MANOTHERMOSONICATION (MTS) AND PH-SHIFTING COMBINED TREATMENT FOR MODIFYING THE FUNCTIONAL PROPERTIES OF PLANT PROTEINS: PROTEIN-STABILIZED NANOCARRIERS AND PROTECTION OF DOCOSAHEXAENOIC ACID (DHA)

BY

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DISSERTATION

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ABSTRACT

Plant proteins are low cost, non-toxic, natural, biocompatible, and biodegradable polymers. In recent years, plant proteins have drawn increasing attention from the food and pharmaceutical industries as an alternative to animal proteins due to increased consumer concerns over the safety of animal-derived products (e.g., prion diseases). Plant protein-based nanocarriers have been investigated for use as delivery systems to encapsulate, protect, and release bioactive components. Many studies have attempted to improve the functional properties of plant proteins as they are inherently less effective emulsifying agents compared to animal proteins, with various degrees of success. The goal of this study is to explore the use of mano-thermo-sonication (MTS), a new sonication method and its combination with pH-shifting to modify the functionality of soy and pea proteins for preparing nanocarriers to encapsulate and protect docosahexaenoic acid (DHA).

In study one, a new physical and chemical combined treatment for protein modification, i.e. mano-thermo-sonication (MTS) with and without pH-shifting was developed. This method was tested using soy protein isolate (SPI) as a model protein. The soluble soy protein nano-aggregates produced by this method were used to make SPI-stabilized nanoemulsions. The effect of treatments (pH shifting, MTS, and high pressure homogenization) on the physicochemical (e.g., solubility, surface hydrophobicity, free sulfhydryl and disulfide bond (S-S) content, antioxidant capacity, and total phenols), interfacial (e.g., rheology), and emulsifying (e.g., droplet size, turbidity, emulsion stability, and emulsion activity indexes) properties of SPI nano-aggregates were evaluated for the development of SPI nanostructures.

In study two, a plant protein and polysaccharide complex was developed to address the low solubility of plant protein in the vicinity of pI encountered for soy protein nano-structures

developed in Study 1. Specifically, pea protein isolate (PPI) and soy protein isolate (SPI) nano-aggregates produced with pH 12-MTS were used to form complexes with modified starch, gum Arabic, or pectin. The physicochemical (e.g., solubility, surface hydrophobicity, free sulfhydryl, and disulfide bond (S-S) content), interfacial (e.g., rheology), and emulsifying (e.g., droplet size, turbidity, emulsion stability, and emulsion activity indexes) properties of the protein-polysaccharide complexes were examined to choose the best combination for producing the most stable wall material for the encapsulation. Overall, the PPI and modified starch complex was identified as the best combination due to its high solubility, hydrophobicity, and improved emulsifying properties.

In study three, the effect of wall material and the presence of surfactant (Tween 20 and SDS) in emulsion formula on protection of DHA against oxidation was examined. A total of 4 wall materials including pea protein isolate (PPI), pea protein isolate - modified starch (PPI-MS) complex, Tween 20, and sodium dodecyl sulfate (SDS) were used for microemulsion preparation with canola oil containing DHA. The microemulsions were dried with a lab-scale freeze dryer. The freeze-dried powders were characterized with respect to physicochemical characteristics, oxidative stability, and release properties. The PPI-MS complex as a natural polymeric wall material exhibited similar or better encapsulation efficiency and desirable level of peroxide value (<5 meq/kg) compared to the synthetic surfactants (Tween 20 and SDS).

In summary, the MTS and pH-shifting combined treatment provided a new and effective protein modification method that can be used to produce protein nano-aggregates with significantly improved functional properties. Especially, the PPI nano-structures produced by this new method exhibited good physicochemical and functional properties representing a promising alternative to animal-derived proteins. The PPI and modified starch nano-complex was shown to

be a stable wall material that can be used to encapsulate DHA to fortify omega-3 fatty acids in food products and supplements.

To my family, for their love and support

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CHAPTER 1

INTRODUCTION

Proteins are widely used in the food industry as foaming and emulsifying agents (Wilde et al. 2004). They stabilize oil-in-water emulsions by forming an interfacial membrane where the adsorbed proteins unfold and rearrange their secondary and tertiary structures to expose hydrophobic residues to the hydrophobic phase (MacRitchie, 1978). Plant proteins are low cost, non-toxic, natural, biocompatible, and biodegradable polymers. In recent years, plant proteins have drawn increasing attention from the food and pharmaceutical industries as an alternative to animal proteins due to increased consumer concerns over the safety of animal-derived products (e.g., prion diseases) (Liu et al., 2010). Among plant proteins, soybean proteins are the most used and researched pulse proteins on the market (Nunes et al. 2003; Hua et al. 2005; Caillard et al., 2009; Gómez-Mascaraque and López-Rubio, 2016). The alternative legume proteins that are being researched are the ones that are believed to possess the same, or similar, functional properties and nutritional qualities as soy protein. The proteins should also have a price competitive to that of soybeans. One alternative pulse protein demonstrating potential for food applications is pea protein (*Pisum sativum* L.). Besides the good functional properties of pea proteins, they are also said to be containing less in anti-nutritional substances than soy protein, and are not classified as an allergen (as are soy and egg proteins) (Sahoo & Labhasetwar, 2008; Torchilin, 2008). Nevertheless, compared to animal proteins, such as whey protein, plant proteins are normally less soluble and less effective as an emulsifier. This necessitates the need to modify functional properties of plant proteins.

A number of chemical and physical methods have been proposed to modify the native structures of plant proteins for improving their functional properties. New physical methods reported to improve plant protein properties include high-pressure and heat combined treatments (Molina et al., 2001; Puppo et al., 2005;), extreme pH treatment (Elizondo et al., 2011), high pressure processing (HHP) (Manassero et al., 2015), pulsed electric field (PEF) (Li et al., 2007), enzyme hydrolysis (Tsumura et al., 2005), subcritical water treatment (Zhang et al., 2015), and ultrasonication (Hu et al., 2013). Among the chemical treatments, pH-shifting is a relatively new method that adjusts the pH of a protein solution to extreme basic or acidic conditions to unfold the protein, followed by changing the pH back to neutral to refold the protein. This unfolding-refolding process has been reported to effectively modify the protein functional properties (Jiang et al., 2010, Lee et al., 2016). High intensity ultrasound or power ultrasound refers to sonic waves that are at frequencies higher than sound audible to the human ear, with sound intensities in the range of 0.1-1 W/cm^2 (Feng and Yang, 2011). The mode of action of ultrasound-induced protein-structure modification is often attributed to acoustic cavitation. The physical forces produced by cavitation, such as shear forces produced by micro-streaming and normal impingement from the water jets at the solid-liquid interfaces, help to break down the protein particles or aggregates in dispersions (Lee et al., 2016). Conventional ultrasonic modification of protein functionality is performed at relatively low cavitation intensities. Consequently, a relatively long treatment time, i.e. 15-30 min for soy proteins (Hu et al., 2013) is required to produce meaningful changes in protein structures. There is a need to reduce the treatment times of ultrasound processes, thereby increasing throughput, lowering costs, and making their applications practical. Ultrasonication in combination with low hydrostatic pressure and mild heat, a process termed mano-thermo-sonication (MTS), has been reported to enhance acoustic cavitation activity (Kuldiloke, 2002).

Significant process enhancement by MTS has been reported in microbial inactivation tests. For instance, it took 15.9 min to achieve a 5-log cycle (99.999%) reduction in the population of *E. coli* K12 in apple cider, a benchmark set by USFDA for microbial inactivation in juices, in an ultrasound-alone treatment (Ugarte-Romero, et al., 2006). The time was reduced to 1.4 min when an MTS treatment at 400 kPa and 59°C was applied to the juice to obtain the same 5-log reduction (Lee et al., 2013).

The use of plant proteins and polysaccharides as emulsifiers is of great interest to the food and beverage industry because of their nutritional properties, safety, and low cost. Modified plant proteins possess good emulsifying capacity, but the emulsions stabilized by proteins are sensitive to environmental conditions, such as pH, ionic strength, and thermal processing (Phillips & Williams, 2001). Several polysaccharides, such as pectin, soybean polysaccharide, and gum Arabic, are naturally conjugated with hydrophobic proteins, and therefore can also be used as emulsifiers. Compared with the emulsions produced from proteins, the emulsions produced from polysaccharides have better stability against unfavorable environmental conditions because the carbohydrate part tends to stretch into the aqueous phase, thus preventing the droplets from aggregation and coalescence. Consequently, protein-polysaccharide covalent conjugates and protein/polysaccharide electrostatic complexes have been exploited as emulsifiers in an attempt to take advantages of the properties of both the protein and polysaccharide (Ray et al., 1995; McNamee et al., 1998; Garti, 1999).

Docosahexaenoic acid (DHA, 22 carbons and 6 double bonds) is a polyunsaturated fatty acid (Shahidi, 2008). DHA has positive effects on the prevention and treatment of various diseases. It is a fundamental compound for a healthy nutrition and have many beneficial effects, including neurological benefits (Dyall and Titus, 2008), anti-depressive effects and cardiovascular benefits

(Bays, 2008 and Wang et al., 2006). However, due to its unsaturated nature, DHA is chemically unstable and susceptible to oxidative deterioration and readily produce free radicals and unpleasant tastes, which have affected the shelf-life, sensory properties and overall acceptability of food products negatively. To minimize oxidation, an encapsulation process is normally used to protect this compound.

The overall objective of this project was to explore the use of MTS and pH-shifting combined treatment to modify the functional properties of soy and pea proteins for developing protein-mediated nanocarriers to protect and deliver docosahexaenoic acid (DHA). Specific objectives of this research were: a) to develop a MTS and pH shifting combined method for the production of nano-sized soy protein isolate (SPI) aggregates and nano-emulsions, and to investigate their physicochemical, interfacial, and emulsifying properties; b) to create protein-polysaccharide complexes using pH 12-MTS treaded soluble proteins (SPI and PPI) and polysaccharides (Modified Starch, Gum Arabic, and Pectin), and to investigate their physicochemical, interfacial, and emulsifying properties; c) to encapsulate docosahexaenoic acid (DHA) using pea protein isolate (PPI), pea protein isolate - modified starch (PPI-MS) complex, Tween 20, and SDS, and to investigate the encapsulation properties, physical properties, release properties, and storage stability of the freeze-dried powders.

The hypotheses of this study were: a) the use of a new physical and chemical combined treatment, i.e. MTS and pH-shifting would significantly improve the functional properties of selected plant proteins; b) protein-polysaccharide complexes help to overcome the low solubility problem at pH values around isoelectric point (PI); and c) the PPI/starch complexes are effective in encapsulation, protection, and delivery of DHA.

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CHAPTER 2

LITERATURE REVIEW

2.1 Plant protein

2.1.1 Food applications of plant protein

Plant proteins are inexpensive, non-toxic, biocompatible and biodegradable biopolymers that have recently been studied as an alternative to animal proteins for some specific applications (Baniel, Caer, Colas, & Gueguen, 1992; Chel-Guerrero, Perez-Flores, Betancur-Ancona, & Davila-Ortiz, 2002; Gennadios & Weller, 1990; Mateos-Aparicio, Redondo Cuenca, Villanueva-Suárez, & Zapata-Revilla, 2008; Vliet, Martin, & Bos, 2002). Proteins are great sources for wall forming material in an emulsion system due to their good film formation ability, resistance against oils or organic solvents, and gas barrier properties (De Graaf, Harmsen, Vereijken, & Monikes, 2001). Good water and fat holding capacity make plant protein a good ingredient in meat products. The protein content in legumes varies from 17% to 30% depending on their origins, and the proteins are presented as globulins (60-90%) and albumins (10-20%) (Sathe et al., 1984). Proteins are widely used in food products to improve the nutritional properties as well as texture (Taherian et al., 2011). With consumers' growing demand for nutritional and health food products, plant proteins especially legume proteins have gained increased interest in the food industry. Protein as a natural emulsifier leads to less interfacial tension between oil/water and air/water phase, working as an important ingredient in food systems like bread, ice cream, dressings, and milk-like beverages (Taherian et al., 2011).

Soybean proteins are the most used and researched plant proteins both in the academia and on the market. There is an increasing interest in the functional properties and nutritional quality of unconventional legume proteins as an ingredient in new food products (Chavan et al., 2001; Butt & Batoola, 2010). The alternative legume proteins are expected to have similar functional and nutritional qualities as soy protein. They should also be competitive to soybean for the price (Marcone et al., 1998; Vose, 1980). One such alternative plant protein that has a good potential for food applications is pea protein (*Pisum sativum* L.) (O'Kane et al., 2004). Pea protein obtained from field peas by wet extraction has high nutrition and relatively good functionalities (Gharsallaoui et al., 2009). Besides functional properties, they also contain less amounts of anti-nutritional substances than soy protein (Gwiazda et al., 1979) and are not classified as an allergen such as soy and egg proteins. Therefore, the allergic reaction related to peas is not common in humans (San Ireneo et al., 2000).

Plant proteins from soy, pulses such as pea, chickpea and lentil, and cereals such as wheat, barley and zein have been used previously for encapsulating bioactive compounds due to their amphiphilic nature, their ability to stabilize oil-in-water emulsions, and their film forming ability (McClements et al., 2007). The globular nature and large size of plant proteins enable thick viscoelastic films to form around the oil droplets at the oil-water interface to enhance their stability during processing.

2.1.2 Animal versus plant protein

Plant proteins are sustainable and environment friendly. Compared with raising livestock to get animal proteins, people can produce approximately five times more protein when growing vegetables or grains on the same area of farmland. Therefore, with the increasing interest of dietary protein due to the growth of world population, plant proteins especially legume proteins become more important. Furthermore, for economic reasons, it is less expensive to use plant proteins than to use milk proteins (Kent & Doherty, 2014).

