Polytron PT 2100 (Kinematica AG, Switzerland) at 13,000 rpm for 3 min to form an emulsion. Immediately afterwards, emulsions were poured into a small aluminum tin (diameter 60.18 mm, depth 18.82 mm) and frozen (-30^{o}C). Samples were then freeze dried. Each of the aforementioned experiments were conducted in triplicate for each set of wall materials for each oil concentration (n=3).

4.3.6 Microcapsule properties

All freeze dried final products were crushed into a powder form using a spatula and their moisture content, water activity, surface oil content and encapsulation efficiency were determined as outlined below (n=3).

Moisture content and water activity: Moisture content of the freeze dried microcapsules was examined gravimetrically after drying the capsules in an Isotemp forced air oven (Fisher Scientific, ON, Canada) at 102^{o}C for ~16 h. Water activity of the capsules was determined using

a Decagon CX-1 water activity meter (Decagon Devices, Inc., Pullman, WA, USA) at room temperature.

Surface oil and encapsulation efficiency: Surface oil content of the freeze dried microcapsules was determined according to the method of Liu et al. (2010). Briefly, about 1 g of microcapsules were dispersed in 30 mL of hexane and vigorously shaken for 30 s. The solvent including microcapsules were then filtered through Whatman Gr. 1 paper into a 100 mL beaker. The beaker and solvent were left in a fumehood overnight to allow for the evaporation of the solvent. The surface oil of the microcapsules was determined gravimetrically following heating of the beaker at 102°C for 30 min to remove residual solvent and cooling in a desiccator for 20 min. The total oil content was assumed to be at the initial levels added that is 10.0, 20.0, and 30.0%. Encapsulation efficiency (EE) was then calculated from the following equation (Anwar & Kunz, 2011):

$$EE = \frac{(Total\ oil - Surface\ oil)}{Total\ oil} \times 100\% \qquad \text{(eq. 4.1)}$$

Droplet size distribution: The droplet size distribution was measured for all three wall materials employing only the 10.0% flaxseed oil content samples using fresh and reconstituted emulsions (i.e., freeze dried microcapsules). The latter was prepared by dispersing 0.5 g of freeze dried microcapsules in 4 mL of water, followed by stirring at 500 rpm for 5 min at room temperature. The droplet size distribution of all emulsions were analyzed using a Mastersizer 2000 laser light scattering instrument (Malvern Instruments LTD., Worcestershire, United Kingdom) with a Hydro 2000S sample handling unit (containing water). Immediately after homogenization, a sample was drawn from the bottom of the 50 mL centrifuge tube. This sample was loaded into the Hydro 2000S wet sample unit under continuous stirring (850 rpm). All measurements were taken at an obscuration level of ~14% by the addition of water. Droplet sizes were calculated by the instrument in accordance with the Mie Theory using the refractive index difference between the droplets and the dispersing medium to predict the intensity of the scattered light. The ratio of the refractive index of flaxseed oil (1.479) to that of the dispersion medium (1.330) was 1.112. Droplet size measurements were reported as volume-length mean diameters ($d_{4,3}$) which is expressed as:

$$d_{4,3} = \frac{\sum_{i=1} n_i \cdot d_i^4}{\sum_{i=1} n_i \cdot d_i^3} \qquad \text{(eq. 4.2)}$$

where n_i is the number of droplets of diameter (d_i) (McClements, 2005a).

Light microscopy: The microstructures of fresh emulsions were examined immediately after homogenization using a Nikon Eclipse E400 light microscope equipped with a Nikon DS-FiL color camera and a 40X objective lens and condenser (Nikon Instruments Inc., Melville, NY). A drop from the emulsion was mounted onto a slide with a cover slip.

4.3.7 Oxidative stability

Oxidative stability of free and encapsulated oils within the n-LPI-maltodextrin microcapsules only were investigated over a 30 d period at room temperature by measuring the peroxide value (measures primary oxidative products) and 2-thiobarbituric acid reactive substances (TBARS) content (measures secondary oxidative products). Freeze dried microcapsules (~4 g/bottle) and free oil (~3 mL/bottle) were transferred into 10 mL amber glass bottles, flushed with N_2, and individually sealed for storage. Oxidative tests were performed on both encapsulated and free oil at days 0, 10, 20 and 30 using separate unopened bottles. For each day, 3 bottles were prepared for both the free oil and the microcapsules in order for values to be reported in triplicate. Prior to each oxidative test, oil was extracted from the microcapsules using a modified method of Klinkesorn et al. (2006). In brief, 4 g of capsules were dispersed within 16 mL of water, and then stirred for 2 min at 300 rpm. Oil was then extracted by adding 100 mL of hexane/isopropanol (3:1 v/v), followed by stirring at 300 rpm for 15 min. The solutions were then centrifuged within 250 mL centrifuge tubes (using a Sorvall RC-6 Plus centrifuge (Thermo Scientific, Asheville, NC, USA)) at 5,000 x *g* for 5 min. The clear organic phase was decanted and filtered through Whatman Gr. 1 filter paper containing anhydrous Na_2SO_4 into a 125 mL Erlenmeyer flask and the organic solvent was evaporated under a steady stream of N_2 gas.

Peroxide value (PV): In brief, ~0.2 g of extracted flaxseed oil was weighed into a 125 mL Erlenmeyer flask, followed by the addition of 10 mL of 3:2 acetic acid/chloroform (v/v) solution and 200 µL of saturated potassium iodide (KI). After stirring at speed 3 for 1 min using an IKA five position stirrer, 10 mL of water was added to this mixture. The mixture was then titrated with 0.001 N sodium thiosulfate ($Na_2S_2O_3$) until a faint yellow colour was observed and

then the 0.5 mL aliquot of 1.0% (w/v) starch indicator was added to the mixture, and the titration was continued until the purple colour disappeared. Sample PV was calculated as:

$$PV = \frac{(S - B) \times N \times 1000}{W} \qquad \text{(eq. 4.3)}$$

where *S* is the volume of $Na_2S_2O_3$ added to the sample, *B* is the volume of $Na_2S_2O_3$ of the blank, *N* is the normality of the $Na_2S_2O_3$ solution, and *W* is the sample weight (g) (Pegg, 2005).

2-thiobarbituric acid reactive substances (TBARS): In brief, ~40 mg of extracted flaxseed oil was weighed into a 10 mL volumetric flask and was dissolved and brought to volume with n-butanol. To a 2.0 mL Eppendorf tube, the following was added, 50 µL of 8.1% (w/v) SDS, 375 µL of 20% acetic acid (~ pH 3.5), 375 µL of 0.8% (w/v) 2-thiobarbituric acid (TBA), 8.25 µL of 0.02% (w/v) butylated hydroxytoluene (BHT) (in dimethyl sulfoxide (DMSO)) and 200 µL of the oil-butanol mixture. A standard curve was prepared using malondialdehyde (MDA) at a concentration range of 1.25-50.00 µM under the same experimental conditions. Samples and standards were then heated at 95°C for 1 h. After cooling in cold water (5 min), 0.9 mL of n-butanol/pyridine (15:1, v/v) was added, followed by vigorous shaking for 30 s. Samples and standards were centrifuged (Eppendorf Centrifuge 5424, Hamburg, Germany) at 4,000 × *g* for 10 min, and the upper organic layer was transferred to a 1.5 mL cuvette and the absorbance at 532 nm was measured against a butanol blank. TBA values were expressed as mg MDA eq/mg oil, which equates to the MDA content (nmol)/sample oil weight (mg) (Pegg, 2005; Akhlaghi & Bandy, 2010).

4.3.8 Statistical analyses

A two-way analysis of variance (ANOVA) with a Scheffe post hoc test was used to determine statistical differences among: a) surface oil and encapsulation efficiency for microcapsules prepared with different material types (n-LPI, u-LP and h-LPI) and percent oil concentrations (10.0, 20.0 and 30.0%); and b) droplet size D[4,3] for microcapsules prepared using different wall materials (n-LPI, u-LPI and h-LPI) with 10.0% oil from fresh or reconstituted emulsions. A paired student t-test was used to determine statistical differences between encapsulated vs. free oil during oxidative testing (PV and TBARS) on each day and over the duration of the oxidative storage study. All statistical analyses were performed with SPSS version 17.0 software (SPSS Inc., 2008, Chicago, IL, USA).

4.4. Results and Discussion

4.4.1 Material characterization

The proximate composition of LPI was determined to be 87.00% (wet basis, w.b.) protein, 2.58% moisture, 4.15% (w.b.) ash, 1.22% lipid (w.b.) and 5.05% (w.b.) carbohydrate. Protein levels were comparable to other LPI products produced by isoelectric precipitation (Alsohaimy et al., 2007; Can Karaca et al., 2011a; Joshi et al., 2011). For the present study, the protein powder was deemed an isolate since it contained >85% protein (89.30%, d.b.). Although there is no current universal classification scheme to properly distinguish a protein concentrate from an isolate as it concerns legume proteins, Pearson (1983) proposed the minimum requirement for a soy protein isolate was to have levels of 85% (d.b.) using a 6.25 nitrogen conversion factor.

In the present study, flaxseed oil was encapsulated using a native LPI (n-LPI), a heated and unhydrolyzed LPI (u-LPI), and a heated and hydrolyzed LPI (h-LPI). The addition of a heat treatment (85^{o}C for 20 min) acts to induce some level of denaturation of the lentil proteins resulting in aggregation, and terminates the hydrolysis reaction by denaturing trypsin. The addition of trypsin cleaves polypeptides at the carboxyl terminal of the amino acids lysine and arginine, however if proline is present on the carboxyl side of the cleavage site there will be no cleavage (Ventureira et al., 2010). The h-LPI used in this study had a total degree of hydrolysis after 40 min of enzymatic reaction of ~18.37 ± 0.20%. An SDS-PAGE analysis of the n-LPI, u-LPI, and h-LPI samples is shown in Figure 4.1. The n-LPI (lane 2) shows multiple bands ranging between approximately 60 and 100 kDa, as well as distinct bands with molecular weights of approximately 50, 40, 35, 22, 14 and 10 kDa. For the u-LPI (lane 3) material, higher molecular weight bands between approximately 60 and 100 kDa disappear with the exception of the one at ~66 kDa. Bands at ~40 and ~50 kDa were notably fainter after the heat treatment, whereas the band at ~35 kDa disappeared. Bands at approximately 22, 14 and 10 kDa remained unchanged relative to the n-LPI material. Changes to the molecular weight bands between the n-LPI and the u-LPI are proposed to reflect heat induced conformational changes in the proteins and subsequent aggregation. Protein aggregates were presumed to be larger than the molecular weight range of the gel selected. Similarly, high molecular weight bands ranging between 50 and 100 kDa disappeared for the h-LPI (lane 4) material. Also bands at approximately 40, 35, 22, 14