Several studies have attempted to incorporate plant proteins in the formulation of meat products. Addition of pulse flour increased protein content and improved fat and moisture retention ability of restructured meat (Dzudie, Scher, & Hardy, 2002; Modi, Mahendrakar, Narasimha Rao, & Sachindra, 2004; Serdaroglu, Serdaroglu, Yildiz Turp, & Abrodímov, 2005). Adding protein to fortify gluten-free foods improved the nutritional value with no negative effect on product flavor (Kent & Doherty, 2014).

2.1.3 Soy protein

Soy protein is a heterogeneous mixture of storage proteins, which are generally classified through their separation by using centrifugal sedimentation in sucrose gradients. There are four major fractions in soy protein, which are classified in relation to their sedimentation coefficients, 2S (15%), 7S (34%), 11S (42%), and 15S (9%) (Liu, 1997; Kinsella,1979). Two major soy proteins, glycinin (in 11S) and b-conglycinin (in the 7S fraction), affect the processing functionality of soy protein ingredients. These glycinin and β-conglycinin proteins have a similar structure as both of them derived from X-ray crystallography (Moreira et al., 1979; Adachi., 2003).

Whole soybean can be processed into several products such as roasted soy nuts, soy flour, defatted flakes, soy protein concentrates, and soy protein isolates (SPI) (Liu, 1997).

Soy protein isolate (SPI) is obtained by isoelectric precipitation of storage proteins, and it is the most highly refined soy protein product containing more than 90% protein. Soy protein isolate mainly consists of glycinin and b-conglycinin, and these two protein fractions often account for 34% and 27% of total protein content of SPI, respectively, (Iwabuchi and Yamauchi, 1987 a, b). 11S has a hexameric structure with a molecular weight of 320-360 kDa and an isoelectric point of 4.6. On the other hand, 7S has a structure which is similar to canavalin and phaseolin with a molecular weight of 140-180 kDa and an isoelectric point of 4.9 (Riblett, Herald, Schmidt, & Tilley, 2001). Processed soy protein products usually have lower solubility and less efficient emulsifying capacity compared with animal proteins, such as casein and whey proteins (Palazolo, Sorgentini, &Wagner, 2005; Santiago, Gonzalez, Remondetto, & Bonaldo, 1998). The relatively low solubility of soy proteins might be caused by the complexity of the tertiary and quaternary structures of the 7S and 11S, globular compact conformation, and high molecular sizes (Wang et al., 2006).

2.1.4 Pea protein

Pea protein is a plant protein commonly obtained from field peas (*Pisum sativum*) by wet extraction. The protein content in peas is generally 18-30% similar to other legumes (Shand, Ya, Pietrasik, & Wanasundara, 2007).

Pea protein has a balanced amino acid profile. As a complete protein, pea protein contains all the essential amino acid for the daily needs of people. It is thus an ideal protein source for vegetarians and vegans as well as those who have allergies to animal protein and soy protein. The

nutritional value of dietary proteins is mostly based on the amino acid composition. Pea protein is rich in branched-chain amino acids such as isoleucine and valine. It was reported that proteins with high content of branched-chain amino acids have health benefits such as lowering cholesterol and blood pressure (Roy et al., 2010). In addition, they play important roles in the formation of collagen, bones, skin and tendons. They also support the development and maintenance of a healthy immune system. Consuming significant peas in the diet may reduce the incidence of colon cancer, type-2 diabetes, LDL-cholesterol and heart disease effectively (Roy et al., 2010). The digestibility of pea protein is higher than that of soybean and other pulses (Dahl et al., 2012).

2.2 Protein functional properties

Functionality can be described as any property of a protein besides its nutritional value, which affects its utilization. According to Hurrell (1980), proteins are the most reactive food components and are able to react with reducing sugars, fats, and polyphenols. These reactions may cause a reduction in nutritive value, browning, flavor formation, and sometimes toxicity, yet some of these interactions are essential to functionality of proteins in foods.

Functional properties influence the behavior of proteins in foods during processing, storage, and preparation. They have a very important role on food quality and acceptance (Matil, 1971). Functional properties include the followings:

- Sensory and kinesthetic properties such as flavor, odor, color, and texture,
- Solubility, hydration, dispersibility, and swelling,
- Surface-active properties such as emulsification, and foaming,
- Rheological properties which include gelation and texturization, and

- Other properties such as adhesive, cohesive, dough making, film and fiber making (Nakai and Powrie, 1981).

2.2.1 Modification to improve functionality

Protein functionality is generally related to changes in secondary and tertiary structure, i.e. heat denaturation for gelation. Therefore, several treatments such as high pressure and ultrasound have been used to alter the structure and functional properties (Galazka, Dickinson, & Ledward, 2000; López-Fandiño, 2006). The amino acid lysine contains an amino group which contributes to the net charge and nucleophilic properties, which in turn allows for reactions with carbonyl compounds found in foods. The most exploited approach is to use the start of the Maillard reaction to either:

1) modify protein charge and/or

2) attach a sugar or oligosaccharide and form a conjugated molecule (Campbell, Raikos, & Euston, 2003; Oliver, Melton, & Stanley, 2006), which in turn affects functionality.

Protein charge and isoelectric point can also be changed by deamidation (Hamada, 1994; Wright, 1991). Another approach is to form covalent links between proteins by enzymatic or chemical reactions making polymers of individual or a mixture of proteins (Buchert et al., 2010). This can be used to make modified ingredients or combined with forming specific structure (i.e., adding cross- links in gels). An opposite approach is to enzymatically hydrolyze the protein to varying degrees (Kilara & Panyan, 2003; Kristinsson & Rasco, 2000). In these and other modifications, the significance of the modification is usually based on how it changes the results of specific tests for protein functionality.

2.3 Application of ultrasound in food processing

2.3.1 Ultrasound

High intensity ultrasound (20 to 100 kHz) is a cost-effective and easy to use technology that has been used to modify the structural and functional properties of globular proteins (Jambrak et al., 2008; Mason et al., 1996). The mode of action in ultrasound treatment is achieved by the chemical, mechanical, and physical effects of acoustic cavitation (Figure 2.1). This includes the formation, growth, and violent collapse of small bubbles in liquid as a result of acoustic pressure fluctuation. It has been suggested that cavitation could modify protein conformation and structures by affecting hydrogen bonds and hydrophobic interactions, disrupting the quaternary and/or tertiary structure of globular proteins, and breaking down protein aggregates (Mason et al., 1996). Many studies have reported the use of ultrasound to improve the emulsifying properties of soy proteins (Puppo et al., 2008; Molina et al., 2001).

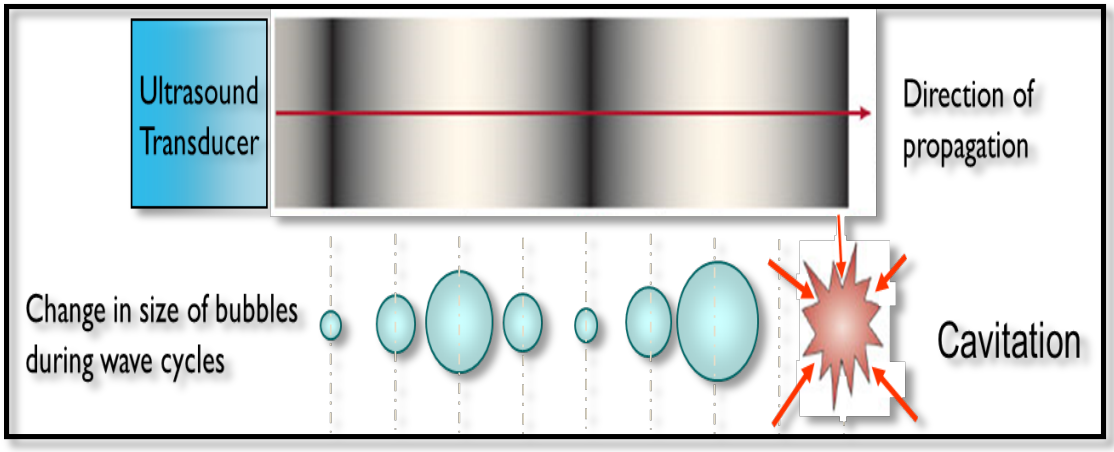

Figure 2.1. Acoustic cavitation phenomena

Bench-top sonicators are widely used in research laboratories for producing nanoemulsions. These devices consist of an ultrasonic probe that contains a piezoelectric crystal, which converts input electrical waves into mechanical vibration. The probe is dipped into the

sample to be homogenized, and generates intense disruptive forces at its tip through a combination of cavitation, turbulence and interfacial waves (Kentish et al., 2008). Emulsification by ultrasonic technology mainly occurs through two mechanisms. Firstly, the use of an acoustic field produces interfacial waves which become unstable, eventually resulting in the eruption of the oil phase into the water medium in the form of droplets. Secondly, the application of low frequency ultrasound causes acoustic cavitation, that is, the formation and subsequent collapse of microbubbles by the pressure fluctuations of a simple sound wave. Each bubble collapse (an implosion on a microscopic scale) event causes extreme levels of highly localized turbulence (Figure 2.2). The turbulent micro-implosions act as an very effective method of breaking up the primary droplets of dispersed oil into droplets of sub-micron size (Forster, 1997).

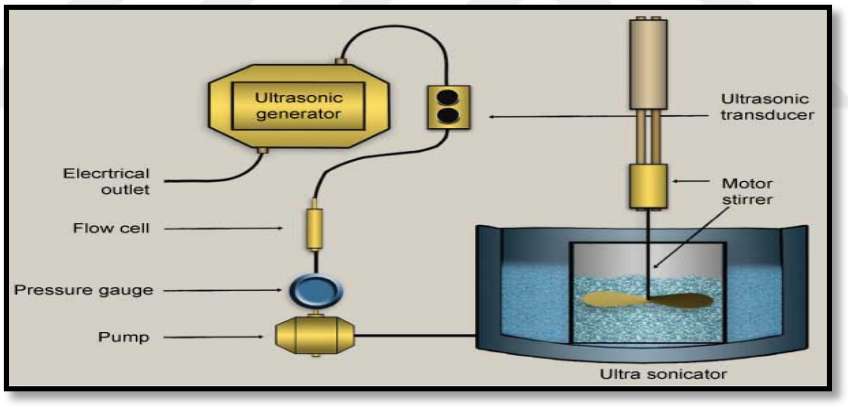

Figure 2.2. Emulsification by ultrasonic technology (Forster, 1997).

2.3.2 Mano-thermo-sonication

Ultrasonication in combination with low static pressure and low heat is a process called mano-thermo-sonication (MTS). Figure 3.1 shows a laboratory scale MTS system**.** In the MTS system, a VC-750 ultrasound generator is used to deliver acoustic energy into a jacketed reactor. The frequency of the generator is 20 kHz and the peak-to-peak amplitude at the tip of the probe is

124 micros. The temperature inside the reactor is monitored by a thermal couple and controlled to ±1 ^{o}C of the target temperatures by circulating water is regulated by mixing tap water with that from a water bath set a pre-determined temperature. A feedback control facilitated and interfaced by LabView version 7.1 is used to adjust the opening of a water valve to the tap water stream. Nitrogen gas is used as the pressure source to pressurize the MTS system (Lee et al., 2009). MTS has been reported to enhance acoustic cavitation activity which is effective in performing a target food processing operation (Kuldiloke., 2002).

2.4 pH-shifting process

A number of attempts have been made to modify the native structure of soy proteins to improve emulsifying capacity (Elizalde et al., 1996). Changing the pH of a protein solution is one of the oldest methods utilized to unfold proteins. An improved charge repulsion forces the proteins to a partially unfolded state (Kristinsson & Hultin, 2003). Several studies have reported that globular proteins might be partly unfolded under extreme pH conditions, mostly at low pH levels. This dynamic structure is referred to as the "molten globule" structure (Goto et al., 1989 & Goto et al., 1990). This structure maintains a relatively compact structure such as retention of most secondary structure, however has a tendency to lose some of the tertiary structure (Goto et al., 1990). Studies have used myosin (Kristinsson & Hultin, 2003), egg albumin (Liang & Kristinsson, 2007), and hemoglobin (Kristinsson & Hultin, 2004) and exposed them to extreme pH levels (pH 2 or pH 12) followed by readjustment of the pH back to neutrality (pH 7), a process known as pH shifting, to produce MG state proteins. In the MG state, proteins show enhanced functional properties, especially emulsifying and foaming activities.

The pH-shifting process was initially designed to better recover of proteins from animal based materials. Significant functionality enhancements caused by structural changes have been observed in muscle proteins and egg albumen following acid or alkali treatments (Liang e al., 2007 & Raghavan et al., 2008). pH-shifting was tested as a simple and cost-effective method to extract proteins (Fu, Wu, & Li, 2012; Özyurt, Simsek, Karakaya, Aksun, & Yesilsu, 2015), and improve the protein functional properties (Jiang, Xiong, Newman, & Rentfrow, 2012). Specifically, alkaline pH shifting (pH 12.0) was outlined to significantly increase the solubility of soy protein isolate (Jiang, Xiong, & Chen, 2010).