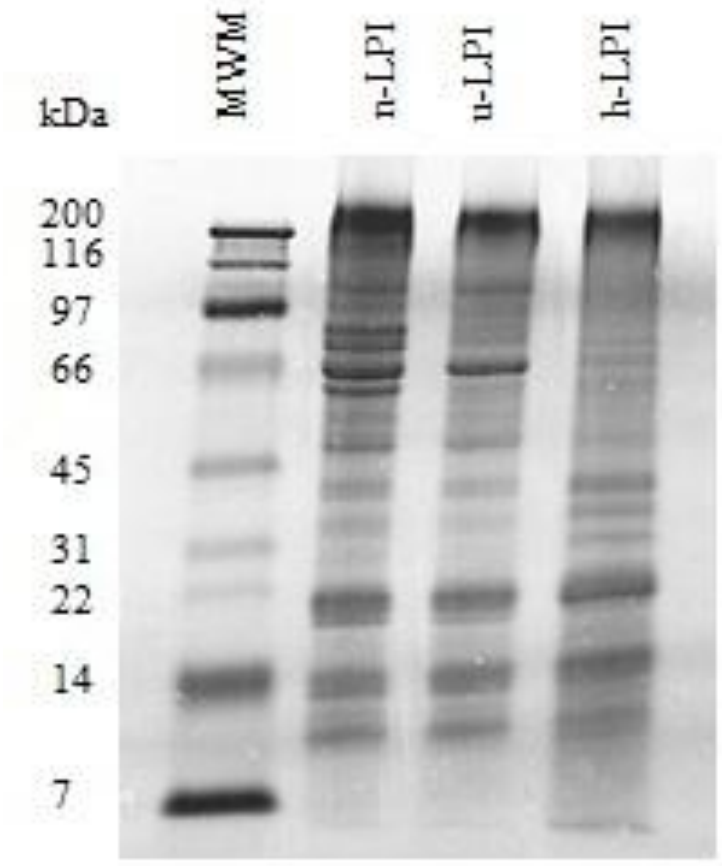

Figure 4.1 SDS PAGE (non-reducing) for native (n-LPI), unhydrolyzed (u-LPI), and hydrolyzed (h-LPI) lentil protein isolates (Lanes: (1) molecular weight marker (MWM); (2) n-LPI, (3) u-LPI, and (4) h-LPI.

and 10 kDa became darker and thicker in the h-LPI when compared to n-LPI and u-LPI, as well as a new band appeared at ~31 kDa; suggesting that trypsin was acting to cleave proteins or protein subunits to produce lower molecular weight species. Protein aggregation is believed to be responsible for the loss of high molecular weight fractions, similar to that observed in u-LPI.

4.4.2 Physical characterization of microcapsules

All capsules, regardless of the protein wall material used (n-LPI, u-LPI or h-LPI) and oil concentration were found to have low water activities (<0.2) (Figure 4.2A) and moisture levels (<6.0%) (Figure 4.2B). Moisture levels of 3-4% are typically used by the food industry to

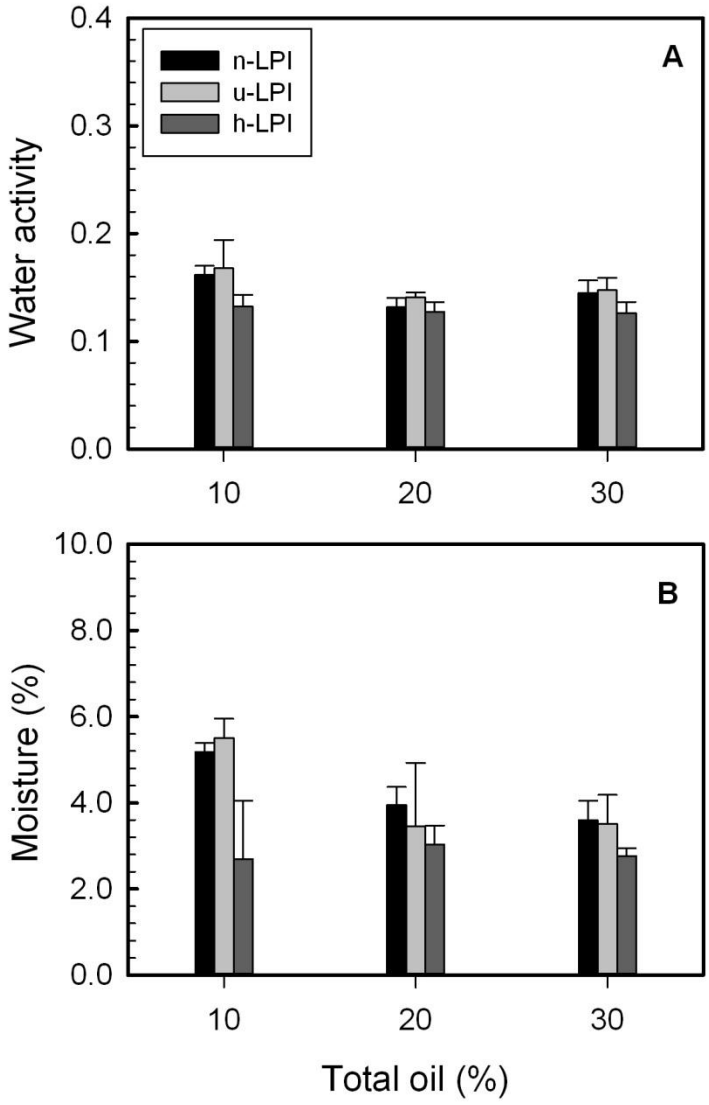

Figure 4.2 Mean water activity (A) and percent moisture (B) for native (n-LPI), unhydrolyzed (u-LPI), and hydrolyzed (h-LPI) lentil protein isolate (LPI; 4.0% w/w) and maltodextrin (36.0% w/w) microcapsules as a function of flaxseed oil concentration (percent of the total solids). Data represent the mean ± one standard deviation (n=3).

characterize a shelf-stable dried powder (Klinkesorn et al., 2006). Water activity is often related to final product stability and rates of deteriorative reactions of food systems regarding microbial growth, chemical/biochemical reaction rates and physical properties (Beristain et al., 2002). At

water activities of <0.2, microbial growth is severely limited and lipid oxidation is slowed (Fennema, 1996).

Surface oil contents refers to the amount of unencapsulated oil spatially located on the surface of the capsules after the drying process, caused by discontinuities in the wall material due to an initially less stable emulsion (Thies, 2003; Klinkesorn, et al., 2006). High surface oil levels (>2%) are undesirable as it reduces the shelf life of the powdered product due to oxidation and ensuing off-flavour and odour. The surface oil and encapsulation efficiencies (EE) of the produced microcapsules as a function of protein type and oil content are shown in Figures 4.3A and 3B, respectively. An analysis of variance of surface oil data showed that both protein material ($p<0.001$) and oil concentration ($p<0.001$) were highly significant factors, however their interaction (protein material x oil content) was not ($p>0.05$). Overall, surface oil for the n-LPI (9.4%) and u-LPI (9.7%) materials were similar, and lower than that observed for h-LPI (11.7%). Also, overall surface oil was found to increase linearly with increasing oil concentration, where surface oil content was found to be 3.7, 9.6 and 17.5% for the 10.0, 20.0 and 30.0% oil concentrations, respectively. In contrast, EE indicates the amount of oil that has been contained within the wall material, relative to the amount of oil on its surface (Klinkesorn et al., 2006). An analysis of variance of EE data found that both protein material ($p<0.001$) and oil content ($p<0.001$) were highly significant factors, however their interaction (protein material x oil content) was not ($p>0.05$). Overall, EE was greater for n-LPI (EE ~62.8%), followed by u-LPI (EE ~51.9%) and then h-LPI (EE ~41.8%). In contrast, overall EE was similar for oil concentrations of 10.0 (EE ~56.1%) and 20.0% (EE ~55.0%) oil present, and then decreased significantly at the 30.0% level (EE ~45.5%).

Surface oil content is a major determining factor for the encapsulation efficiency of microcapsule formulations. It should be noted that the amount of surface oil determined for capsules in powder form is highly dependent on the extraction method and conditions used (Klinkesorn et al., 2006). A common trend found among studies involved in encapsulating oil has been that as the oil load increases within the encapsulated powder, there is a corresponding increase in surface oil content and a decrease in EE (Kagami et al., 2003; Quispe-Condori et al., 2011; Tonon et al., 2011). The method of drying has also been shown to play a role in

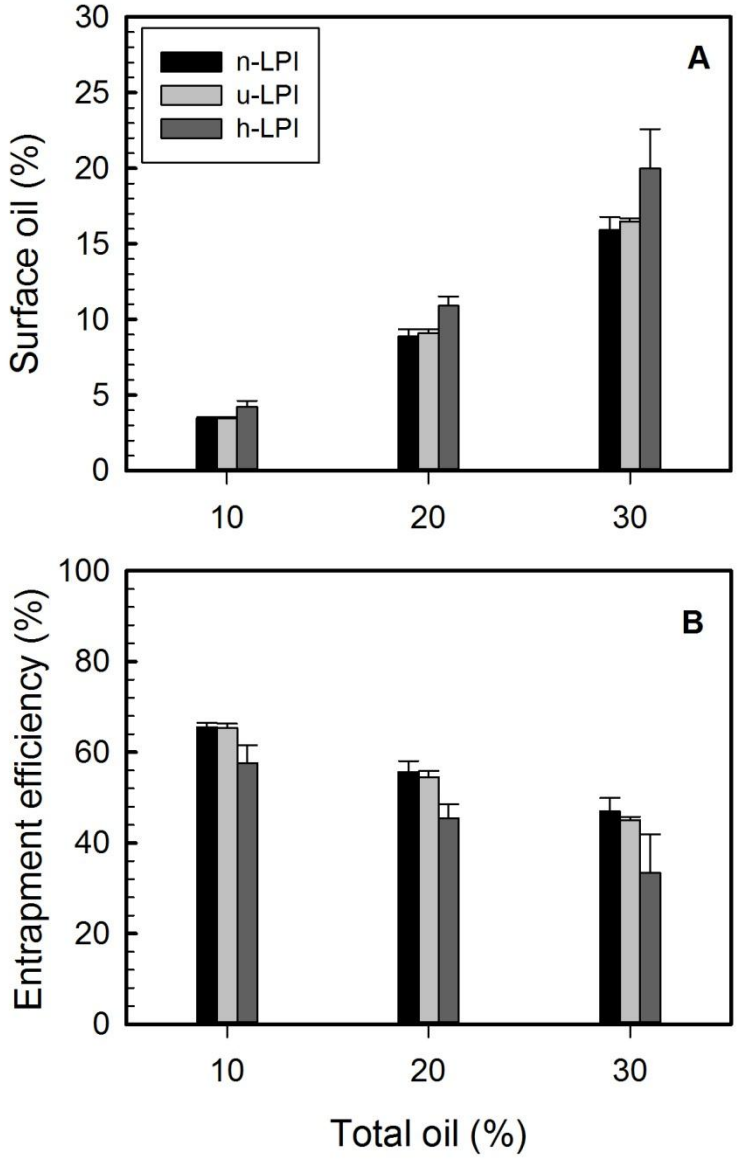

Figure 4.3 Percent surface oil (A) and entrapment efficiency (B) for native (n-LPI), unhydrolyzed (u-LPI), and hydrolyzed (h-LPI) lentil protein isolate (LPI; 4.0% w/w) and maltodextrin (36.0% w/w) microcapsules as a function of flaxseed oil concentration (percent of the total solids). Data represent the mean ± one standard deviation (n=3).

influencing surface oil and EE. For example, Quispe-Condori and co-workers (2011) used both spray and freeze drying to encapsulate flaxseed oil within zein protein at oil loading levels of 8 and 20%. The authors found that the surface oil contents in the spray and freeze dried powders were 90 and 79%, and 60 and 33%, for capsules with oil loads of 8 and 20%, respectively.

In the present study, microcapsules prepared from h-LPI showed significantly higher surface oil and lower EE than both the n-LPI and u-LPI wall materials. Furthermore, as the oil content increased, surface oil levels increased and EE decreased because there was insufficient protein coverage. A prerequisite for oil encapsulation is the formation of a stable oil-in-water emulsion. In Chapter 3 of this thesis, it was found that h-LPI displayed inferior emulsifying properties compared to u-LPI. As a consequence, h-LPI may not be able to stabilize the oil-water interface as well as the other wall materials (n-LPI and u-LPI) used in this study, leading to the formation of larger droplets due to coalescence during the time period following homogenization and before freezing (~60 min). Drusch et al. (2012) examined sodium caseinate and hydrolyzed casein for the encapsulation of fish oil and found that hydrolyzed casein resulted in lower EE than the unhydrolyzed material. The authors hypothesized that the hydrolyzed casein material would experience a greater loss of water during spray drying, causing shrinkage of the viscoelastic protein film surrounding the oil droplets. As a result, the film was weaker resulting in higher surface oil and lower EE in the final dried product.

Light microscopy images of protein isolate-maltodextrin stabilized emulsions immediately after homogenization with a 10.0% oil concentration are shown in Figures 4.4A-C. Although droplet size distributions were not measured from the images, overall the h-LPI (Figure 4.4C) appears to have larger oil droplets than the n-LPI (Figure 4.4A) and u-LPI (Figure 4.4B), which appear similar. The mean size distribution and volume weighted diameters (D[4,3]) for oil droplets within a fresh and reconstituted (i.e., microcapsules were re-dissolved) oil-in-water emulsions were measured by light scattering and are shown in Figures 4.5 and 4.6, respectively. In all cases, sized distribution as a function of oil droplet diameter ranged from ~1 to 30 μm and ~1to 45 μm for the n-LPI/u-LPI and h-LPI materials, respectively (Figure 4.5). In all cases, distributions were skewed towards lower diameters with the majority of droplets being <10 μm (Figure 4.5). Also, the droplet size distributions measured from reconstituted emulsions were shifted slightly towards larger sizes for all materials suggesting that coalescence may have

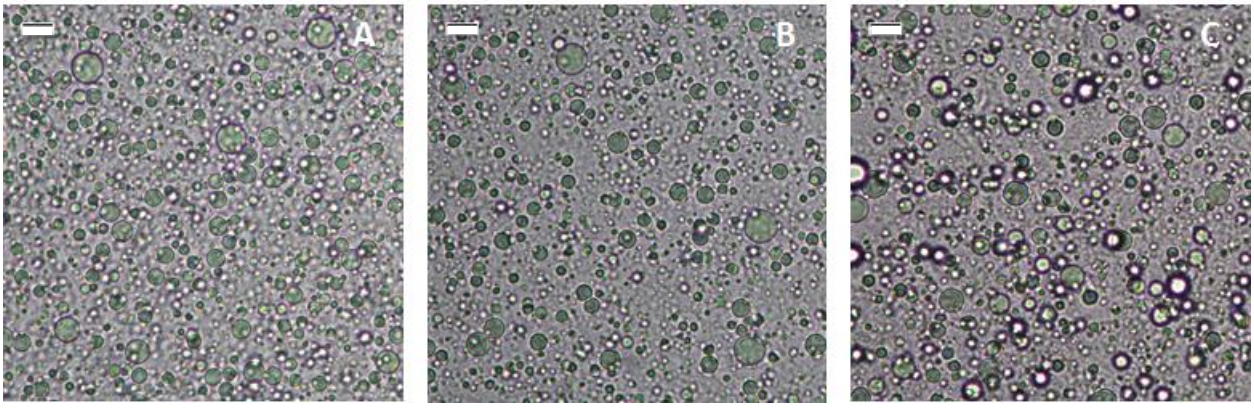

Figure 4.4 Light microscopy images of emulsions stabilized by native (A), unhydrolyzed (B), and hydrolyzed (C) lentil protein isolate (LPI; 4.0% w/w) and maltodextrin (36.0% w/w) wall formulations. Scale bars are equivalent to 10 µm.

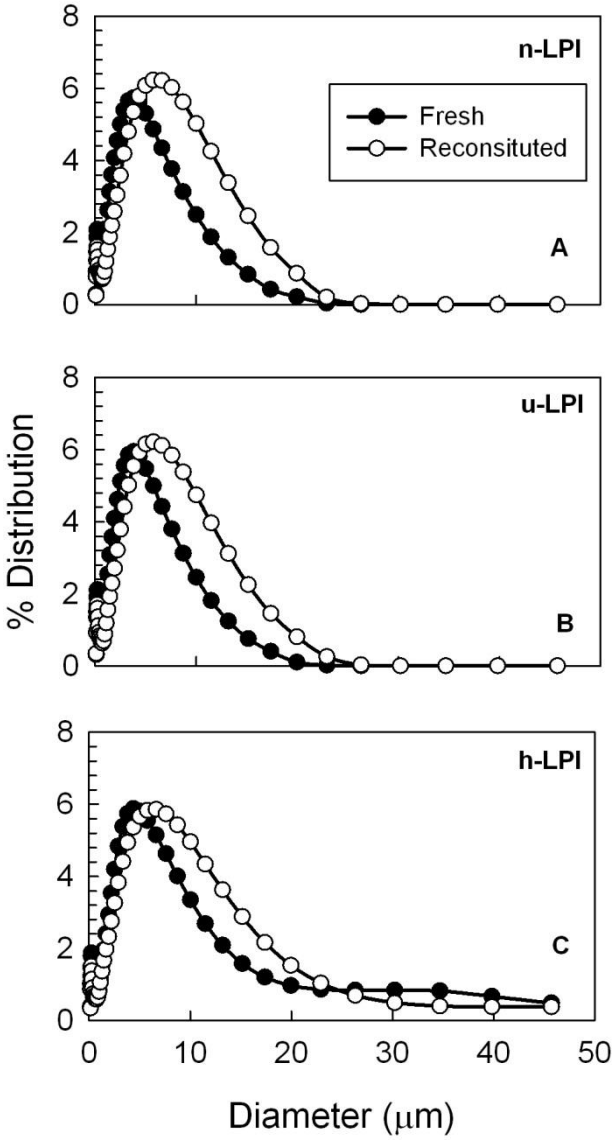

Figure 4.5 Mean size distributions of oil droplets prepared from fresh and reconstituted emulsions stabilized by native (n-LPI), unhydrolyzed (u-LPI), and hydrolyzed (h-LPI) lentil protein isolate (LPI; 4.0% w/w) and maltodextrin (36.0% w/w) wall formulations (n=2).

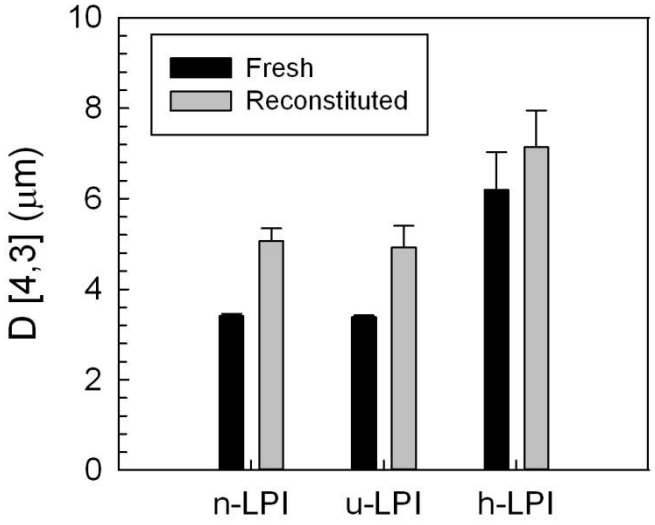

Figure 4.6 Mean droplet diameter (D [4,3]) of oil droplets prepared from fresh and reconstituted emulsions stabilized by native (n-LPI), unhydrolyzed (u-LPI), and hydrolyzed (h-LPI) lentil protein isolate (LPI; 4.0% w/w) and maltodextrin (36.0% w/w) wall formulations (n=2).

progressed during the time lapse between homogenization and freezing (~60 min) or possibly the powder was not fully reconstituted (Figure 4.5). An analysis of variance of the mean volume weighted diameter indicated that the protein wall material (n-LPI, u-LPI and h-LPI) ($p<0.001$), and differences between droplet sizes prepared from fresh and reconstituted samples ($p<0.01$) were highly significant, whereas their associated interaction was not ($p>0.05$). Overall, D[4,3] was greater (6.7 µm) for emulsions prepared with h-LPI than those with n-LPI (4.2 µm) and u-LPI (4.2 µm) materials, which were similar (Figure 4.6). The larger droplet size observed for h-LPI could be due to the inability of the lower molecular weight proteins/polypeptides of this material to sufficiently cover the surface of the oil droplets, allowing for the oil to flocculate and coalesce. Results also show that the D[4,3] measured for droplets from reconstituted microcapsules for all protein wall materials were larger overall (5.7 µm) than those observed using the fresh emulsion (4.3 µm) (Figure 4.6). These findings support the hypothesis that the oil-in-water emulsions were less stable when prepared with h-LPI than either n-LPI or u-LPI.

Based on the surface oil, EE and droplet size data obtained as a function of oil content and protein wall material, only the n-LPI-maltodextrin wall material and 10.0% oil content formulation was carried forward for oxidative testing over a 30 d room temperature storage period versus free oil.