2.5 Protein-stabilized emulsions

Emulsion preparation is a crucial initial step for microencapsulation of oils. During emulsion formation, oil droplets become dispersed through the input of mechanical energy (i.e., homogenization) within an aqueous continuous phase containing an emulsifier (i.e., protein) (McClements, 2005). Emulsions are considered to be thermodynamically unfavorable systems, which tend to break down over a period of time by a variety of destabilization mechanisms such as gravitational separation, aggregation, and coalescence The role of emulsifiers is to prevent this instability by adsorbing to the interface to form a protective barrier around the oil droplets and can provide both electrostatic (at pHs away from the protein's isoelectric point) and steric stabilizing forces. During emulsion formation, proteins partially unfold to align at the oil-water interface and place their hydrophobic and hydrophilic parts towards the oil and aqueous phases, subsequently. Then, the proteins at the interface aggregate to form a viscoelastic film which then may or may not be crosslinked using a fixative (i.e., transglutaminase) based on the strength of the film (Morris and Gunning, 2008). It was observed that flexible proteins such as casein have more disordered

structures, and are the most surface-active proteins. On the other hand, globular proteins such as β-lactoglobulin or pea legumin are more compact and rigid, and need some levels of unfolding before adsorbing to the interface (Dickinson, 1986). Several intrinsic characteristics of the proteins including flexibility, surface hydrophobicity, solubility, surface charge, and molecular size have an impact on the formation and stabilization of emulsions. For example, good solubility in the aqueous phase is a prerequisite for the proteins to be used as emulsifiers, because system figures out the amount of proteins available to migrate to the oil-water interface to stabilize the oil droplets (Sikorski, 2001). Surface hydrophobicity also plays an important role; as the hydrophobic areas of proteins increases, the greater absorption and retention at the oil-water interface (Sikorski, 2001). Due to non-toxicity, wide availability, and friendly labeling, proteins such as soy proteins are widely used to stabilize emulsions to further produce microcapsules. Emulsion viscosity is another important parameter to control during encapsulation, because it may affect the stability and flow behavior of emulsions during drying. For example, high viscosities of a feed emulsion might interfere the atomization during spray drying, prolong the drying process, and cause air inclusion in the particles (Rosenberg et al., 1990). Finally, emulsions should be stable over a certain period of time before drying, so smaller droplet sizes are needed to prevent destabilization and air inclusion in the particles (Drusch, 2006).

2.6 Microencapsulation technology

Microencapsulation has been used in various areas such as pharmaceuticals, biotechnology, agriculture and food to carry and protect essential oils, colorants, flavors, vitamins and microorganisms for different applications (i.e., shelf-life and stability) (Ray et al., 2016). Microencapsulation is described as a process to isolate or embed bioactive particles by building a

physical barrier to produce small capsules with different morphologies and diameters (0.2 and 5,000 μm). Basically, the encapsulated substances are known as the core, or internal phase, whereas the outer protective materials are defined as the wall, or external phase. In general, microencapsulation can be applied for below purposes:

1) protecting the core material from surrounding environmental changes (i.e., pH, temperature, oxygen, light, and humidity),
2) controlling the volatility and release properties of the core material,
3) masking the unpleasant flavor and taste of the core material,
4) transforming liquid compounds into solids for easy handling, and
5) diluting the core material when only very small amounts are needed (Desai and Park, 2005; Nesterenko et al., 2013; Bakry et al., 2016).

2.6.1 Core materials

A core material can be any bioactive compound that is either hydrophobic or hydrophilic. One such example is omega fatty acids. The health benefits of omega fatty acid-rich oils have proven through extensive studies that show their ability to prevent coronary artery disease, hypertension, diabetes, arthritis, inflammatory and autoimmune disorders (Connor, 2000; Tur et al., 2012). Studies have recommended a daily intake of omega fatty acids (e.g., omega-3 and omega-6 fatty acids) by pregnant and lactating women to support the healthy development of both the retina and brain of the infant (Connor, 2000; Koletzko et al., 2011). Omega fatty acids are the unsaturated fatty acids, which have at least one double bond within the carbon chain. They include omega-3 (i.e., α-linolenic acid, eicosapentaenoic acid (EPA), and docosahaxaneoic acid(DHA)), omega-6 (i.e., linoleic acid, arachidonic acid, docosapentaenoic acid), and omega-9 fatty acids

(i.e., oleic acid). Because of their unsaturated nature, omega fatty acid-rich oils are chemically unstable and susceptible to oxidative deterioration. They produce free radicals and unpleasant tastes which affect the shelf-life, sensory properties and overall acceptability of food products in a negative way (Velasco et al., 2003). For this reason, microencapsulation is utilized as a feasible method to maintain and improve the biological and functional characteristics of the oils, and further develop healthy food products fortified with omega fatty acids (e.g., bread, milk, and yogurt).

2.6.2 Wall materials

Selection of appropriate wall materials is an important parameter in microcapsule design, because wall materials affect their stability, entrapment efficiency and the degree of protection. An ideal wall material should have such characteristics as low viscosity under high concentrations, good emulsifying properties to stabilize the core material, non-reactivity with the core material, ability to hold the core material within the capsules, desired controlled release characteristics, ability to provide maximum protection to core material against environmental conditions (i.e., oxygen, heat, light, and humidity), excellent solubility (in water or ethanol), and being economically viable (Desai and Park, 2005).

Specifically, biopolymers (i.e., carbohydrates and proteins) are most commonly used wall materials for microencapsulation in the food industry (Dubey et al., 2009; Nesterenko et al., 2013). Polysaccharides have been studied as wall materials, including starches, maltodextrins, gum arabic, pectins, chitosan, and sodium alginate (Liu et al., 2010; Sun-Waterhouse et al., 2011; Nesterenko et al., 2013). Maltodextrins are often used as a secondary material in the wall material due to their excellent solubility and low viscosity at high concentrations (Gharsallaoui et al., 2007).

The major advantage of polysaccharides is their excellent water solubility. However, they tend to have poor emulsifying properties that are important for microencapsulation (Nesterenko et al., 2013). On the other hand, proteins from animal sources such as whey and casein (Devi et al., 2012; Sanguansri et al., 2013; Silva et al., 2016) and plant sources (i.e., pulses, oilseeds and cereals) have also been used for oil microencapsulation (Wang et al., 2011; Can Karaca et al., 2013; Tang and Li, 2013), due to their excellent emulsifying properties. Plant proteins are emerging in the food industry as an alternative to animal proteins, because of the safety concerns related to the consumption of animal-derived proteins (i.e., bovine spongiform encephalopathy), lower cost, and abundant supply (Choi et al., 2010; Li et al., 2012; Nesterenko et al., 2013). According to Nesterenko et al. (2013), no single wall material can posess all the desired properties, therefore a combination of polysaccharides and proteins is most commonly studied to develop microcapsules. Utilization of proteins/polysaccharides mixtures leads to incorporation of specific properties of each polymer, to further improve their emulsion stability and produce microcapsules with the better oxidative stability of the core material (Young et al., 1993; Gharsallaoui et al., 2010).

2.6.3 Microencapsulation techniques

A number of technologies have been developed to produce microcapsules using, including spray drying, freeze drying, coacervation, extrusion, and fluidized-bed coating (Bakry et al., 2016). Selection of a suitable technique depends on different parameters such as the type of core and wall materials, the size of final microcapsules, the desired physicochemical properties of microcapsules, the release profile, and the cost (Desai and Park, 2005; Ray et al., 2016).

2.6.3.1 Freeze drying

Freeze drying is commonly used for dehydration of heat-sensitive materials. For microencapsulation, the oil is homogenized with coating materials to form an emulsion and is then frozen to be freeze dried (Heinzelmann et al., 2000). Frozen water is directly sublimated from the solid phase to a gas under a vacuum (Oetjen and Haseley, 2004). Freeze drying is a popular drying method for microencapsulation due to the simple operating procedures and reduced temperatures. However, high energy use, long processing time, high production cost, and poor ability to protect encapsulated oils from oxidation (because of the porous structure of the microcapsules) are major concerns for industrial application (Desobry et al., 1997). In their work with encapsulated fish oil with sodium caseinate, Heinzelmann and co-workers (2000) observed that the microcapsules with highest core entrapment efficiency did not show better storage stability for the encapsulated oil, since other characteristics such as moisture and particle size were also important. Avramenko and co-workers (2016) produced flaxseed oil microcapsules using native and pre-treated (heat and enzymatic hydrolysis) lentil protein isolate in combination with maltodextrin, and found that applying a pre-treatment of the lentil protein isolate was not needed because the pre-treated lentil protein isolate (~47%) produced microcapsules with decreased entrapment efficiency compared with native lentil protein isolate (~63%). Finally, Calvo et al. (2011) found that the fatty acid composition in walnut oil was not affected by freeze drying and wall materials.

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CHAPTER 3

FUNCTIONALIZING SOY PROTEIN NANO-AGGREGATES WITH PH-SHIFTING AND MANO-THERMO-SONICATION (MTS)

3.1 Introduction

Proteins are widely used in the food industry as foaming and emulsifying agents (Wilde et al. 2004). They stabilize oil-in-water emulsions by forming an interfacial membrane on which the adsorbed proteins unfold and rearrange their secondary and tertiary structures to expose hydrophobic residues to the hydrophobic phase (MacRitchie, 1978). Plant proteins are low cost, non-toxic, natural, biocompatible, and biodegradable polymers. In recent years, they have drawn increasing attention from the food and pharmaceutical industries as an alternative to animal proteins due to increased consumer concerns over the safety of animal-derived products (e.g., prion diseases) (Liu et al., 2010). Among plant proteins, soy proteins have been widely studied for numerous applications (Nunes et al. 2003; Hua et al. 2005; Caillard et al., 2009; Gómez-Mascaraque and López-Rubio, 2016). Soy protein is an ample byproduct of the soybean oil industry. Whole soybeans can be processed into a number of products such as roasted soy nuts, soy flours, soybean oil, defatted flakes, soy-protein concentrates, and soy-protein isolates (SPI) (Pujara et al., 2017). The main storage protein in soybean are globulins, which are mostly insoluble

in water. The poor solubility of globulins in soy makes it a less effective emulsifier than dairy proteins, such as casein (Nishinari et al., 2014).

A number of attempts have been made to modify the native structures of soy proteins in order to improve their functional properties, including high-pressure and heat combined treatments (Puppo et al., 2005; Molina et al., 2001), extreme pH treatment (Elizondo et al., 2011), high pressure processing (HHP) (Manassero et al., 2015), pulsed electric field (PEF) (Li et al., 2007), enzyme hydrolysis (Tsumura et al., 2005), subcritical water treatment (Zhang et al., 2015), and ultrasonication (Hu et al., 2013). Among the chemical treatments, pH-shifting is a relatively new method that adjusts the pH of a protein solution to extreme basic or acidic conditions to unfold the protein, followed by changing the pH back to neutral to refold the protein. This unfolding-refolding process was reported to effectively modify the protein functional properties (Jiang et al., 2010, Lee et al., 2016). *High intensity ultrasound* or *power ultrasound* refers to sonic waves with frequencies higher than sound audible to the human ear, with sound intensities in the range of 0.1-1 W/cm^2 (Feng and Yang, 2011). The mode of action of ultrasound-induced protein-structure modification is often attributed to acoustic cavitation. The physical forces produced by cavitation, such as shear forces produced by micro-streaming and normal impingement from the water jets at the solid-liquid interfaces, help to break down the protein particles or aggregates in dispersions (Lee et al., 2016). Ultrasound treatment was reported to enhance protein solubility and surface hydrophobicity, and modify protein subunits (Tang et al., 2009; Jiang et al., 2017). Conventional ultrasonic modification of protein functionality is performed at relatively low cavitation intensities. Consequently, relatively long treatment times, i.e. 15-30 min for soy proteins (Hu et al., 2013) and 20 min for whey protein (Arzeni et al., 2012), are required to produce meaningful changes in

protein structures. There is a need to reduce the treatment time of ultrasound processes, thereby increasing throughput, lowering costs, and making their applications practical.

Ultrasonication in combination with low hydrostatic pressure and low heat, a process termed mano-thermo-sonication (MTS), has been reported to enhance acoustic cavitation activity (Kuldiloke, 2002). Significant process enhancement by MTS has been reported in microbial inactivation tests. For instance, it took 15.9 minutes to achieve a 5-log cycle (99.999%) reduction in the population of *E. coli* K12 in apple cider, a benchmark set by USFDA for microbial inactivation in juices, in an ultrasound-alone treatment (Ugarte-Romero, et al., 2006). The time was reduced to 1.4 minutes when an MTS treatment at 400 kPa and 59°C was applied to the juice to obtain the same 5-log reduction (Lee et al., 2013). However, MTS has not been reported for use in protein modification. This study documents the first attempt to utilize MTS to enhance the functional properties of soy-protein isolate. Furthermore, we combined pH-shifting with MTS to further improve the efficacy of the treatment. The MTS was applied when the protein was unfolded by an extremely basic pH treatment, as the open structure of the protein made it more susceptible to the ultrasound treatment. The pH-shifting and MTS combined treatment was optimized with Response Surface Methodology, and compared with high pressure homogenization (HPH). The physicochemical, interfacial, and emulsifying properties of the treated soy protein were evaluated.

3.2. Materials and methods

3.2.1 Soy protein isolate (SPI)

Soy protein isolate (SPI, Pro-Fam® 955, pH 5.0-5.5) was provided by Archer Daniels Midland Company (ADM, Decatur, IL, USA). The Pro-Fam® 955 contains 90% soy protein on dry basis and is a water washed intact protein (without hydrolysis with proteases).

3.2.2 pH-shifting, high pressure homogenization, and MTS

Mano-thermo-sonication treatment was conducted using a laboratory scale MTS system (Fig. 3.1). In continuous mode, the protein dispersion in a sample bottle placed in ice was pumped by a peristaltic pump (750 W, Model 7523-20, Masterflex, Vermon Hill, IL, U.S.A) into a custom-made sono-reactor. The treated samples were collected from the two outlets of the sono-reactor and cooled for analysis. A preliminary test was performed to determine the optimal MTS treatment conditions. In MTS tests, 9 g SPI were dispersed in 300 mL DI water by agitation with a stirrer for 30 min, and the dispersion was pumped through the MTS unit. The MTS treatment time (or residence time of the sample in the sono-reactor) was determined by the flow rate (mL/s) and the volume (L) of the treatment chamber. The MTS parameters include 3 temperatures (40, 45, and 50^{o}C), 3 sonication times (30, 45, and 60 s) and 3 absolute pressures (100, 200, and 400 kPa).