4.4.3 Oxidative stability

The oxidative storage stability of free and encapsulated (4.0% n-LPI, 36.0% maltodextrin, 10.0% oil) flaxseed oil was determined over a 30 d storage period at room temperature by measuring the primary and secondary oxidative products using the PV and TBARS assays, respectively (Figure 4.7). Unexpectedly, the encapsulation process resulted in significantly higher PV values ($p<0.01$) for the encapsulated versus the free oil at day zero of 15.58 ± 0.43 and 5.66 ± 0.86 meq. active 0_2/kg, respectively. In addition, the PV value for the encapsulated oil increased to 37.33 ± 1.04 meq. active 0_2/kg at day 30, whereas the free oil value remained relatively constant at 5.76 ± 0.20 meq. active 0_2/kg. These results indicated that the encapsulation process led to the generation of primary oxidation products (Figure 4.7A). Possible reasons for the higher levels of primary oxidation products in the encapsulated oil include: (1) the presence of transition metals in the LPI, which can act as catalysts to promote lipid oxidation (Nuchi et al., 2001); (2) the capsule drying process, which may result in the formation of surface pores for increased permeability of oxygen (Heinzelmann & Franke, 1999; Heinzelmann et al., 2000; Quispe-Condori et al., 2011); and (3) the grinding of the freeze dried sample prior to analysis, which could have resulted in the exposure and incorporation of oxygen into the released flaxseed oil.

In contrast, TBARS values for the free and encapsulated oil samples were found to be similar ($p>0.05$) at 0.41 ± 0.09 and 0.54 ± 0.13, respectively (Figure 4.7B) at day zero and remained so throughout the 30 d testing period. These results indicated that the presence of secondary oxidation products, such as aldehydes, remained relatively constant throughout the 30 d storage period for both the free and encapsulated flaxseed oil samples.

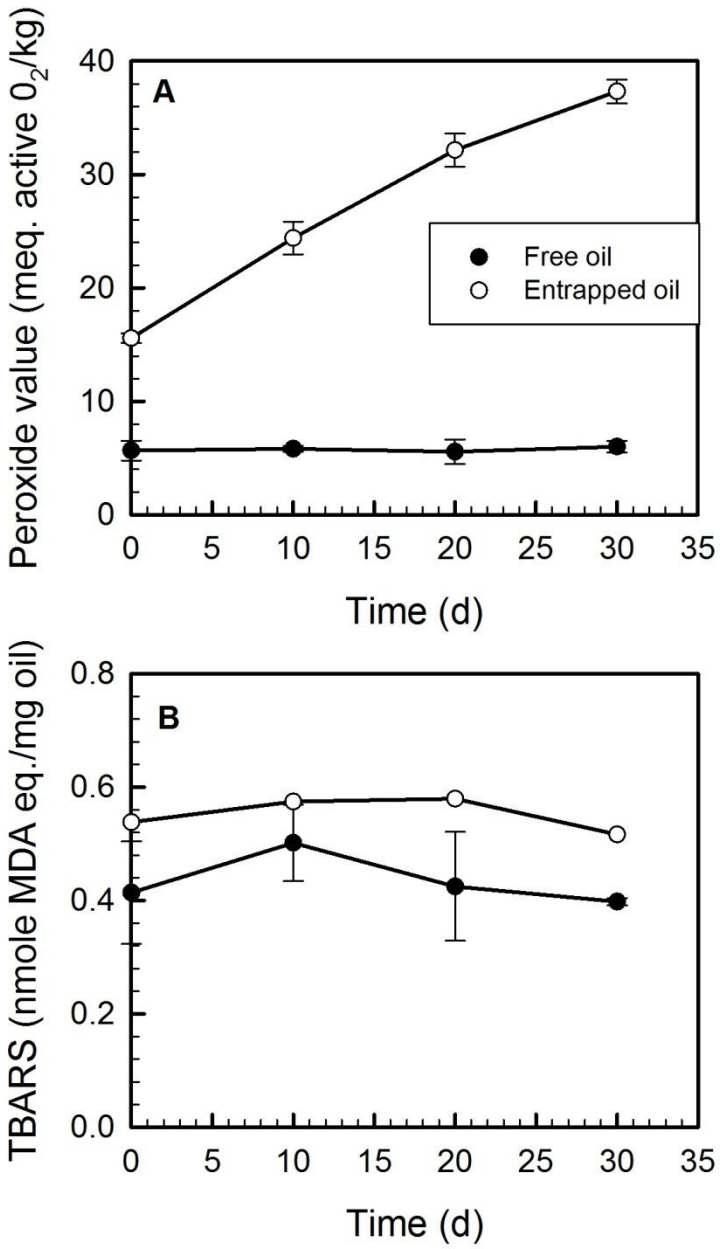

Figure 4.7 Changes in a) peroxide value (PV) and b) thiobarbituric acid-reactive substances (TBARS) for free and microencapsulated flaxseed oil prepared using a wall material of 4.0% n-LPI and 36.0% maltodextrin. Data represent the mean ± one standard deviation (n=3).

The free radical or autooxidation theory is the most acknowledged mechanism for lipid oxidation, which consists of three phases: initiation, propagation and termination (Frankel, 2005; Kolanowski et al., 2007; Lagarde, 2010; Indrasena & Barrow 2011). In the first phase, known as initiation, a reaction between singlet state oxygen and a site of unsaturation on a lipid molecule occurs resulting in the formation of unstable peroxides which rapidly decompose to produce reactive oxygen species such as the hydroxyl ($^{\cdot}$OH), hydroperoxyl ($^{\cdot}$OOH) and alkoxyl ($^{\cdot}$OOR) radicals. These compounds react with unsaturated lipids in the sample in the propagation phase so as to produce more free radicals. The propagation reaction continues until the concentration of the radical species are sufficient to result in reactions amongst themselves so as to produce non-radical species (termination phase) such as aldehydes and ketones (Kubow, 1992; McClements & Decker, 2000). The presence of primary and secondary oxidation products in oils rich in PUFA's may result in oils which have poor sensory characteristics, have lower nutritional value, and may result in health disorders for consumers due to high levels of free radical species (Babu & Wiesenfled, 2003; Bera et al., 2006; Ahn et al., 2008b; Bozan & Temelli, 2008). Based on the oxidative stability results observed, the encapsulation formulation and process employed in this study would not result in a commercially acceptable final product.

4.5 Conclusions

In this study, the physicochemical properties of lentil protein isolate-maltodextrin formulated microcapsules with encapsulated flaxseed oil were investigated using native and pre-treated lentil protein isolates as a function of oil load concentration. All final free flowing powders had moisture contents and water activities characteristic of dried powders. Surface oil increased and EE decreased as the oil load concentration increased from 10.0 to 30.0% for all formulations. Overall, a formulation of 4.0% n-LPI with 36.0% maltodextrin with a 10.0% oil load had the lowest surface oil content and highest EE values. This formulation was subjected to an oxidative storage stability study conducted at room temperature over a 30 d period. Results from this study indicated that the encapsulation process itself induced oxidation in the encapsulated oil that resulted in higher primary oxidative products than those observed in free oil. Further investigation is required to improve the oxidative stability of flaxseed oil through the encapsulation process, possibly by the addition of antioxidants to the oil itself.

5. GENERAL CONCLUSIONS

The overall aim of this research was to use a modified lentil protein isolate (LPI)-maltodextrin mixture for the successful encapsulation of ω-3 fatty acid rich flaxseed oil, by providing protection to its sensitive core material from oxidation. Specifically, the effect of limited protein hydrolysis and/or a heat treatment on microcapsule moisture content, water activity, size, surface oil content, encapsulation efficiency, and oil oxidative stability were investigated.

In the first stage of this research (Chapter 3), the effects of limited enzymatic (trypsin) hydrolysis on the physicochemical and emulsifying properties of a lentil protein isolate (heated, hydrolyzed; h-LPI) was studied. Overall, trypsin-catalyzed hydrolysis of LPI yielded a degree of hydrolysis (% DH) ranging between 4 and 20, and resulted in a product with reduced surface hydrophobicity (S_o-ANS) and intrinsic fluorescence relative to heated unhydrolyzed-LPI (u-LPI). The observed surface characteristic changes are postulated to be due to the initial exposure of reactive aromatic amino acids, which is followed by protein aggregation to re-bury these hydrophobic sites. Similarly, interfacial tension between a flaxseed oil-water interface was reduced for h-LPI materials relative to u-LPI (heated, unhydrolyzed). In contrast, surface charge of the u-LPI and h-LPI materials was found to be similar. Furthermore, the emulsifying properties, as described by the emulsification activity and stability indices also were reduced for the h-LPI materials relative to the u-LPI, suggesting the limited hydrolysis of LPI had a detrimental effect on its emulsifying properties. No differences were observed for any of the physicochemical tests conducted and emulsifying properties as a function of degree of hydrolysis (i.e., 4, 9 and 20%). The findings from this study indicated that formed emulsions were less stable using LPI with a limited degree of hydrolysis than u-LPI. Therefore the use of h-LPI as a possible wall material for the encapsulation of flaxseed oil could result in a finished product with a high surface oil content and low encapsulation efficiency when compared to u-LPI

The second stage of this research (Chapter 4) investigated the physicochemical properties of LPI-maltodextrin microcapsules containing flaxseed oil as the core material produced by

freeze drying employing native (n-LPI), u-LPI and h-LPI (% DH 18) as a function of oil concentration (10.0, 20.0 and 30.0%). The moisture content (<6.0%) and water activity (<0.2) for all capsule formulations displayed characteristics that were typical and industrially acceptable for dried powder food ingredients. Light microscopy imaging and dynamic light scattering of the emulsion droplets, revealed that the h-LPI had slightly larger oil droplets than the n-LPI and u-LPI, which both appeared similar. Overall, microcapsules prepared from h-LPI showed significantly higher surface oil and lower EE than those produced with n-LPI and u-LPI. These experimental results supported the hypotheses generated from the work reported in Chapter 3, where the h-LPI materials produced emulsions with poorer stability. Also, as the oil concentration in the formulation was raised from 10.0 to 30.0%, microcapsule surface oil increased and EE decreased. Based on experimental results, microcapsules prepared using a formulation of n-LPI (4.0%, w/w)-maltodextrin (36.0%, w/w) and 10.0% flaxseed oil was found to have the lowest surface oil content (~3.7%) and highest EE (~62.8%), and was taken forward for a 30 d room temperature oxidative stability test versus free oil. Unfortunately, the encapsulation process induced autooxidation leading to the production of a greater amount of primary oxidative products than what was observed in the free oil.

Lentil protein isolates represent a promising protein ingredient for the food industry that could compete with soy and animal-derived proteins, based on its nutritional and functional importance. Further refinement of the encapsulation process is required prior to the use of this protein as a wall material.