Five treatments were applied to modify SPI: MTS (at optimal conditions), pH shifting, pH 12-MTS, HPH, and pH 12-HPH (Table 3.1). In the pH alone treatments, 3 g SPI was dissolved in 100 mL deionized (DI) water and stirred at room temperature for 30 min, resulting in protein dispersions. The pH of the protein dispersions was adjusted to pH 12 with 2M sodium hydroxide (NaOH). Sample was stored at room temperature for 1 h, followed by adjusting the pH back to pH 7 with 2M hydrochloric acid (HCl). In the pH 12-MTS treatment, the SPI dispersions were adjusted to pH 12 with 2M NaOH and then treated by MTS (50^{o}C, 200 kPa and 60 s) before the pH was adjust to neutral. For the pH12-HPH treatment, the SPI dispersions were adjusted to pH 12 with 2M NaOH and then treated by HPH followed by changing the pH of the dispersion to pH 7. Neutralized protein dispersions were centrifuged (1,200 g and 20^{o}C) for 15 min and the supernatants were collected as soluble soy protein for further experiments. The HPH treatment was conducted using a high-pressure homogenizer (APV two stage homogenizer; SPX Flow

Technology, Denmark) at 8,000 psig for 3 min with a sample size of 500 mL containing 15 g SPI. The sample with no treatment but only stirred 30 min under room temperature was used as the Control.

3.2.3 Protein solubility

Soluble protein content was determined with a Bio-Rad Protein Assay based on the method of Bradford (Bradford, 1976). Bovine serum albumin (BSA) (Bio-Rad 500-0007) was used as the standard. Dye reagent was prepared by diluting 1 part of dye reagent concentrate (Bio-Rad 500-0006) into 4 parts of DI water, and filtered through Whatman #1 filter paper. Diluted dye reagent was added to soluble SPI. Protein concentration of soluble SPI was measured using a spectrophotometer (Lambda 1050 UV/VIS/NIR Spectrometer, PerkinElmer, Waltham, MA, USA) under the wavelength of 595 nm (Bradford, 1976). Protein solubility was calculated as the percentage of the soluble protein content in the supernatant over the total protein added in the dispersion as shown in equation 3.1.

$$\text{Recovery of soluble protein (\%)} = \frac{\textbf{Protein concentration in soluble SPI}}{\textbf{Initial protein concentration}} \times 100 \qquad (3.1)$$

3.2.4 Water solubility profile

Three grams of SPI was dispersed into 100 mL deionized (DI) water. The pH of the protein dispersion was adjusted to 2, 3, 4, 5, 6, 7, 8, 9, and 10 with either 2N HCl or 2N NaOH. The protein dispersion was stirred for 1 h. 25 mL of the dispersion was put into 50 mL centrifuge tubes and centrifuged at 10,000 × g for 10 min at 20°C. Protein content of supernatant was determined with

a Bio-Rad Protein Assay (Bradford, 1976). Bovine serum albumin (BSA) (Bio-Rad 500-0007) was used as the standard. The solubility was calculated as:

$$\text{Protein Solubility (\%)} = \frac{\textbf{Protein in supernatant (g)}}{\textbf{Protein in starting material (g)}} \times 100 \quad (3.2)$$

3.2.5 Particle size and turbidity

Volume-weighted mean diameters (D [4,3]) of the soluble protein and soy protein-stabilized nanoemulsions were measured by dynamic light scattering (DLS) using a NICOMP 380 DLS instrument (Santa Barbara, CA, USA). For soy protein-stabilized nanoemulsion samples, the stability of the droplets was evaluated by measuring the droplet size over a period of 21 days at 4^{o}C. Samples were diluted 500-fold with distilled water before DLS measurement. All measurements were performed at a fixed scattering angle of 90° with 658 nm wavelengths at 25°C. The mean particle size was determined as the average of three runs in which each run was performed for 1 min. Turbidity of the soluble protein and soy protein-stabilized nanoemulsions was measured using a spectrophotometer (Lambda 1050 UV/VIS/NIR Spectrometer, PerkinElmer, Waltham, MA, USA), followed the method of Lee et al. (2016). Distilled water was used as the blank. The absorbance at 600 nm of each sample represented the turbidity.

3.2.6 Free sulfhydryl and disulfide bond (S-S) content

Concentrations of the free sulfhydryl groups (SH, mmoles/g soluble solids) were determined using a cysteine standard as reported by Lee et al. (2016). A set of cysteine standards were prepared by dissolving cysteine hydrochloride monohydrate at different concentrations (1.50, 1.25, 1.00, 0.75, 0.50, 0.25, and 0.00 Mm) in reaction buffer (0.1M sodium phosphate containing

1mMEDTA). A set of test tubes containing 50 mL of Ellman's reagent solution and 2.5 mL of reaction buffer were prepared. 250 mL of each standard was added in above test tubes. The solution in the test tubes was mixed and incubated at room temperature for 15 min. The absorbance at 412 nm was measured. The values obtained for the standards were plotted to generate a standard curve, and the sample concentrations were determined from this curve. For total SH content analysis, 4 mL of Tris-Gly buffer (0.086 M Tris, 0.09 M glycine, 0.04 M EDTA, pH 8.0) was added to 1 mL of the protein solution. The mixture was incubated 1 h at room temperature. Then, the mixture was centrifuged at 5,000 g for 10 min. 0.04 mL of Ellman's reagent solution was added to 4 mL of this solution, and the absorbance was measured at 412 nm. Half of the value after subtracting the free SH value from the total SH value was defined as the disulfide bond (SS) content:

$$\text{SS Content} = \frac{\textbf{Total SH} - \textbf{Free SH}}{\textbf{2}} \left(\frac{\boldsymbol{\mu}\textbf{mol}}{\textbf{g}}\right) \quad (3.3)$$

3.2.7 Surface hydrophobicity

Surface hydrophobicity (Ho) of soluble soy protein was measured as reported by Lee et al. (2016). The fluorescent probe utilized was 1-anilino-8-naphthalenesulfonate (ANS) (Sigma Aldrich, St. Louis, MO, USA). ANS stock solution (8 mM) was prepared in phosphate buffer (0.01 M, pH7). Five soy protein concentrations, 0.04-0.2 mg/mL, were also prepared with phosphate buffer (0.01M, pH7) (final volume 4 mL). Twenty mL ANS stock were added to protein solutions and fluorescence intensity was measured (Synergy™2, BioTek Instrument Inc., Winooski, VT, USA) at 340 nm (excitation) and 440 nm (emission). Initial slopes of fluorescence intensity vs. protein concentration were used as surface hydrophobicity (Ho) of proteins.

3.2.8 Emulsifying properties

Emulsifying properties were measured by the method of Pearce and Kinsella (1979). Oil-in-water (o-w) emulsions were prepared by adding 1 mL of canola oil in 3 mL of the SPI samples. The oil concentration was 0.25% (w/w) in this test. Ultrasound (750 W, Model 7523-20) was used to generate the SPI nanoemulsions. The mixture of oil and SPI solution was stirred strongly for 5 min and then sonicated for 5 min. After emulsion formation, the absorbance was measured at 500 nm at 0 (A_0) and 10 min (A_{10}), respectively. Emulsifying activity index (EAI) and emulsion stability index (ESI) were calculated by using the following equations:

$$\text{EAI } (m^2/g) = 2T\ A_0 \times \text{dilution factor}/c \times \Phi \times L \times 10\ 000 \quad (3.4)$$

$$\text{ESI (min)} = A_0 / (A_0 - A_{10}) \times 10 \text{ (min)} \quad (3.5)$$

where T = 2.303; dilution factor = 100, c – weight of protein per unit volume (g/mL), L – width of the optical path (0.01 m), and Φ – oil volumetric fraction (0.25).

3.2.9 Antioxidant capacity

1,1-diphenyl-2picryl hydrazyl radical (DPPH) was used to determine the antioxidant capacity of treated SPI (Ebrahimzadeh et al., 2009). The sample extracts were prepared in different concentration (w/w) (0.02 %, 0.04 %, 0.06 %, 0.08 %, and 0.10%). The DPPH solution was added to sample extracts, vortexed, and incubated in the dark at room temperature for 15 min. The absorbance of the solutions was then read at 517 nm with a spectrophotometer (Lambda 1050 UV/VIS/NIR Spectrometer, PerkinElmer, Waltham, MA, USA). Absorbance of DPPH radical without sample was used as a control. The antioxidant activity was calculated as the following:

Activity (%) = $(A_c - A_t)/(A_c) \times 100$ (3.6)

where A_c is the absorbance of control and A_t is the absorbance of samples.

3.2.10 Total phenols

Total phenolic content of treated SPI was determined using a modified colorimetric method (Singleton, 1974). The method involves the reduction of Folin-Ciocalteau reagent (Sigma Chemical, St. Louis, Missouri, USA) by phenolic compounds, with a concomitant formation of a blue complex. 0.1 mL of aqueous solutions was transferred to test tubes and their volumes adjusted to 0.5 ml with distilled water. 0.25 ml Folin-Ciocalteu reagent and 1.25 ml 20 % aqueous sodium carbonate solution were added; tubes were vortexed; and absorbance of blue colored mixtures recorded at 725 nm after 40 min. The measurement was compared to a standard curve prepared with catechin solution. Total phenolic content was expressed as mg catechin/g dry plant material.

3.2.11 Rheological measurements

Rheological measurements were carried out using a TA ARES-G2 Rheometer (TA Instruments, New Castle, DE, USA), according to the method of Sittikijyothin et al. (2010) with some modifications. Protein dispersions of SPI were prepared in distilled water. All rheological measurements were performed at 25^{o}C. Concentric cylinder geometry was used for the samples. A flow curve analysis was performed using a steady state flow ramp with the shear rate ranging between 1 to100 s^{-1}. The shear rate was recorded point by point with consecutive 3 min steps at constant shear rate. Three minutes were used because this time allowed getting constant shear rates for each point. The viscosity was then determined for each point and used to build the flow curve.

3.2.12 Nanoemulsion preparation

Oil-in-water (O/W) nanoemulsions were prepared with canola oil and soluble nano-SPI. Canola oil (0.125 g and 0.25 g) was added to 50 mL soluble SPI (10 mg/mL) and vigorously stirred 5 min with a magnetic stirrer. To achieve fine homogenization, samples were sonicated (5 min) using a VC-750 ultrasound unit (20 kHz, Sonics & Materials, Inc., Newtown, CT, USA) in an ice bath.

3.2.13 Lipid oxidation

Lipid hydroperoxide formation during storage of soy protein-stabilized nanoemulsions was determined as reported by Min et al. (2003). Soy protein-stabilized nanoemulsions (5 mL) were placed in lightly sealed screw-cap test tubes and allowed to oxidize at 37°C in the dark. Lipid hydroperoxides were measured by mixing 0.3 mL of emulsion with 1.5 mL of isooctane/2-propanol (3:1, v/v) by vortexing (10 s, 3 times) and isolation of the organic solvent phase by centrifugation at 1,000 g for 2 min. The organic solvent phase (200 μL) was added to 2.8 mL of methanol/1-butanol (2:1, v/v), and followed by 15 μL of 3.97 M ammonium thiocyanate and 15 μL of ferrous iron solution (prepared by mixing 0.13 M $BaCl_2$ and 0.144 M $FeSO_4$). After 20 min, the absorbance of the solution was measured at 510 nm. Lipid hydroperoxides of the nanoemulsions were measured at 1, 24, 48, 96, 120, 144, and 168 h.

3.2.14 Transmission electron microscopy (TEM)

Morphology of the soy protein-stabilized nanoemulsions was examined by TEM (Voltage: 20.00 kV; Magnification: 1 200 ×). A drop of the solution dispersed in fixative solution (2% glutaraldehyde and 2.5% paraformaldehyde) was put onto a parafilm ® sheet. The copper coated

side of the TEM sample holder grid was gently laid on top of the droplet and incubated for 10 min. A drop of a 7% uranyl acetate solution was added and held for 5 min. The grid was then removed from the droplet and allowed to dry at room temperature before presenting it to the TEM. Samples were observed using a Philips CM200 transmission electron microscope (FEI company, Hillsboro, Oregon), and the sample images were captured by a Peltier-cooled Tietz (TVIPS) 2k × 2k CCD camera.

3.2.15 Statistical analysis

Three replications for each treatment were used for all measurements, unless otherwise stated. The results were analyzed by analysis of variance using the General Linear Models (PROC GLM) procedure in SAS (version 9.3, SAS Institute, Inc., Cary, North Carolina, USA). Differences among the mean values were obtained by Fisher's least significant difference (LSD) test at alpha = 0.05.

3.3. Results and discussion

3.3.1 Protein solubility

Table 3.2 tabulates the protein solubility values and particle sizes obtained in the preliminary test, where the SPI samples were treated by 13 combinations of temperature (40, 45, and 50^{o}C), sonication time (30, 45, and 60 s.) and pressure (100, 200, and 400 kPa) determined by the Response Surface Methodology. The optimal treatment condition (highest solubility and smallest particle size) was found to be sonication at 50^{o}C, 200 kPa, for 60 s (Table 3.2), and this condition was used in all the following experiments when MTS was used.

The soluble protein contents of the six treatments (Control, pH shifting, MTS, pH12-MTS, HPH, and pH12-HPH) are shown in Table 3.3. The highest protein solubility was 82.5% from the pH12-MTS treatment, while the lowest protein solubility (9.1%) was observed in the Control (untreated SPI). The HPH and pH12-HPH treated samples had solubility significantly higher the Control and pH-shifting alone, but was significantly lower than the MTS and pH12-MTS treatments. Improvement in SPI protein solubility after an ultrasonic treatment was reported in previous studies (Chen et al., 2006; Jambrak et al., 2009). Lee et al. (2016) found that the solubility of the SPI samples was significantly increased by pH-shifting at pH 12 and further enhanced by the application of ultrasound for 5 min (pH 12 + US5). They reported that the pH12 + US5 treated SPI increased SPI solubility from 1.49 % for the control to 82.73 %, which is slightly higher than the highest solubility (82.5%) obtained in this study. The difference may be caused by the long ultrasonication time (5 min) used in the work of Lee at el. (2016) compared to 60 s used in this work.