6. GENERAL DISCUSSIONS

Greater consumption of ω-3 rich flaxseed oil, in our diet, as a preventative means for reducing the risks of cardiovascular diseases, stroke and some types of cancer would have a tremendous impact on the health and wellbeing of Canadians. However, greater technology and product innovation is required in order to mask off-flavours/odours arising from oxidation, its taste, and to reduce any losses in bioavailability as a result of oxidative degradation. Encapsulation technology enables an oil to be encased within an amphiphilic dry powder to increase its miscibility in aqueous food products, effectively widening its potential product/markets for utilization. Lentil proteins, also serve as promising alternative to animal-derived ingredients (e.g., gelatin, casein, whey and ovalbumin) or other plant proteins with known allergens (e.g., soy and wheat). Lentil proteins have excellent nutritional (Alsohaimy et al., 2007; Lee et al., 2007; Boye et al., 2010) and functional (Can Karaca et al., 2012a,b) properties. Lentil proteins used in combination with flaxseed oil, could produce a high valued ingredient that provides new demands and niche markets for producers to capitalize on economically.

Design of an encapsulated powder with a hydrophobic core involves first the formation of a stable emulsion, in which, as was the case in this research, involves the migration of a protein emulsifier to the oil-water interface, followed by conformational changes to align hydrophobic amino groups towards the oil phase and hydrophilic amino groups towards the aqueous phase (Dalgleish, 2004). The process acts to lower interfacial tension, which upon homogenization under high shear creates micron-sized oil droplets dispersed within the continuous aqueous phase (McClements, 2005d). The proteins create a viscoelastic film surrounding the droplets to resist against rupturing from mechanical shear (Dalgleish, 2004). The film also creates electrostatic repulsion (depending on pH) between neighboring droplets in solution to resist against flocculation or aggregation and coalescence, and creates steric hindrance from tails or loops of protein chains that extend in the aqueous phase to reduce flocculation/aggregation (Damodaran, 2005; McClements, 2005b). In contrast to protein

emulsifiers, other lower molecular weight compounds, such as polysorbate 80, Tween 20-80, lecithin, etc. can be used as an alternative. These compounds can be more effective at diffusing to the interface and lowering interfacial tension than bulkier proteins, but the thickness of the viscoelastic film is reduced making it more susceptible to rupturing, and lacks tails or loops of material on a coated oil surface droplet that partakes in steric hindrance (Damodaran, 2005; Kralova & Sjoblom, 2009).

In the present research, the limited hydrolysis of LPI was hypothesized to lead to greater exposure of surface active moieties, such as the buried hydrophobic amino groups. This was the case for limited hydrolysis of faba bean legumin with trypsin (Henning et al., 1997), soy protein isolate with pancreatin (Hettiarachchy & Kalapathy, 1997), trypsin treated oat bran protein (Guan et al., 2007), trypsin-treated sunflower protein isolate (Karayannidou et al., 2007), and trypsin treated amaranth protein (Ventureira et al., 2010). These could then lead to greater integration into the oil-water interface and enhanced emulsifying properties (Hettiarachy & Kalapathy, 1997; Guan et al., 2007; Karayannidou et al., 2007). Cleaved peptides through the hydrolysis process would also generate small molecular weight surface active molecules, which perceivably could diffuse quicker to the interface to produce droplets smaller in size. However, the hypothesis proposed in the current study was rejected, where limited hydrolysis of LPI resulted in fewer hydrophobic amino groups (e.g., tryptophan, phenylalanine and tyrosine) becoming exposed relative to u-LPI, as the result of rapid protein-protein aggregation via hydrophobic interactions. Similar findings were reported for soy flour (Jung et al., 2005), soy protein isolate (Ortiz & Wagner, 2002), extruded/unextruded soy protein concentrate (Surowka et al., 2004), and spent grain protein (Celus et al., 2007). The LPI with limited levels of hydrolysis also resulted in no changes in surface charge, and a lower interfacial tension than u-LPI indicating that the cleaved off peptides potentially helped to stabilize the interface. Interfacial characteristics (charge, thickness, density, and hydrophobicity) greatly affect the formation of the emulsion and its stability (McClements, 2007a). Application of heat (used to denature the enzyme) has been related to conformational changes and protein aggregation, which alters the molecular weight, solubility, number of ionizable groups, and exposure ratio of hydrophilic: hydrophobic groups (Panyam & Kilara, 1996; Lamsal et al., 2007; Pinterits & Arntfield, 2007). Unfortunately, in the present study, the lowering of interfacial tension did not

translate into improved emulsification and stability of flaxseed oil. In the present study, differences between the physicochemical and functional properties as a function of degree of hydrolysis were similar between 4 and 20%. Molecular weight bands of h-LPI materials as a function of the degree of hydrolysis (Figure 3.2) showed little difference among the h-LPI materials. It is perceivable that trypsin hydrolysis did not have as great as an effect on the lower molecular weight bands as the higher molecular weight ones.

Foundational knowledge gained on the effects of limited hydrolysis on the physicochemical and emulsifying properties of LPI provided a good understanding, in the further design of LPI-maltodextrin microcapsules prepared by freeze drying. Microcapsules were prepared using an n-LPI (native), u-LPI (heated, unhydrolyzed) and h-LPI (heated, hydrolyzed) combined with maltodextrin as a drying aid. During encapsulation, higher amounts of LPI were used to increase yield and effectiveness as emulsifiers. McClements (2004) reported sufficient emulsifier concentration within a system is necessary in order for the oil droplet to be fully covered and to avoid droplet aggregation. Findings from the current study indicated that only the n-LPI resulted in capsules with low surface oil content (3.7%) and high encapsulation efficiencies (62.8%). Both the u-LPI and h-LPI resulted in higher surface oil levels and lower encapsulation efficiencies. Furthermore, an increase in surface oil and reduction in encapsulation efficiencies were seen as the oil load concentration increased in the final dried product. Powders with high surface oil contents tend to agglomerate more (becoming less free flowing) and are more susceptible to surface oxidation, which can lead to off-flavours/odours and reduced shelf life (Thies, 2003; Drusch & Mannino, 2009).

Oxidative testing over a 30 d storage period was investigated for n-LPI-maltodextrin based capsules with encapsulated flaxseed oil versus free oil, with primary and secondary oxidative products being measured every 10 d. Peroxide value (measures primary oxidative products, e.g. peroxides which rapidly decompose to produce reactive oxygen species: hydroxyl ($^{\cdot}$OH), hydroperoxyl ($^{\cdot}$OOH) and alkoxyl ($^{\cdot}$OOR) radicals) measured over time for the free oil found no changes due to oxidation. In contrast, PV values were higher than in free oil at day 0 for the encapsulated product indicating that the process itself induced oxidation. TBARS data (measures secondary oxidative products) also was slightly higher at day 0, and then remained unchanged over the duration of the study. This was different from that reported by Can Karaca

(2012) where free oil showed increased PV and TBARS values near day 15-20, whereas the encapsulated oil remained constant. The present study and that of Can Karaca (2012) used CDC Greenland and CDC Grandora lentils, respectively. It was perceivable that the different varieties may have different levels of micronutrients and minerals, including those that are considered to be prooxidants (e.g. iron and copper) (McClements & Decker, 2000; Chen et al., 2012). The latter acts as a catalyst to spur on the autooxidation process (McClements & Decker, 2000; Chen et al., 2012). Onset of oxidation of the material during the encapsulation process may be associated with the homogenization process (not under nitrogen), the grinding process for transitioning the freeze dried material into a free flowing powder, and during the oil extraction procedure.

Although further work as it relates to the encapsulation process is needed, potential exists for the use of LPI as a plant-based protein emulsifier or as an encapsulating agent. Oxidative degradation of the oils could be prevented through the addition of antioxidant (e.g., α-tocopherol, ascorbyl palmitate, carnosol) compounds (McClements & Decker, 2000). The use of plant-based proteins as encapsulating agents is especially attractive due to the relative low cost associated with them, and the market demand away from using animal-derived products.

7. FUTURE STUDIES

Gaining further foundational knowledge relating to the physicochemical and functional properties of plant proteins, and external environmental or processing factors that can influence their performance as ingredients is of utmost importance. This type of knowledge, along with market costs, supply, and processing requirements would provide decision makers in industry the information needed to start switching out animal-derived proteins or soy for alternatives. Lentil proteins are primarily sold as whole seeds, however the potential exists based on their amino acid profile (major amino acids are glutamic acid, aspartic acid, arginine, leucine and lysine) (Bhatty et al., 1976; El-Nahry et al., 1980; Lee et al., 2007), high protein content (~24-30%) (Alsohaimy et al., 2007; Lee et al., 2007) and functionality (Can Karaca et al., 2011a,b) for their production into an isolate product. Alsohaimy et al. (2007) reported one of the limitations associated with protein ingredients is the ability for acquiring a consistent product. Especially, since processing can have a significant impact on protein functionality and nutritional quality.

Building on from the current study, various treatments could be explored as a way to optimize the emulsion stability when using LPI. For instance, a) consideration to pre-treatment time-temperature conditions to induce aggregation; b) improved control over the enzymatic hydrolysis process using a pH-STAT instrument; c) the testing of various enzymes alone or in combination; d) changes in enzyme-substrate ratio and concentrations; e) emulsification conditions (e.g. high pressure homogenization or ultra-fluidization, function of time-temperature of shear); and f) the addition of ingredients within the emulsion formulation (e.g., lower molecular weight surfactants). In addition, various methods for producing LPI could be explored, such as salt extraction, isoelectric precipitation or micellular precipitation. Each method could produce an isolate product with unique functionality based on selecting one class of proteins over another. Furthermore, since this research works primarily with an isolate product, further purification into a globulin-rich isolate or further into a legumin- or vicilin-rich isolate could provide greater control during the hydrolysis process. However, purification can be disadvantageous since low yields become an issue.

In terms of encapsulation, further work is needed in order to provide additional protection to the encapsulated oil, and to discern differences found during the oxidative storage test between the present study and work by Can Karaca (2012). A comparative encapsulation study using isolates prepared from CDC Greenland (Chapter 4) and CDC Grandora-type (Can Karaca, 2012) lentils would help distinguish possible differences raw materials may have on oxidation (e.g., levels of prooxidants). Capsules could also be re-formulated using additional materials such as gums (e.g., gum Arabic) to enhance emulsion stability or through the addition of antioxidant compounds (e.g., α-tocopherol) for improved shelf life. Furthermore, spray drying should be the focus of future work in order to produce prototype powders closer to industry needs. Spray drying could be used alone or in combination with fluidized bed coating to produce multi-shell capsules with enhanced abilities to protect against oxidation. The advantage to spray drying is the capability to tailor the processing conditions to give the desired powder properties such as, particle size, moisture content, bulk density, flowability, dispersability, appearance, and structural strength (Reineccius, 2001; Augustin & Sanguansri, 2008). And finally, the performance of powders within various food processing operations and/or products needs to be evaluated prior to moving the technology to industry.

8. REFERENCES

Abuzaytoun, R. & Shahidi, F. (2006). Oxidative stability of flax and hemp oils. *Journal of the American Oil Chemists' Society, 83*, 855-861.

Adler-Nissen, J. (1979). Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. *Journal of Agricultural and Food Chemistry, 27*, 1256-1262.

Ahn, J. H., Kim, Y. P., Lee, Y. M., Seo, E. M., Lee, K. W., & Kim, H. S. (2008a). Optimization of microencapsulation of seed oil by response surface methodology. *Food Chemistry, 107,* 98-105.

Ahn, J., Kim, Y., Seo, E., Choi, Y., & Kim, H. (2008b). Antioxidant effect of natural plant extracts on the microencapsulated high oleic sunflower oil. *Journal of Food Engineering, 84*, 327-334.

Akhlaghi, M. & Bandy, B. (2010). Dietary broccoli sprouts protect against myocardial oxidative damage and cell death during ischemia-reperfusion. *Plant Foods for Human Nutrition, 65*, 193–199.

Alsohaimy, S. A., Sitohy, M. Z., & El-Masry, R. A. (2007). Isolation and partial characterization of chickpea, lupine and lentil seed proteins. *World Journal of Agricultural Sciences, 3,* 123-129.

Aluko, R. E. & Yada, R. Y. (1995). Structure-function relationships of cowpea (*Vigna unguiculata*) globulin isolate: influence of pH and NaCl on the physicochemical and functional properties. *Food Chemistry, 53*, 259-265.

Anwar, S. H. & Kunz, B. (2011). The influence of drying methods on the stabilization of fish oil microcapsules: Comparison of spray granulation, spray drying, and freeze drying. *Journal of Food Engineering, 105*, 367-378.

AOAC (2003). *Official Methods of Analysis of AOAC International, 17th Edition, Revision 2*. Gaithersburg: Association of Official Analytical Chemists, Inc.

Augustin, M. A. & Hemar, Y. (2009). Nano-and micro-structured assemblies for encapsulation of food ingredients. *Chemical Society Reviews, 38,* 902-912.

Augustin, M. A, Sanguansri, L., & Bode, O. (2006). Maillard reaction products as encapsulants for fish oil powders. *Journal of Food Science, 71,* 25-32.

Augustin, M. A. & Sanguansri, L. (2008). Encapsulation of Bioactives. In J. M. Aguilera & P. J. Lillford (Eds.), *Food Materials Science: Principles and Practice* (pp. 577-601). New York, NY: Springer Science.

Babu, U. S. & Wiesenfled, W. (2003). Nutritional and hematological effects of flaxseed. In L. U. Thompson & S. C. Cunnane (Eds.), *Flaxseed in Human Nutrition Second Edition* (pp.150-173). Champaign, IL: AOCS Press.

Barbana, C. & Boye, J. I. (2011). Angiotensin I-converting enzyme inhibitory properties of lentil protein hydrolysates: Determination of the kinetics of inhibition. *Food Chemistry, 127*, 94-101.

Bera, D., Lahiri, D., & Nag, A. (2006). Studies on a natural antioxidant for stabilization of edible oil and comparison with synthetic antioxidants. *Journal of Food Engineering, 74*, 542-545.

Beristain, C. I., Azuara, E., & Vernon-Carter, E. J. (2002). Oxidation of spray-dried encapsulated orange peel oil using mesquite gum (*prosopis juliflora*) as wall material. *Journal of Food Science: Food Engineering and Physical Properties, 67,* 206-211.

Beristain, C. I., Garcia, H. S., & Vernon-Carter, E. J. (2001). Spray-dried encapsulation of cardamom (*Elettaria cardamomum*) essential oil with mesquite (*Prosopis juliflora*) gum. *LWT-Food Science and Technology, 34,* 398-401.

Bhatty, R. S. (1986). Protein subunits and amino acid composition of wild lentil. *Phytochemistry, 25,* 641-644.

Bhatty, R. S., Slinkard, A. E., & Sosulski, F. W. (1976). Chemical composition and protein characteristics of lentils. *Canadian Journal of Plant Science, 56,* 787-794.

Bora, P. S. (2002). Functional properties of native and succinylated lentil (*Lens culinaris*) globulins. *Food Chemistry, 77,* 171-176.

Bouyer, E., Mekhloufi, G., Rosilio, V., Grossiord, J., & Agnely, F. (2012). Proteins, polysaccharides, and their complexes used as stabilizers for emulsions: Alternatives to synthetic surfactants in the pharmaceutical field? *International Journal of Pharmaceutics, 436,* 359-378.

Boye, J., Fatemeh, Z., & Pletch, A. (2010). Pulse proteins: Processing, characterization, functional properties and applications in food and feed. *Food Research International, 43,* 414-431.

Bozan, B. & Temelli, F. (2008). Chemical composition and oxidative stability of flax, safflower and poppy seed and seed oils. *Bioresource Technology, 99,* 6354-6359.

Braudo, E. E., Danilenko, A. N., Guslyannikov, P. V., Kozhevnikov, G. O., Artykova, G. P., Lapteva, N. A., Vaintraub, I. A., Sironi, E., & Duranti, M. (2006). Comparative effects of limited tryptic hydrolysis on physicochemical and structural features of seed 11S globulins. *International Journal of Biological Macromolecules, 39,* 174-178.

Calderon de la Barca, A. M., Ruiz-Salazar, R. A., & Jara-Marini, M. E. (2000). Enzymatic hydrolysis and synthesis of soy protein to improve its amino acid composition and functional properties. *Journal of Food Science, 65,* 246-253.

Can Karaca, A. (2012). *Encapsulation of flaxseed oil using plant proteins* (Doctoral Thesis). University of Saskatchewan, Saskatoon, SK.

Can Karaca, A., Low, N., & Nickerson, M. (2011a). Emulsifying properties of chickpea, faba bean, lentil and pea proteins produced by isoelectric precipitation and salt extraction. *Food Research International, 44,* 2742-2750.

Can Karaca, A., Low, N., & Nickerson, M. (2011b). Lentil and chickpea protein-stabilized emulsions: optimization of emulsion formulation. *Journal of Agricultural and Food Chemistry*, *59*, 13203-13211.

Carneiro, H. C. F., Tonon, R. V., Grosso, C. R. F., & Hubinger M. D. (2013). Encapsulation efficiency and oxidative stability of flaxseed oil microencapsulated by spray drying using different combination of wall materials. *Journal of Food Engineering, 115*, 443-451.

Celus, I., Brijs, K., & Delcour, J. A. (2007). Enzymatic hydrolysis of brewers' spent grain proteins and technofunctional properties of the resulting hydrolysates. *Journal of Agricultural and Food Chemistry, 55*, 8703-8710.

Chabanon, G., Chevalot, I., Framboisier, X., Chenu, S., & Marc, I. (2007). Hydrolysis of rapeseed protein isolates: Kinetics, characterization and functional properties of hydrolysates. *Process Biochemistry, 42*, 1419-1428.

Champagne, C. P. & Fustier, P. (2007). Microencapsulation for the improved delivery of bioactive compounds into foods. *Curr**e**nt Opinion in Biot**ec**hnology, 18,* 184-190.

Chang, C., Leung, T., Lin, S., & Hsu, C. (2006). Release properties on gelatin-gum Arabic microcapsules containing camphor oil with added polystyrene. *Colloids and Surfaces B: Biointerfaces, 50*, 136-140.

Chen, B., Panya, A., McClements, D. J., & Decker, E. A. (2012). New insights into the role of iron in the promotion of lipid oxidation in bulk oils containing reverse micelles. *Journal of Agricultural and Food Chemistry, 60*, 3524-3532.

Coskuner, Y. & Karababa, E. (2007). Some physical properties of flaxseed (*Linum usitatissimum* L.*). Journal of Food Engineering, 78*, 1067-1073.

Dalgleish, D. G. (2004). Food emulsions: Their structures and properties. In S. E. Friberg, K. Larsson, & J. Sjoblom (Eds.), *Food Emulsions: Fourth Edition, Revised and Expanded* (pp. 1-44). New York, NY: Marcel Dekker.

Damodaran, S. (2005). Protein stabilization of emulsions and foams. *Journal of Food Science, 70*, 54-66.

de Vos, P., Faas, M. M., Spasojevic, M., & Sikkema, J. (2010). Encapsulation for preservation of functionality and targeted delivery of bioactive food components. *International Dairy Journal, 20,* 292-302.

del Toro, M. A. N. & Garcia-Carreno, F. (2005). Evaluation of the progress of protein hydrolysis. In R.E. Wrolstad, T.E. Acree, E.A. Decker, M. H. Penner, D. S. Reid, S. J. Schwartz, C. F. Shoemaker, D. Smith, & P. Sporns (Eds.), *Handbook of Food Analytical Chemistry* (pp. 141-154). Hoboken, NJ: John Wiley and Sons, Inc.

Desai, K. G. H. & Park, H. J. (2005). Recent developments in microencapsulation of food ingredients. *Drying Technology, 23,* 1361-1394.

Dickinson, E. (2003). Hydrocolloids at interfaces and the influence on the properties of dispersed systems. *Food Hydrocolloids, 17,* 25-39.

Dickinson, E. (2009). Hydrocolloids as emulsifiers and emulsion stabilizers. *Food Hydrocolloids, 23,* 147-1482.

Dickinson, E. (2010). Flocculation of protein-stabilized oil-in-water emulsions. *Colloids and Surfaces B: Biointerfaces, 81*, 130-140.

Drusch, S. & Mannino, S. (2009). Patent-based review on industrial approaches for the microencapsulation of oils rich in polyunsaturated fatty acids. *Trends in Food Science & Technology, 20,* 237-244.

Drusch, S., Serfert, Y., Berger, A., Shaikh, M. Q., Ratzke, K., Zaporojtchenko, V., & Schwarz, K. (2012). New insights into the microencapsulation properties of sodium caseinate and hydrolyzed casein. *Food Hydrocolloids, 27,* 332-338.

Ducel, V., Richard, J., Popineau, Y., Boury, F. (2004a). Adsorption kinetics and rheological interfacial properties of plant proteins at the oil–water interface. *Biomacromolecules, 5*, 2088-2093.

Ducel, V., Richard, J., Popineau, Y., & Boury, F. (2005). Rheological interfacial properties of plant protein-arabic gum coacervates at the oil-water interface. *Biomacromoleclues, 6,* 790-796.

Ducel, V., Richard, J., Saulnier, P., Popineau, Y., & Boury, F. (2004b). Evidence and characterization of complex coacervates containing plant proteins: application to the microencapsulation of oil droplets. *Colloids and Surfaces A: Physicochemical Engineering Aspects, 232,* 239-247.