The increase in protein solubility an ultrasound treatment is often attributed to acoustic cavitation related activities. Acoustic cavitation produces physical forces such as hydrodynamic shear forces that may cause physical damage contributing to increasing protein solubility (Khanal et al., 2007). Sonication at high intensity may break the covalent and non-covalent bonds and enhance SPI solubility (Hua et al., 2005). Acoustic cavitation has an effect on the three-dimensional structure of globular proteins. This effect leads to increased numbers of charged groups. These conditions cause electrostatic forces to be higher, so more water molecules interact with the protein molecules, the polarity increases, and hence solubility increases (Gülseren et al., 2007). During an ultrasonic treatment, the increase in solubility may also be caused by conformational changes so that hydrophilic amino acid residues are oriented against water (Morel

et al., 2000) and formation of soluble protein aggregates from insoluble protein aggregates (Tang et al., 2009). Moreover, the particle size of SPI can be reduced by an ultrasonic treatment that increases the protein-water interactions and thus results in increased protein solubility (Arzeni et al., 2012). Particularly, ultrasound treatment at elevated pressure and low static pressure, i.e. MTS enhances all the physical effects of ultrasonication by intensifying the cavitation intensity. From our unpublished data, cavitation intensity in a MTS treatment was 3 to 6 times higher than that in an ultrasound alone treatment. A static pressure of above 1 atm will help to enhance the implosion power of cavitating bubbles. In addition, elevated temperature in a MTS treatment will lower activation energy of biochemical reactions involved in the process thereby enhancing the treatment.

3.3.2 Water solubility profile

Protein solubility as a function of pH serves as a useful indicator on how well the protein performs if it is incorporated into a food or drug system. Extrinsic factors such as pH, temperature, and ionic strength affect protein solubility (Bolontrande et al., 2013). The effect of pH on soy protein solubility was reported to show a u-shaped curve, with high solubility shown to be on both sides of the isoelectric point, ~pH 4.5 (Lee et al., 2013). The protein pH profiles of the untreated, MTS alone and pH 12-MTS of SPI are shown in Fig. 3.2. All treatments exhibited the typical U-shaped solubility profile with the lowest solubility near the isoelectric pH (4-5) except the Control. The control SPI has low solubility at all pH values as it had the largest particle size (Table 3.4) making its hydration difficult. Under the alkaline conditions, both the MTS and pH12-MTS samples showed higher solubility than that under the acidic conditions. The pH12-MTS treatment enhanced SPI solubility compared to the MTS alone treatment, as shown by the higher solubility

and narrower pH range where SPI had very low solubility. Protein carries positive or negative zeta potential based on the pH of the medium. All treatments including control, MTS, and pH12-MTS were positively charged at pH below pI and negatively charged at pH above pI, as shown by the Zeta potential values listed in Table 3.5. At pH 5.0, there were almost no net charges (~3 mV) for all treatments indicating that proteins were near the pI values. A practical implication is that the pH12-MTS treatment SPI can be used in a liquid food or drug system with pH of <3.5 or > 5.5 with high solubility and less precipitations.

3.3.3 Particle size and turbidity

The sizes (D [4, 3]) of the soluble SPI aggregates from the 6 treatments are shown in Table 3.4. All the treatments produced soluble SPI aggregates with sizes of <100 nm except the Control. The pH 12-MTS treated samples had smallest size (27.1 nm) of the soluble protein aggregates, while the D [4, 3] for the Control was 192 nm. In the pH 12-MTS treatment, the MTS (50^{o}C, 200 kPa, for 60 s) was applied after unfolding of globular soy proteins with high pH treatment (pH 12); the unfolded SPI became more susceptible to breakdown by physical forces produced by acoustic cavitation. This may contribute to the smallest particle size observed in the pH 12-MTS samples. The reduction of soy protein particle sizes by ultrasound alone treatments was reported by a number of studies (Arzeni et al., 2012; Hu et al., 2003). Karki et al. (2010) found that the particle size of defatted soy flakes was reduced nearly 10-fold by ultrasonic treatment at high-power density (up to 2.56 W/mL). Using the same ultrasound generator as this study, Lee et al (2016) reported a reduction of SPI aggregate size from 376.1 nm in the Control to 21.8 nm after sonication for 5 min in a pH-shifting (pH 12) and sonication combined treatment. Since the breakdown of aggregates in the dispersion is caused by the physical forces produced by acoustic cavitation (Lee

et al., 2016), a process that enhances the cavitation activity, such as MTS, will contribute to enhance ultrasound treatment resulting in more effective particle size deduction. There was also a significant decrease in particle size of samples treated with the pH12-HPH (45.3 nm) compared to the Control. This is in agreement with the observation of Song et al. (2013) compared to the pH12-MTS and MTS samples

Turbidity of protein dispersion is determined by both the number concentration and size of the soluble protein aggregates (Gregory, 1998). The number concentration is directly related to protein solubility. The turbidity values of the treated SPI are shown in Table 3.4 and their appearance is shown in Fig. 3.3. The pH12-MTS and MTS samples had high number concentration (solubility), but since they also had the small particle sizes, their turbidity was among the lowest (0.09 and 0.27, respectively) and the sample was almost transparent (Fig. 3.3). The Control looked cloudy as it had the largest particle sizes (192 nm). The HPH sample also looked translucent as it had a relatively small particle size (72.9 nm) compared to the Control (192 nm) with moderate number concentration (solubility).

3.3.4 Free sulfhydryl content (SH) and disulfide bond (S-S) content

Free sulfhydryl (SH) and disulfide bond (S-S) contents of the treated SPI samples are shown in Table 3.3. The SH and S-S contents of the pH12-MTS samples were the highest among all other treatments. The lowest SH and S-S contents are observed in the Control, followed by the HPH samples. The MTS treatment also had high SH and S-S values, only second to the pH12-MTS samples. A higher SH content indicates the exposure of internal SH groups due to protein unfolding, or the cleavages of the S–S bonds in native proteins. Therefore, the surface SH content is closely related to conformation changes and protein unfolding, indicating the exposure of SH

groups or the breakdown of disulfide bonds (Jiang et al., 2017). The increase in SH may also be caused by reduction of SPI particle sizes after MTS and HPH treatment, which allows the buried SH groups in SPI to be exposed to the surface. The higher SH and S-S content in the pH12-MTS samples compared with the MTS treatment actually highlighted the advantage of pH12-MTS. The unfolding-sonication-refolding process, or exposing SPI to pH 12 + MTS + adjusting the pH to neutral process, enhanced the treatment making it more effective in modifying the structures and thus functional properties of SPI. An increase in free sulfhydryl (SH) and disulfide bond (S-S) contents also reported by Lee et al. (2016) in the pH12-ultrasound treated SPI samples compared to the ultrasound alone and pH-shifting alone SPI. Similar enhancement of SH or S-S content after an ultrasound treatment was also reported by Hu et al. (2013). From Table 3.3, the pH12-MTS treated sample had the highest protein solubility, showing that the increase in SH content and double bonds contributed to increase solubility.

3.3.5 Surface hydrophobicity (Ho)

Surface hydrophobicity (Ho) values of the SPI samples are listed in Table 3.3. While the lowest surface hydrophobicity (124.0) was observed for the untreated samples (Control), the highest Ho (241.0) was observed for the SPI dispersions treated with pH12-MTS. Compared to the MTS treated SPI samples, the HPH treated samples showed significantly lower Ho (180.2). An increase in Ho of soy protein by ultrasonication was also reported in a few other studies (Hu et al., 2013; Chen et al. 2011). Since protein hydrophobicity is determined by the number of hydrophobic groups on the surface that is in contact with the polar aqueous environment, an increase in Ho might be caused by the new surfaces formed by ultrasonic breakdown of large protein aggregates or by relocation of hydrophobic groups that were previously embedded in

protein to its surfaces (Lee et al., 2016). The significantly higher Ho of the pH12-MTS sample indicated a more aggressive modification of SPI structure compared to other treatments. It may be caused by the superimposing of the MTS and pH-shifting treatments, as both when applied individually significantly improved the Ho of proteins, as shown in Table 3.3.

A positive correlation can be observed between solubility and Ho (Table 3.3). For instance, the pH12-MTS treated samples had the highest solubility (82.5%), thus having the highest Ho (241.0). Similarly, The Control had the lowest solubility (9.1%), and its Ho (124.0) was also the lowest. These findings confirmed that protein solubility is not exclusively determined by surface hydrophobicity. The way how the protein aggregates is prepared plays an important role. Dissociation of native protein structures into individual subunits is thought to be the driving force for the increased solubility. This observation is in agreement with the observation of Hu et al. (2013) who found a positive relationship between solubility and Ho of SPI. It was also confirmed by the early work of Wagner and Gueguen (1995).

3.3.6 Emulsifying properties

Emulsifying properties are indication of the ability of a protein to absorb to the oil-water interface and can be evaluated via protein's emulsion stability index (ESI) and emulsion activity index (EAI) (Zhang et al., 2014). The ESI is the measure of the stability of the emulsion over a certain time span and EAI is a measurement of how much oil a protein can emulsify per unit protein (Boye et al., 2010). Table 3.4 shows the EAI and ESI of the treated SPI samples. Among the treatments, the pH12-MTS treated samples had the highest EAI (326 m^2/g) and ESI (49 min) whereas the untreated samples (control) showed the lowest EAI (128 m^2/g) and ESI (22.0 min), followed by the HPH sample. The MTS alone treatment showed the second highest EAI (291 m^2/g)

and ESI (36 min). Similar improvement in the emulsifying properties of soy proteins after an ultrasound treatment was confirmed in the reports of Karki et al. (2010) and Zhang et al. (2014). Since the emulsifying properties are related to protein solubility, Ho, and molecular flexibility, measures that improve protein solubility and Ho will introduce improvement in ESI and EAI (Zhang et al., 2014). This can be seen clearly when comparing Table 3.3 with Table 3.4. The SPI samples with high solubility and Ho, such as the pH12-MTS and MTS samples also had high ESI and EAI.

The significant improvement in the emulsifying properties of the pH12-MTS and MTS samples can be attribute to the synergy of pH-shifting and cavitation activities produced by the MTS treatment. The pH-shifting treatment alone was shown to enhance the emulsifying properties of soy protein (Jiang et al. 2009). As previously discussed, the MTS provided a strong cavitation activity compared to an ultrasound alone treatment. Thus, the use of MTS enabled enhanced sonication treatment, especially when combined with pH-shifting, to facilitate protein structure modification, resulting in improvement in physicochemical properties (solubility, Ho, etc.) and emulsifying properties (ESI and EAI). As previously discussed, the MTS provided a strong cavitation activity compared to an ultrasound alone treatment. Thus, the use of MTS enabled enhanced sonication treatment, especially when combined with pH-shifting, to facilitate protein structure modification, resulting in improvement in physicochemical properties (solubility, Ho, etc.) and emulsifying properties (ESI and EAI).

3.3.7 Antioxidant activity

Total antioxidant activity of the SPI solutions is shown in Table 3.3. Total antioxidant activity of the SPI solution was significantly increased by the pH12-MTS treatment, almost three-

fold higher than the Control. The increase was insignificant for other treatments including HPH treatment. It was reported that an increased in the levels of free amino acids and small peptides enhanced soy protein antioxidant capacity (Yoo and Chang, 2016). The free-SH groups might also be responsible for the antioxidant activity of soy proteins (Elahi and Mu, 2017). A significant increase in the free sulfhydryl (SH) content was observed in this study (Table 3.3) for the pH shifting-MTS sample. A breakdown of peptide bonds in protein with sonication is difficult, but not impossible if the energy input from the ultrasonic treatment is high (Weiss et al., 2011). MTS is by now the most powerful ultrasound treatment, as it can achieve cavitation intensity not previously possible by an ultrasound treatment. As the first experiment using MTS to treat a protein, a more powerful ultrasound treatment was achieved in this study that might have caused release of some free amino acids or even breakdown some peptide bonds contributing to the nearly 3-fold increase in the antioxidant activity. The enhanced sonication should have also contributed to the enhancement of antioxidant activity in the pH shifting-MTS treatment.

3.3.8 Total phenols

Similar to the antioxidant activity, a significant increase in the total phenolic content (TPC) was observed in the pH12-MTS treated samples, while the increase in all other treatments was not significant (Table 3.3). The highest TPC (2.9 catechin/g) was recorded in the pH12-MTS sample while the Control had the lowest TPC (2.2 mg catechin/g). The high values of both total phenols (Table 3.3) and antioxidant activity (Table 3.3) could help to decrease protein oxidation. The antiradical mechanism in protein aggregates can be attributed to the ability of phenolic compounds to transfer oxidative damage from one phenolic site to other, which protects proteins from oxidation (Viljanen et al., 2005). The phenolic compounds in plant tissues can act as antioxidants

by retarding protein oxidation reaction or by binding to the proteins. Degradation of isoflavones, the major phenolic constituents in soybean seeds, has been shown to occur in soy and soy products during food processing and storage, resulting in changes in their bioactivities. An increase in total phenolic contents are beneficial for antioxidant properties of soybean seeds due to polymerization of phenolic constituents and also cross-linking and fragmentation, which are the key reactions controlling the properties of macromolecules such as proteins.

3.3.9 Rheological measurements

The flow curves (apparent viscosity versus shear rate) of the SPI samples are presented in Fig. 3.4. The data were collected at 25^{o}C at six shear rates (1–100 s^{-1}). The Power law coefficients (apparent viscosity (n_{app}), flow behavior indices (n), consistency coefficients (k)) and the regression coefficients (r^2) are shown in Table 3.6. It can be seen in Fig. 3.4 that the apparent viscosity of the treated SPI decreased with increasing shear rate, suggesting that the suspensions had a shear-thinning or pseudo-plastic behavior. This is confirmed by their flow behavior indices. In Table 3.6, the SPI dispersions of all the treatments had $n<1$, indicating a pseudo-plastic behavior. The apparent viscosity of the pH12-MTS treated SPI samples was much higher than other treatments, including the pH12-HPH samples at all shear rates. This might be caused by the high solubility and thus high number density (concentration) of the pH12-MTS sample. Shear thinning is often caused by the breakdown of protein aggregates, alignment of the aggregates in the direction of flow, or disruption of covalent bonds and non-covalent interactive forces (such as hydrogen bonds, hydrophobic interactions, and electrostatic interactions), which could cause modification of SPI network structures (Song et al., 2013). The shear-thinning behavior of the SPI suspension caused by the MTS can be related to the strong cavitation activity that facilitate the

above changes in the SPI dispersions.

3.3.10 Nanoemulsions characterization (droplet size, turbidity, and emulsion stability)

Turbidity and droplet sizes of the soy protein-stabilized nanoemulsions, using the SPI nano-aggregates produced by the pH12-MTS treatment as the wall material, at two oil concentrations (0.25% and 0.50%, w/w) were measured. The turbidity was found to increase with increasing oil concentration. While the turbidity was 0.71 for the sample with 0.25% oil, it was 1.9 for the nanoemulsion with 0.50% oil. Similarly, the droplet size of soy protein-stabilized nanoemulsions increased with increasing oil concentration. The droplet size of the nanoemulsions with 0.5% oil was relatively large (148.4 nm) compared with the nanoemulsions with 0.25% oil (92.9 nm). The stability of the soy protein-stabilized nanoemulsions was examined by measuring droplet sizes over a 21-day period at 4 ^{0}C, as shown in Fig. 3.5. The droplet size of the nanoemulsions slightly increased from 92.9 nm on Day 0 to 100.3 nm on Day 21 for the sample with 0.25% oil, while that for the nanoemulsions made with 0.5% oil increased from 148.4 nm to 173.9 nm in 21 days (Fig. 3.5). The soy protein-stabilized nanoemulsions at the two oil concentrations both exhibited relatively good stability.

3.3.11 Lipid oxidation

Lipid hydroperoxide values of the nanoemulsions at two oil concentrations (0.25% and 0.50%) during a 7-day (168 hours) storage at 37^{o}C are shown in Fig. 3.6. No lipid oxidation was detected until day 5 for the nanoemulsion with 0.50% oil and until day 6 for the samples with 0.25% oil. A significant increase in lipid oxidation from day 6 to day 7 for the nanoemulsions at both oil concentrations could be observed. The ability of soy protein acting as a chemical barrier

to retard lipid oxidation is evidenced in the first 5 days of storage. It is known that amino acids such as cysteine in protein can be more readily oxidized than lipids (Hu et al., 2003). The high free SH groups in the soy protein isolates treated with MTS may delay the lipid hydroperoxide development. Therefore, encapsulation of bioactive compounds with the soy protein-mediated nanoemulsion should be performed within 120 hours after the preparation of the nanoemulsion, before oxidation of the oil used in the emulsion.

3.3.12 Transmission electron microscopy (TEM)

The morphology and size of soy protein-stabilized nanoemulsion droplets at two oil concentrations (0.25% and 0.50%) are shown with the TEM images in Fig. 3.7. The droplets in the nanoemulsions exhibited a spherical morphology. There is clear evidence that the droplet diameters were depended on oil concentration, in agreement with the DLS analysis (Table 3.6). The droplets in the nanoemulsion made with 0.25% oil had smaller sizes (< 100 nm) compared to that in the nanoemulsion with 0.50% oil (>100 nm).

3.4. Summary and conclusions

A novel physical and chemical combined treatment, i.e. sequential pH-shifting and mano-thermo-sonication (MTS), was proposed and tested in this study to significantly modify soy protein functional properties and to produce functionalized nano-sized soy protein aggregates. Compared with other treatments, such as high-pressure homogenization (HPH), this method provided significantly improved the physicochemical (solubility, free sulfhydryl and disulfide bond (S-S) content, surface hydrophobicity, and antioxidant activity), interfacial (rheology), and emulsifying (particle size, emulsifying activity index, and emulsion stability index) properties of commercial

soy protein isolates (SPI). The soy protein nano-aggregates (<100 nm) produced by this method were used to make soy protein-stabilized nanoemulsions that showed good stability during storage at 4^{o}C for 21 days and less lipid oxidation. The findings in this study demonstrated the potential of the pH-shifting and MTS combined treatment as an effective alternative to commonly used methods, such as HPH, for protein modification. The functionalized soy protein nano-aggregates produced by this method can be used as an excellent wall material in the place of animal-based proteins for encapsulation of hydrophobic bioactive compounds to meet the consumer need for plant protein-based food products with enhanced nutrition.

3.5 References

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3.6 Figures and tables

Table 3.1. Treatments used to modify soy protein isolates (SPI).

Treatment	MTS	pH adjustment (pH 12)	MTS	HPP	RT for 1 hour	pH adjustment (pH 7)
Control	x	x	x	x	x	x
pH shifting	x	o	x	x	o	o
MTS	o	x	x	x	x	x
pH12-MTS	x	o	o	x	o	o
HPH	x	x	x	o	x	x
pH12-HPH	x	o	x	o	o	o

MTS: mano-thermo-sonication, HPP: high-pressure homogenization, RT: Room temperature storage; o: represents the steps that were applied during the process; and x: represents the steps that were not applied during the process

Table 3.2. Response Surface treatment conditions and test results

Treatment	Soluble Protein Recovery (%)	Particle size (nm)
Untreated SPI	9.08 ± 0.02^{c}	192.0 ± 3^{a}
40^{0}C		
30 s, 200 kPa	74.2 ± 0.14^{ab}	58.7 ± 3^{c}
45 s, 100 kPa	74.8 ± 0.03^{ab}	58.0 ± 2^{c}
45 s, 400 kPa	65.6 ± 0.22^{b}	73.0 ± 1^{b}
60 s, 200 kPa	68.1 ± 0.78^{b}	67.0 ± 2^{b}
45^{0}C		
30 s, 100 kPa	72.1 ± 0.20^{ab}	55.0 ± 3^{c}
30 s, 400 kPa	72.6 ± 0.18^{ab}	53.3 ± 2^{c}
45 s, 200 kPa	79.5 ± 0.62^{a}	39.2 ± 2^{d}
60 s, 100 kPa	76.7 ± 0.74^{ab}	42.4 ± 4^{d}
60 s, 400 kPa	77.4 ± 0.58^{a}	40.2 ± 1^{d}
50^{0}C		
30 s, 400 kPa	78.1 ± 0.02^{a}	35.7 ± 3^{d}
45 s, 100 kPa	78.8 ± 0.31^{a}	33.9 ± 2^{de}
45 s, 400 kPa	81.2 ± 0.44^{a}	32.5 ± 2^{de}
60 s, 200 kPa*	**82.5 ± 0.42^{a}**	**27.1** $\pm 1^{e}$

[a-e] Means within temperature treatments with the same letter in each sample are not significantly different ($p<0.05$) (Three replications).
*MTS at 50°C, 200 kPa, and 60 s was determined as a best treatment conditions based on recovery and particle size.

Table 3.3. Physicochemical properties of the untreated (Control) and the treated SPI samples.

Treatment	Soluble protein recovery (%)	Free SH (μmol/g)	S-S content (μmol/g)	Surface Hydrophobicity (Ho)	Antioxidant activity (%)	Total phenols (mg catechin/ g dry weight)
Control	9.1 ± 0.02^{d}	4.22 ± 0.17^{d}	14.82 ± 0.08^{d}	124.0 ± 0.4^{d}	6.2 ± 0.01^{b}	2.2 ± 0.11^{b}
pH shifting	57.0 ± 0.04^{c}	5.09 ± 0.22^{bc}	15.96 ± 0.02^{bc}	183.0 ± 0.6^{c}	6.7 ± 0.02^{b}	2.3 ± 0.34^{b}
MTS	71.4 ± 0.02^{b}	5.95 ± 0.12^{b}	16.72 ± 0.12^{b}	193.0 ± 0.6^{b}	7.9 ± 0.02^{b}	2.4 ± 0.18^{b}
pH 12-MTS	82.5 ± 0.42^{a}	7.68 ± 0.08^{a}	21.97 ± 0.06^{a}	241.0 ± 0.7^{a}	17.0 ± 0.05^{a}	2.9 ± 0.09^{a}
HPH	60.3 ± 0.61^{c}	4.72 ± 0.06^{c}	15.31 ± 0.08^{c}	182.0 ± 0.6^{c}	8.4 ± 0.03^{b}	2.5 ± 0.15^{b}
pH 12-HPH	69.7 ± 0.13^{b}	5.45 ± 0.06^{b}	16.38 ± 0.04^{b}	185.0 ± 0.8^{c}	8.7 ± 0.02^{b}	2.4 ± 0.22^{b}

$^{a-d}$ Means within treatments with the same letter in each sample are not significantly different ($p<0.05$) (Three replications).

Table 3.4. Emulsifying properties (emulsion activity, emulsion stability, particle size and turbidity) of the untreated and treated SPI samples.

Treatment	EAI (m^2/g)	ESI (min)	Particle size (nm)	Turbidity (abs at 600 nm)
Control	128.0 ± 2^{d}	22.0^{d}	192.0 ± 1^{a}	1.18 ± 0.76^{c}
pH shifting	240.0 ± 2^{c}	33.0^{b}	61.0 ± 2^{b}	0.52 ± 0.45^{bc}
MTS	291.0 ± 4^{b}	36.0^{b}	40.2 ± 2^{c}	0.27 ± 0.56^{b}
pH 12-MTS	326.0 ± 2^{a}	49.0^{a}	27.1 ± 4^{d}	0.09 ± 0.51^{a}
HPH	232.0 ± 3^{c}	27.0^{c}	72.9 ± 1^{b}	0.24 ± 0.87^{bc}
pH 12-HPH	283.0 ± 1^{b}	34.0^{b}	45.3 ± 3^{c}	0.26 ± 0.28^{b}

[a-d] Means within treatments with the same letter in each sample are not significantly different ($p<0.05$) (Three replications).
*EAI: emulsifying activity index, ESI: emulsion stability index

Table 3.5. Zeta potential of SPI at different pH.

Zeta potential (mV)	pH 3	pH 4	pH 5	pH 6	pH 7	pH 8
Control	38	22	2	-18	-30	-35
MTS	40	25	4	-20	-35	-40
pH12-MTS	43	30	3	-15	-40	-42

Table 3.6. Interfacial properties (apparent viscosity (n_{app}), flow behavior indices (*n*), consistency coefficients (*k*) and regression coefficients) of the untreated and treated SPI samples.

Treatments	Apparent viscosity (n_{app}) mPas	Consistency coefficients (*k*) mPas	Flow behavior indices (*n*)	Regression coefficients (r^2)
Untreated SPI	21.0 ± 0.4^{c}	48.1 ± 0.2^{a}	0.841 ± 0.01^{a}	0.998
pH shifting	35.0 ± 0.3^{c}	9.8 ± 0.2^{a}	0.417 ± 0.23^{b}	0.994
MTS	177.0 ± 0.3^{b}	$41\ 10^{3} \pm 1.4^{b}$	0.248 ± 0.41^{b}	0.994
pH12-MTS	246.0 ± 0.2^{a}	$2{,}037 \times 10^{3} \pm 2.1^{b}$	0.261 ± 0.06^{b}	0.991
HPP	154.0 ± 0.3^{b}	$22 \times 10^{3} \pm 2.2^{b}$	0.319 ± 0.07^{b}	0.995
pH12-HPP	222.0 ± 0.2^{a}	$4{,}873 \times 10^{3} \pm 1.7^{b}$	0.299 ± 0.14^{b}	0.991

[a-c] Treatment means within treatments with the same letter in each sample are not significantly different ($p<0.05$) (Three replications).