Duranti, M. (2006). Grain legume proteins and nutraceutical properties. *Fitoterapia, 77,* 67-82.

El-Beltagi, H. S., Salama, Z. A., & El-Hariri, D. M. (2007). Evaluation of fatty acids profile and the content of some secondary metabolites in seeds of different flax cultivars (*Linum usitatissimum* L.*). General and Applied Plant Physiology, 33,* 187-202.

El-Nahry, F. I., Mourad, F. E., Khalik, S. M. A., & Bassily, N. (1980). Chemical composition and protein quality of lentils (*Lens*) consumed in Egypt. *Qualitas Plantarum-Plant Foods for Human Nutrition, 30,* 87-95.

Fang, Z. & Bhandari, B. (2010). Encapsulation of polyphenols-a review. *Trends in Food Science & Technology, 21,* 510-523.

Fennema, O. R. (1996). Water and ice. In O. R. Fennema (Ed.), *Food Chemistry, Third Edition.* (pp. 17-94). New York, NY: Marcel Dekker Inc.

Frankel, E. N. (2005). *Lipid Oxidation,* 2nd Edition, Bridgewater, UK: The Oily Press.

Galazka, V. B., Dickinson, E., & Ledward, D. A. (1999). Emulsifying behaviour of 11S globulin Vicia faba in mixtures with sulphated polysaccharides: Comparison of thermal and high-pressure treatments. *Food Hydrocolloids, 13,* 425-435.

Gouin, S. (2004). Micro-encapsulation: Industrial appraisal of existing technologies and trends. *Trends in Food Science & Technology, 15,* 330-347.

Govindaraju, K. & Srinivas, H. (2006). Studies on the effects of enzymatic hydrolysis on functional and physico-chemical properties of arachin. *LWT-Food Science and Technology, 39,* 54-62.

Grattard, N., Salaun, F., Champion, D., Roudaut, G., & Le Meste, M. (2002). Influence of physical state and molecular mobility of freeze-dried maltodextrin matrices on the oxidation rate of encapsulated lipids. *Journal of Food Science: Food Engineering and Physical Properties, 67,* 3002-3010.

Gu, X., Campbell, L. J., & Euston, S. R. (2009). Effects of different oils on the properties of soy protein isolate emulsions and gels. *Food Research International, 42,* 925-932.

Gu, Y. S., Decker, E. A., & McClements, D. J. (2005). Influence of pH and carrageenan type on properties of β-lactoglobulin stabilized oil-in-water emulsions. *Food Hydrocolloids, 19,* 83-91.

Guan, X., Yao, H., Chen, Z., Shan, L., & Zhang, M. (2007). Some functional properties of oat bran protein concentrate modified by trypsin. *Food Chemistry, 101,* 163-170.

Guzey, D. & McClements, D. J. (2007). Impact of electrostatic interactions on formation and stability of emulsions containing oil droplets coated by β-lactoglobulin-pectin complexes. *Journal of Agricultural and Food Chemistry, 55,* 475-485.

Heinzelmann, K. & Franke, K. (1999). Using freezing and drying techniques of emulsions for the microencapsulation of fish oil to improve oxidation stability. *Colloids and Surfaces B: Biointerfaces, 12,* 223-229.

Heinzelmann, K., Franke, K., & Velasco, J. (2000). Microencapsulation of fish oil by freeze-drying techniques and influence of process parameters on oxidative stability during storage. *European Food Research and Technology, 211,* 234-239.

Henning, T., Mothes, R., Dudek, S., Krause, J. P., & Schwenke, K. D. (1997). Structural and functional changes of faba bean legumin during super-limited tryptic hydrolysis. *Nahrung, 41,* 81-86.

Hettiarachchy, M. QI. N. S. & Kalapathy, U. (1997). Solubility and emulsifying properties of soy protein isolates modified by pancreatin. *Journal of Food Science, 62*, 1110-1115.

Hill, S.E. (1996). Emulsions. In C. M. Hall (Ed.), *Methods of testing protein functionality* (pp. 153-185). London, UK: Blackie Academic & Professional.

Hogan, S. A, McNamee, B. F., O'Riordan, E. D., & O'Sullivan, M. (2001). Emulsification and microencapsulation properties of sodium caseinate/carbohydrate blends. *International Dairy Journal, 11*, 137-144.

Indrasena, W. M., & Barrow, C. J. (2011). Oxidation and stability of food-grade fish oil: Role of antioxidants. In C. Alasalvar, K. Miyashita, F. Shahidi, & U. Wanasundara (Eds.), *Handbook of Seafood Quality, Safety, and Health Applications* (pp. 317-334). Chichester, UK: Wiley-Blackwell.

Joshi, M., Adhikari, B., Aldred, P., Panozzo, J. F., & Kasapis, S. (2011). Physicochemical and functional properties of lentil protein isolates prepared by different drying methods. *Food Chemistry, 129*, 1513-1522.

Jung, S., Murphy, P. A., & Johnson, L. A. (2005). Physicochemical and functional properties of soy protein substrates modified by low levels of protease hydrolysis. *Journal of Food Science C: Food Chemistry and Toxicology, 70*, 180-187.

Kagami, Y., Sugimura, S., Fujishima, N., Matsuda, K., Kometani, T., & Matsumura, Y. (2003). Oxidative stability, structure, and physical characteristics of microcapsules formed by spray drying of fish oil with protein and dextrin wall materials. *Journal of Food Science: Food Engineering and Physical Properties, 68*, 2248-2255.

Kailasapathy, K. (2009). Encapsulation technologies for functional foods and nutraceutical product development. *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources, 4*, 1-19.

Karayannidou, A., Makri, E., Papalamprou, E., Doxastakis, G., Vaintraub, I., Lapteva, N., & Articov, G. (2007). Limited proteolysis as a tool for the improvement of the functionality

of sunflower (*Helianthus annus* L.) protein isolates produced by seeds or industrial by-products (solvent cake). *Food Chemistry*, *104*, 1728-1733.

Kato, A. & Nakai, S. (1980). Hydrophobicity determined by fluorescence probe methods and its correlation with surface properties of proteins. *Biochimica et Biophysica Acta*, *624*, 13-20.

Kim, Y., Morr, C. V., & Schenz, T. W. (1996). Microencapsulation properties of gum Arabic and several food proteins: Liquid orange oil emulsion particles. *Journal of Agricultural and Food Chemistry, 44*, 1308-1313.

Kimura, A., Fukuda, T., Zhang, M., Motoyama, S., Maruyama, N., & Utsumi, S. (2008). Comparison of physicochemical properties of 7S and 11S globulins from pea, fava bean, cowpea, and French bean with those of soybeans-French bean 7S globulin exhibits excellent properties. *Journal of Agricultural and Food Chemistry, 56*, 242-258.

Klassen, D. R. & Nickerson, M. T. (2012). Effect of pH on the formation of electrostatic complexes within admixtures of partially purified pea proteins (legumin and vicilin) and gum Arabic polysaccharides. *Food Research International, 46*, 167-176.

Klaypradit, W. & Huang, Y. (2008). Fish oil encapsulation with chitosan using ultrasonic atomizer. *LWT-Food Science and Technology*, *41*, 1133-1139.

Klinkesorn, U., Sophanodora, P., Chinachoti, P., Decker, E. A., & McClements, D. J. (2006). Characterization of spray-dried tuna oil emulsified in two-layered interfacial membranes prepared using electrostatic layer-by-layer deposition. *Food Research International, 39,* 449-457.

Kolanowski, W., Jaworska, D., Weißbrodt, J., & Kunz, B. (2007). Sensory assessment of microencapsulated fish oil powder. *Journal of the American Oil Chemists' Society, 84,* 37-45.

Kralova, I. & Sjoblom, J. (2009). Surfactants used in food industry: A review. *Journal of Dispersion Science and Technology*, *30,* 1363-1383.

Krog, N. J. & Sparso, F. V. (2004). Food emulsifiers: Their chemical and physical properties. In S. E. Friberg, K. Larsson, & J. Sjoblom (Eds.), *Food Emulsions: Fourth Edition, Revised and Expanded* (pp. 45-92). New York, NY: Marcel Dekker.

Kronman, M. J. & Holmes, L. G. (1971). The fluorescence of native, denatured and reduced-denatured proteins. *Photochemistry and Photobiology, 14,* 113-134.

Kubow, S. (1992). Routes of formation and toxic consequences of lipid oxidation products in foods. *Free Radical Biology & Medicine, 12,* 63-81.

Laemmli, U. K. (1970). Glycine-SDS-PAGE for separation of proteins. *Nature, 227,* 680-685.

Lagarde, M. (2010). Oxygenated metabolites of polyunsaturated fatty acids: Formation and function in blood and vascular cells. *European Journal of Lipid Science and Technology, 112,* 941-947.

Lamsal, B. P., Jung, S., & Johnson, L. A. (2007). Rheological properties of soy protein hydrolysates obtained from limited enzymatic hydrolysis. *LWT-Food Science and Technology, 40,* 1215-1223.

Lee, A. & Hong, Y. (2009). Coacervate formation of α-lactalbumin-chitosan and β-lactoglobulin-chitosan complexes. *Food Research International, 42,* 733-738.

Lee, H. C., Htoon, A. K., & Paterson, J. L. (2007). Alkaline extraction of starch from Australian lentil cultivars Matilda and Digger optimized for starch yield and starch and protein quality. *Food Chemistry, 102,* 551-559.

Lee, S. J. & Ying, D. Y. (2008). Encapsulation of fish oils. In N. Garti (Ed.), *Delivery and Controlled Release of Bioactives in Foods and Nutraceuticals* (pp. 370-403). Boca Raton, FL: CRC Press.

Li, Y. & McClements, D. J. (2011). Controlling lipid digestion by encapsulation of protein-stabilized lipid droplets within alginate-chitosan complex coacervates. *Food Hydrocolloids, 25,* 1025-1033.

Liao, L., Luo, Y., Zhao, M., & Wang, Q. (2012). Preparation and characterization of succinic acid deamidated wheat gluten microspheres for encapsulation of fish oil. *Colloids and Surfaces B: Biointerfaces, 92*, 305-314.

Liu, S., Low, N., & Nickerson, M. (2010). Entrapment of flaxseed oil within gelatin-gum Arabic capsules. *Journal of American Oil Chemists' Society, 87,* 809-815.

Lukaszewic, M., Szopa, J., & Krasowska, A. (2004). Susceptibility of lipids from different flax cultivars to peroxidation and its lowering by added antioxidants. *Food Chemistry, 88,* 225-231.

Madene, A., Jacquot, M., Scher, J., & Desobry, S. (2006). Flavor encapsulation and controlled release-a review. *International Journal of Food Science and Technology, 41*, 1-21.

Martinez, K. D., Sanchez, C. C., Patino, J. M. R, & Pilosof, A. M. R. (2009). Interfacial and foaming properties of soy protein and their hydrolysates. *Food Hydrocolloids, 23*, 2149-2157.

Mauguet, M. C., Legrand, J., Brujes, L., Carnelle, G., Larre, C., & Popineau, Y. (2002). Gliadin matrices for microencapsulation processes by simple coacervation method. *Journal of Microencapsulation, 19*, 377-384.

McClements, D. J. (2004). Protein-stabilized emulsions. *Current Opinion in Colloid & Interface Science, 9,* 305-313.

McClements, D. J. (2005a). Context and background. In F. M. Clydesdale (Ed.), *Food Emulsions: Principles, Practices, and Techniques 2nd Edition* (pp. 1-26). Boca Raton, FL: CRC Press.

McClements, D. J. (2005b). Colloidal interactions. In F. M. Clydesdale (Ed.), *Food Emulsions: Principles, Practices, and Techniques 2nd Edition* (pp. 53-94). Boca Raton, FL: CRC Press.

McClements, D. J. (2005c). Emulsion stability. In F. M. Clydesdale (Ed.), *Food Emulsions: Principles, Practices, and Techniques 2nd Edition* (pp. 269-339). Boca Raton, FL: CRC Press.

McClements, D. J. (2005d). Emulsion formation. In F. M. Clydesdale (Ed.), *Food Emulsions: Principles, Practices, and Techniques 2nd Edition* (pp. 233-268). Boca Raton, FL: CRC Press.

McClements, D. J. (2005e). Interfacial properties and their characterization. In F. M. Clydesdale (Ed.), *Food Emulsions: Principles, Practices, and Techniques 2nd Edition* (pp. 175-231). Boca Raton, FL: CRC Press.

McClements, D. J. (2007a). Critical review of techniques and methodologies for characterization of emulsion stability. *Critical reviews in Food Science and Nutrition, 47,* 611-649.

McClements, D. J. & Decker, E. A. (2000). Lipid oxidation in oil-in-water emulsions: impact of molecular environment on chemical reactions in heterogeneous food systems. *Journal of Food Science: 65,* 1269-1282.

McClements, D. J. & Li, Y. (2010). Structured emulsion-based delivery systems: controlling the digestion and release of lipophilic food components. *Advances in Colloid and Interface Science, 159,* 213-228.

McClements, D. J., Decker, E. A., & Weiss, J. (2007b). Emulsion-based delivery systems for lipophilic bioactive components. *Journal of Food Science R: Concise Reviews/Hypotheses in Food Science, 72,* R109-R124.

McClements, D. J., Decker, E. A., Park, Y., & Weiss, J. (2009). Structural design principles for delivery of bioactive components in nutraceuticals and functional foods. *Critical Reviews in Food Science and Nutrition, 49,* 577-606.

Nesterenko, A., Alric, I., Silvestre, F., & Durrieu, V. (2012). Influence of soy protein's structural modifications on their microencapsulation properties: α-tocopherol microparticle preparation. *Food Research International, 48,* 387-396.

Nesterenko, A., Alric, I., Silvestre, F., & Durrieu, V. (2013). Vegetable proteins in microencapsulation: a review of recent interventions and their effectiveness. *Industrial Crops and Products, 42,* 469-479.

Nuchi, C.D., McClements, D.J., & Decker, E.A. (2001). Impact of Tween 20 hydroperoxides and iron on the oxidation of methyl linoleate and salmon oil dispersions. *Journal of Agricultural and Food Chemistry, 49,* 4912- 4916.

Ortiz, S. E. M. & Wagner, J. R. (2002). Hydrolysates of native and modified soy protein isolates: structural characteristics, solubility and foaming properties. *Food Research International, 35*, 511-518.

Pain, R. H. (2005). Determining the fluorescence spectrum of a protein. In R.E. Wrolstad, T.E. Acree, E.A. Decker, M. H. Penner, D. S. Reid, S. J. Schwartz, C. F. Shoemaker, D. Smith & P. Sporns (Eds.), *Handbook of Food Analytical Chemistry* (pp. 245-265). Hoboken, NJ: John Wiley and Sons, Inc.

Panyam, D. & Kilara, A. (1996). Enhancing the functionality of food proteins by enzymatic modification. *Trends in Food Science & Technology, 7,* 120-125.

Papalamprou, E. M., Doxastakis, G. I., & Kiosseoglou, V. (2010). Chickpea protein isolates obtained by wet extraction as emulsifying agents. *Journal of the Science of Food and Agriculture*, *90*, 304-313.

Partanen, R., Raula, J., Seppanen, R., & Buchert, J. (2008). Effect of relative humidity on oxidation of flaxseed oil in spray dried whey protein emulsions. *Journal of Agricultural and Food Chemistry*, *56*, 5717-5722.

Pearce, K. N. & Kinsella, J. E. (1978). Emulsifying properties of proteins: Evaluation of a turbidimetric technique. *Journal of Agricultural and Food Chemistry, 26*, 716-723.

Pearson, A. M. (1983). Soy proteins. In B. J. F. Hudson (Ed.), *Developments in Food Proteins* (pp. 67-108). London, UK: Applied Sciences Publishers.

Pegg, R. (2005). Measurement of primary lipid oxidation products. In R. E. Wrolstad, T. E. Acree, E. A. Decker (Eds.), *Handbook of Food Analytical Chemistry* (pp. 515–564). New Jersey, NJ: Wiley.

Pegg, R. B. & Shahidi, F. (2007). Encapsulation, stabilization, and controlled release of food ingredients and bioactives. In M. S. Rahman (Ed.), *Handbook of Food Preservation 2nd Edition* (pp. 509-586). Boca Raton, FL: CRC Press.

Pierucci, A. P. T. R., Andrade, L. R., Farnia, M., Pedrosa, C., & Rocha-Leao, M. M. (2007). Comparison of α-tocopherol microparticles produced with different wall materials: Pea protein a new interesting alternative. *Journal of Microencapsulation, 24*, 201-213.

Pinterits, A. & Arntfield, S. D. (2007). The effect of limited proteolysis on canola protein gelation. *Food Chemistry, 102,* 1337-1343.

Quispe-Condori, S., Saldana, M. D. A., & Temelli, F. (2011). Microencapsulation of flax oil with zein using spray and freeze drying. *LWT-Food Science and Technology, 44,* 1880-1887.

Reineccius, G. A. (2001). The spray drying of food ingredients. In P. Vilstrup (Ed.), *Microencapsulation of Food Ingredients* (pp. 151-179). Surrey, UK: Leatherhead Publishing.

Severin, S. & Xia, W. S. (2006). Enzymatic hydrolysis of whey proteins by two different proteases and their effect on the functional properties of resulting protein hydrolysates. *Journal of Food Biochemistry, 30*, 77-97.

Shaw, L. A., Faraji, H., Aoki, T., Djordjevic, D., McClements, D. J., & Decker, E. A. (2008). Emulsion droplet interfacial engineering to deliver bioactive lipids into functional foods. In N. Garti (Ed.), *Delivery and Controlled Release of Bioactives in Foods and Nutraceuticals* (pp.184-206). Cambridge, UK: Woodhead Publishing Limited.

Surowka, K., Zmudzinski, D., & Surowka, J. (2004). Enzymatic modification of extruded soy protein concentrates as a method of obtaining new functional food components. *Trends in Food Science & Technology, 15*,153-160.

Swanson, B. G. (1990). Pea and lentil protein extraction and functionality. *Journal of The American Oil Chemists' Society, 67,* 276-280.

Tcholakova, S., Denkov, N. D., Sidzhakova, D., Ivanov, I. B., & Campbell, B. (2003). Interrelation between drop size and protein adsorption at various emulsification conditions. *Langmuir, 19,* 5640-5649.

Thies. C. (2003). Microcapsules. In B. Caballero, P. Finglas & L. Trugo (Eds.), *Encyclopedia of Food Sciences and Nutrition (Second Edition)* (pp. 3892-3903). San Diego, CA: Academic Press.

Tonon, R. V., Grosso, C. R. F., & Hubinger, M. D. (2011). Influence of emulsion composition and inlet air temperature on the microencapsulation of flaxseed oil by spray drying. *Food Research International, 44,* 282-289.

Tonon, R. V., Pedro, R. B., Grosso, C. R. F., & Hubinger, M. D. (2012). Microencapsulation of flaxseed oil by spray drying: Effect of oil load and type of wall material. *Drying Technology, 30,* 1491-1501.

Turchiuli, C., Fuchs, M., Bohin, M. Cuvelier, M. E., Ordonnaud, C., Peyrat-Maillard, M. N., & Dumoulin, E. (2005). Oil encapsulation by spray drying and fluidised bed agglomeration. *Innovative Food Science and Emerging Technologies, 6,* 29-35.

van Aken, G. A. (2004). Coalescence mechanisms in protein-stabilized emulsions. In S. E. Friberg, K. Larsson, & J. Sjoblom (Eds.), *Food Emulsions: Fourth Edition, Revised and Expanded* (pp. 299-326). New York, NY: Marcel Dekker.

Ventureira, J., Martinez, E. N., & Anon, M. C. (2010). Stability of oil: Water emulsions of amaranth proteins. Effect of hydrolysis and pH. *Food Hydrocolloids, 24,* 551-559.

Wang, R., Tian, Z., & Lingyun, C. (2011). Nano-encapsulations liberated from barley protein microparticles for oral delivery of bioactive compounds. *International Journal of Pharmaceutics, 406,* 153-162.

Weinbreck, F., Minor, M., & de kruif, C. G. (2004a). Microencapsulation of oils using whey protein/gum Arabic coacervates. *Journal of Microencapsulation, 21,* 667-679.

Weinbreck, F., Nieuwenhuijse, H., Robijn, G. W., & de kruif, C. G. (2004b). Complexation of whey proteins with carrageenan. *Journal of Agricultural and Food Chemistry, 52*, 3550-3555.

Yosha, I., Shani, A., & Magdassi, S. (2008). Slow release of pheromones to the atmosphere from gelatin-alginate beads. *Journal of Agricultural and Food Chemistry, 56*, 8045-8049.

Yust, M. D. M., Pedroche, J., Millan-Linares, M. D. C., & Alcaide-Hidalgo, J. M. (2010). Improvement of functional properties of chickpea proteins by hydrolysis with immobilised alcalase. *Food Chemistry, 122*, 1212-1217.

Zhao, G., Liu, Y., Zhao, M., Ren, J., & Yang, B. (2011). Enzymatic hydrolysis and their effects on conformational and functional properties of peanut protein isolate. *Food Chemistry, 127*, 1438-1443.

Zimet, P. & Livney, Y. D. (2009). Beta-lactoglobulin and its nancomplexes with pectin as vehicles for ω-3 polyunsaturated fatty acids. *Food Hydrocolloids, 23*, 1120-1126.