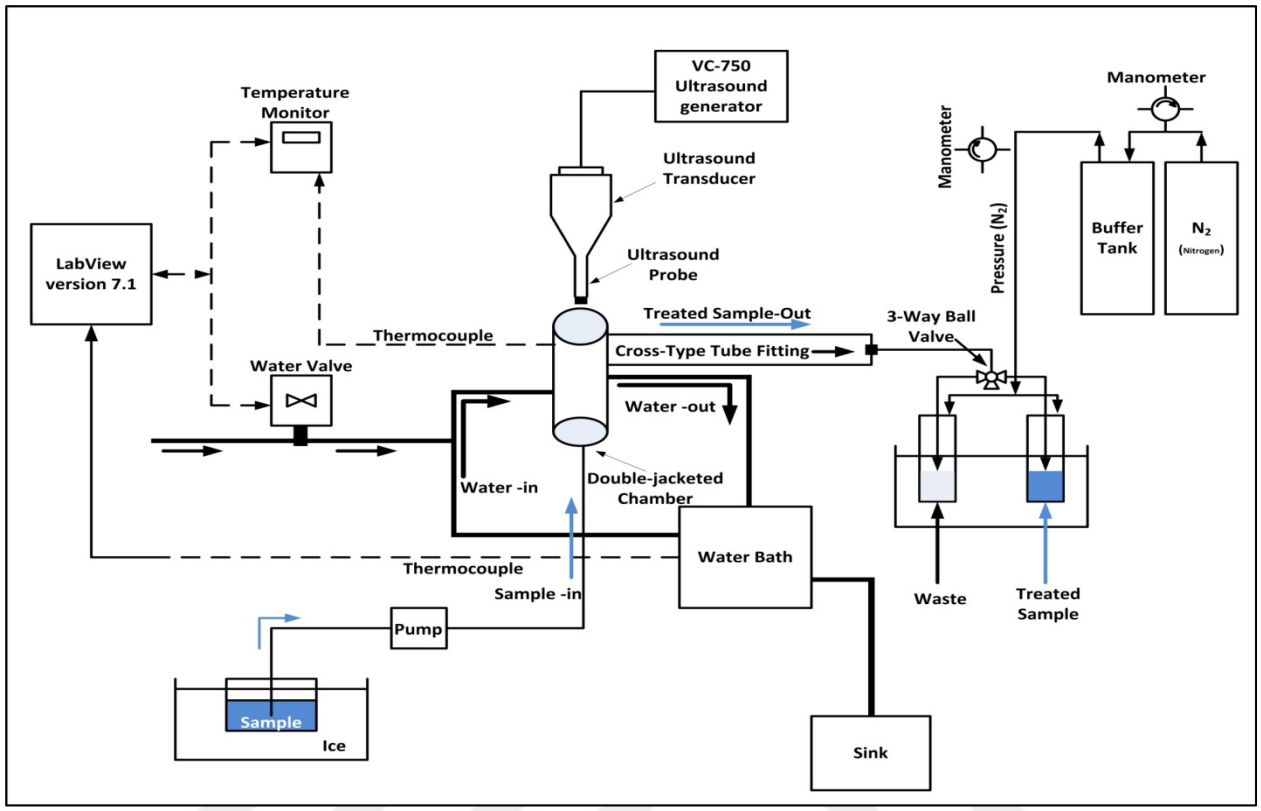

Fig. 3.1. Laboratory scale continuously flow manothermosonication (MTS) system

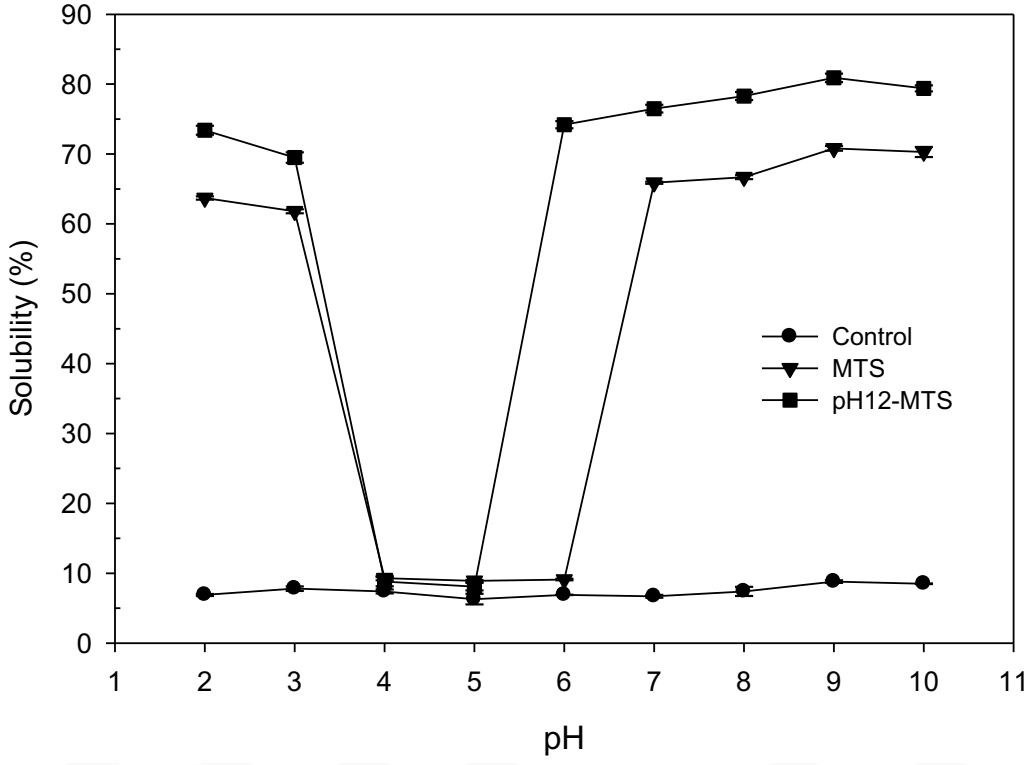

Fig. 3.2. The SPI solubility profile of the Control, MTS, and pH 12-MTS samples

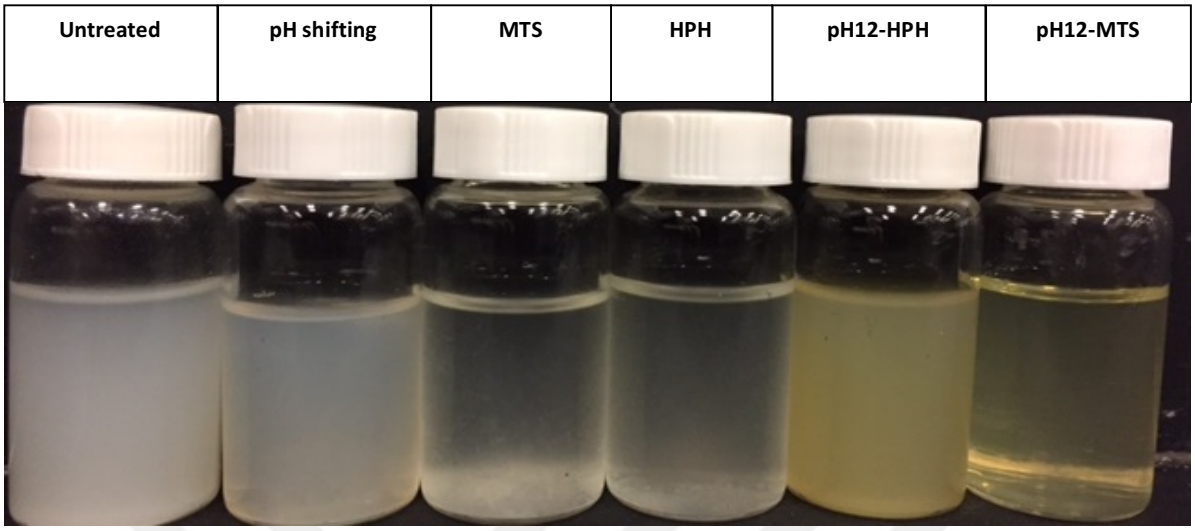

Fig. 3.3. The appearance of the untreated SPI (control) and the SPI treated by pH 12, MTS, HPH, pH12-HPH, and pH12-MTS

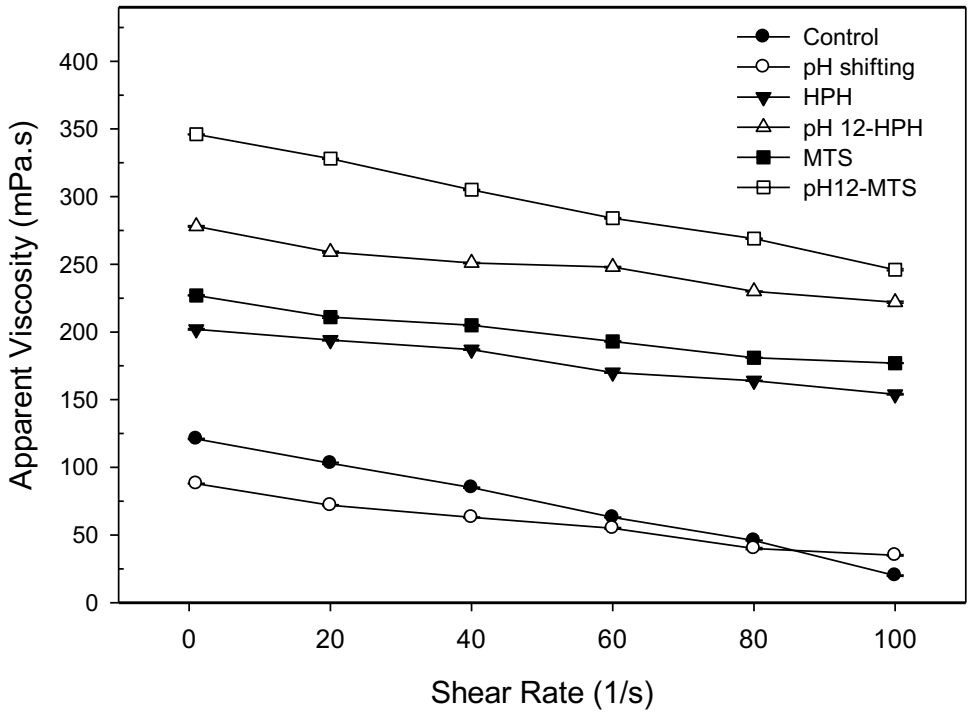

Fig. 3.4. The flow curves (apparent viscosity versus shear rate) of the untreated SPI (control) and the SPI treated by pH 12, MTS, HPH, pH12-HPH, and pH12-MTS

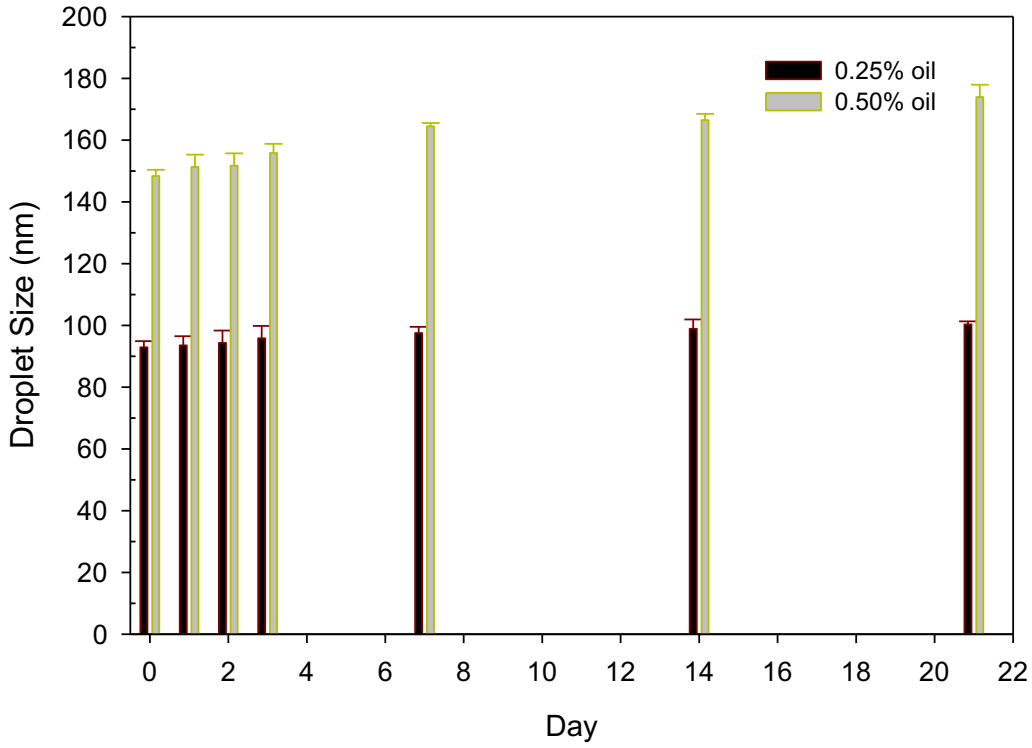

Fig. 3.5. Droplet size of the soy protein-stabilized nanoemulsions during storage at 4^0C for 21 days

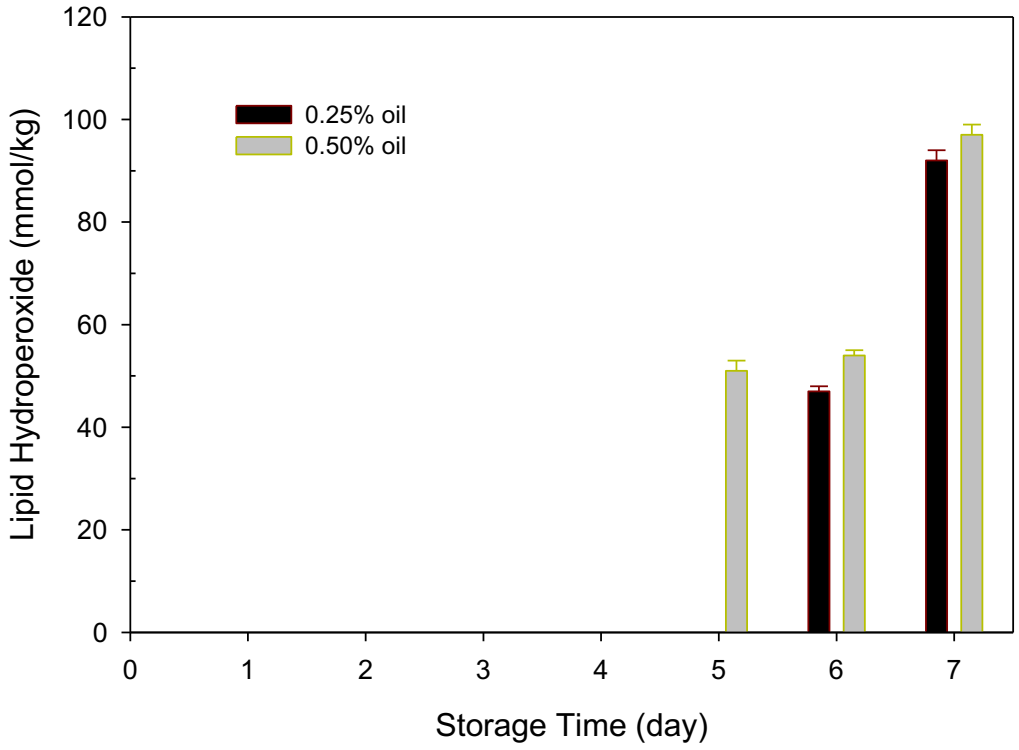

Fig. 3.6. Lipid hydroperoxide values of the soy protein-stabilized nanoemulsions with two oil concentrations (0.25% and 0.50%) during storage at 37^{o}C for 7 days (168 hours)

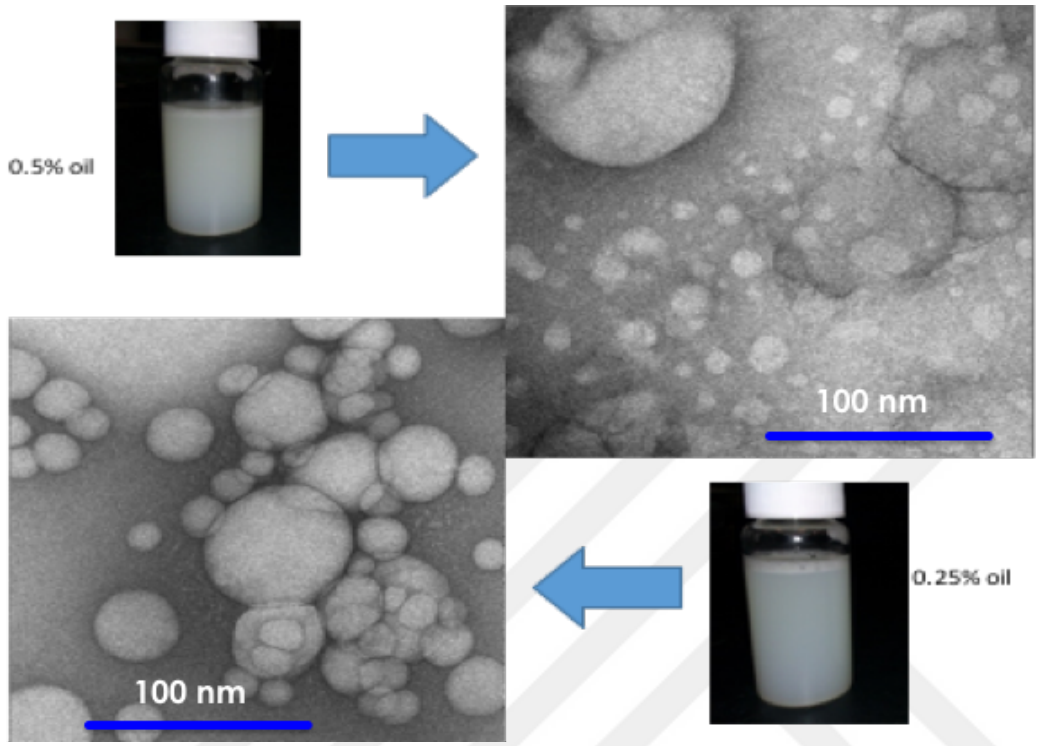

Fig. 3.7. TEM images of the soy protein-stabilized nanoemulsion made with SPI treated with pH12-MTS

CHAPTER 4

STABILIZING OIL IN WATER EMULSIONS PRODUCED FROM PLANT PROTEIN/POLYSACCHARIDE COMPLEXES

4.1 Introduction

In recent years, a growing interest in protein-based acidic beverages has stimulated research and development activities exploring the interactions of proteins with a liquid food system at low pH levels. The use of plant proteins as ingredients in food formulations is notably attractive due to their functionality and nutritional value. However, plant proteins generally have low solubility at acidic pH levels (Phillips & Williams, 2001). A few studies have reported that emulsion stability at low pH can be improved by forming multiple layers around the oil droplets. For instance, the addition of negatively charged polysaccharides that interact with positively charged proteins may form a thick layer at the interface which prevents coalescence (Dickinson, 2009; Mahendran, 2008). High-molecular-weight polysaccharides such as gum arabic, pectin, and modified starch are usually used to stabilize oil-in-water food emulsions (Ray et al. 1995; McNamee et al. 1998; Garti 1999). In emulsions produced from polysaccharides, the carbohydrate part tends to stretch into the aqueous phase, which prevents the droplets from aggregation and coalescence. Consequently, protein-polysaccharide complexes are normally used to form emulsions with good stability in acidic conditions.

The formation of polysaccharide-protein complexes and their solubility rely on several factors such as the charge and nature of the biopolymer, pH, ionic strength, and temperature of the medium (Ghosh & Bandyopadhyay, 2011). At pH values close to the protein isoelectric point (pI), protein/polysaccharide complexes were reported to improve the colloidal stability and interfacial structure of proteins (Jiménez-Castańo, Villamiel, & López-Fandiňo, 2007; Benichou, Aserin, Lutz & Garti, 2007). If the pH of a medium is below the pI of a protein, the net positive charge of the protein will become prominent, which will interact with the negatively charged polysaccharide to form a stable complex. On the other hand, if the solution's pH is above the protein's pI, the net negative charge of the protein will more likely form complex with positively charged polysaccharides (Xia & Dubin, 1994; Dickinson, 2008; Turgeon, Schmitt & Sanchez, 2007). Therefore, one way to address the low solubility and emulsifying properties of a protein in a pH close to the pI is to mix the protein solution with a polysaccharide solution at a higher pH (where the protein is more soluble), and then adjust the mixture to an acidic pH (3.0 or 4.0) to produce the complex (Liu et al., 2010).

Pectin is an acidic polysaccharide containing 80% galacturonic acid and is produced by an aqueous extraction of plant materials, generally apple pomace and citrus peels. It is one of the most frequently used polysaccharides in acidic environments. Similarly, gum arabic (GA) is a negatively charged polysaccharide that is commonly used in the food industry because of its high solubility and good emulsifying properties. Modified starch (MS) is another negatively charged polysaccharide. MS has a number of applications in foods, specifically for thickening, stabilizing and texturing, and is often used in acidic emulsion-based beverages. Nevertheless, insufficient information is currently available on the interactions of negatively charged polysaccharides with soy-protein and pea-protein-based emulsions. The objective of this study is to develop a plant-

protein and polysaccharide-complex system to address the low solubility of the protein near the pI. In particular, the effects of adding modified starch, pectin, and gum arabic to soy-protein or pea-protein isolates to form protein and polysaccharide complexes at pH 4.0 will be evaluated by examining the functional properties of the complexes and their emulsions.

4.2. Materials and methods

4.2.1 Chemicals

Soy protein isolate (SPI, Pro-Fam® 955, 90% soy protein on dry basis) and pea protein isolate (PPI, NUTRALYS® S85F, 85% pea protein based on dry basis) were provided by Archer Daniels Midland Company (ADM, Decatur, IL, USA) and Roquette (Geneva, IL, USA), respectively. The SPI and PPI were stored in a refrigerator at 4°C before use. Branched polysaccharide (gum arabic from acacia tree) and modified starch (hydroxypropylated starch) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA), and pectin was obtained from Fisher Chemical Company (Pittsburgh, PA, USA).

4.2.2 Preparation of pea protein isolate (PPI) and soy protein isolate (SPI) nano-aggregates treated with pH 12-MTS

Three grams of SPI/PPI were dissolved in 100 mL deionized (DI) water and stirred at room temperature for 30 min to get the protein dispersions. The SPI/PPI dispersions were adjusted to pH 12 with 2M sodium hydroxide (NaOH), treated by MTS (50^{o}C, 200 kPa and 60 s), and the samples were stored at room temperature for 1 h, followed by adjusting the pH back to pH 7 with 2M hydrochloric acid (HCl). Neutralized protein dispersions were centrifuged (1,200 g and 20^{o}C)

for 15 min and the supernatants were collected as soluble soy and pea proteins for further experiments.

4.2.3 Preparation of plant protein/polysaccharide complexes

Six grams of polysaccharides (modified starch, pectin, or gum arabic) were dissolved in 100 mL deionized (DI) water and stirred at room temperature for an hour. The soluble SPI/PPI produced with pH 12-MTS was mixed with polysaccharide solutions. After 3 hours stirring at room temperature, the protein/polysaccharide mixtures were sonicated using a VC-750 ultrasound unit (20 kHz, Sonics & Materials, Inc., Newtown, CT, USA) in an ice bath for 5 min and the pH of the mixtures was adjusted to 4.0 to obtain protein/polysaccharide complexes. For the control protein/polysaccharide complexes, untreated SPI and PPI were used instead of the soluble SPI/PPI produced with pH 12-MTS. The control SPI/PPI solutions were produced by dissolving SPI/PPI in distilled water and stirring at room temperature for 30 min. A summary of the treatments is given in Table 4.1.

4.2.4 Solubility

Soluble protein-polysaccharide complexes were determined with a Bio-Rad Protein Assay based on the method described by Bradford (1976). Bovine serum albumin (BSA) (Bio-Rad 500-0007) was used as the standard. Dye reagent was prepared by diluting 1 part of dye reagent concentrate (Bio-Rad 500-0006) into 4 parts of DI water, and filtered through Whatman #1 filter paper. Diluted dye reagent was added to protein-polysaccharide complexes. Soluble protein concentration in protein-polysaccharide complexes was measured using a spectrophotometer

(Lambda 1050 UV/VIS/NIR Spectrometer, PerkinElmer) under the wavelength of 595 nm (Bradford, 1976). The solubility was calculated as below:

$$\textbf{Protein solubility}\ (\%) = \frac{\textbf{Protein concentration in protein}-\textbf{polysaccharide complexes}}{\textbf{Initial protein concentration}} \times 100\% \qquad (4.1)$$

4.2.5 Water solubility profile

The pH of the protein-polysaccharide complexes was adjusted to 2, 4, 6, 8, 10, and 12 with either 2N HCl or 2N NaOH. The protein-polysaccharide dispersion was stirred for an hour. 25 mL of the dispersion was put into 50 mL centrifuge tubes and centrifuged at 10,000 × g for 10 min at 20°C. The supernatant was measured for protein content with a Bio-Rad Protein Assay (Bradford, 1976). Bovine serum albumin (BSA) (Bio-Rad 500-0007) was used as the standard. All samples were analyzed in triplicate. The solubility was calculated as:

$$\textbf{Protein Solubility}\ (\%) = \frac{\textbf{Protein in supernatant (g)}}{\textbf{Protein in starting material (g)}} \times 100 \qquad (4.2)$$

4.2.6 Droplet size and turbidity

Volume-weighted mean diameters (D4, 3) of the protein-polysaccharide complexes and protein-polysaccharide complex emulsions were detected by dynamic light scattering (DLS) using a NICOMP 380 DLS instrument. Samples were diluted 500-fold with DI water before measurement. The measurements were conducted at 23°C. Turbidity of the protein-polysaccharide complexes and protein-polysaccharide complex emulsions was measured using a spectrophotometer (Lambda 1050 UV/VIS/NIR Spectrometer, PerkinElmer, Waltham, MA,

USA). DI water was used as the blank. The absorbance at 600 nm of each sample represented the turbidity.

4.2.7 Free sulfhydryl and disulfide bond (S-S) content

Concentration of the free sulfhydryl groups (SH, mmoles/g soluble solids) was determined using a cysteine standard (Lee et al., 2016). A set of cysteine standards were prepared by dissolving cysteine hydrochloride monohydrate at different concentrations (1.5, 1.25, 1.0, 0.75, 0.5, 0.25, and 0.0 Mm) in a reaction buffer (0.1M sodium phosphate containing 1mMEDTA). A set of test tubes containing 50 mL of Ellman's reagent solution and 2.5 mL of reaction buffer were prepared. 250 mL of each standard was added in above test tubes. The solution in the test tubes were mixed and incubated at room temperature for 15 min. The absorbance at 412 nm was measured. The values obtained were plotted to generate a standard curve, and the sample concentrations were determined from this curve. For total SH content analysis, 4 mL of Tris-Gly buffer (0.086 M Tris, 0.09 M glycine, 0.04 M EDTA, pH 8.0) was added to 1 mL of the of protein-polysaccharide complexes. The mixture was incubated for 1 h at room temperature. Then, the mixture was centrifuged at 5,000 g for 10 min. 0.04 mL of Ellman's reagent solution was added to 4 mL of this solution, and the absorbance was measured at 412 nm.

Half of the value after subtracting the free SH value from the total SH value was defined as the disulfide bond (SS) content:

$$\textbf{SS content} = \frac{\textbf{Total SH} - \textbf{Free SH}}{\textbf{2}} (\boldsymbol{\mu}\textbf{mol/g}) \quad (4.3)$$

4.2.8 Surface hydrophobicity (H_0)

Surface hydrophobicity of the protein-polysaccharide complexes was measured as reported by Lee at et al. (2016) by using 1-anilino-8-naphthalenesulfonate (ANS) (SigmaeAldrich, St. Louis, MO, USA) as the fluorescence probe. ANS stock solution (8 mM) was prepared in phosphate buffer (0.01 M, pH7). Five soy protein concentrations, 0.04-0.2 mg/mL, were also prepared with phosphate buffer (0.01M, pH7) (final volume 4 mL). Twenty mL ANS stock were added to protein-polysaccharide complexes and fluorescence intensity was measured (Synergy™2, BioTek Instrument Inc., Winooski, VT, USA) at 340 nm (excitation) and 440 nm (emission). Initial slopes of fluorescence intensity vs. protein concentration were used as surface hydrophobicity (H_0) of the complexes

4.2.9 Emulsifying properties

Emulsifying properties were measured by the method of Pearce and Kinsella (1979). Oil-in-water (o-w) emulsions were prepared by adding 1 mL of canola oil in 3 mL of protein-polysaccharide complex samples. The oil concentration was 0.25% (w/w) in this test. Ultrasound was used to generate the protein-polysaccharide complex emulsions. The mixture of oil and protein-polysaccharide complexes was stirred strongly for 5 min and then sonicated for 5 min. After emulsion formation, the absorbance was measured at 500 nm at 0 (A_0) and 10 min (A_{10}), respectively. Emulsifying activity index (EAI) and emulsion stability index (ESI) were calculated by using the following equations:

$$\text{EAI } (m^2/g) = 2T\ A_0 \times \text{dilution factor}/c \times \Phi \times L \times 10\ 000 \quad (4.4)$$

$$\text{ESI (min)} = A_0 / (A_0 - A_{10}) \times 10 \text{ (min)} \quad (4.5)$$

where:

T = 2.303, dilution factor = 100, c – weight of protein per unit volume (g/mL), L – width of the optical path (0.01 m), and Φ – oil volumetric fraction (0.25).

4.2.10 Rheological measurements

Rheological measurements were carried out using a TA ARES-G2 Rheometer (TA Instruments, New Castle, DE, USA), according to the method of Sittikijyothin et al. (2010) with some modifications. All rheological measurements were performed at 25^{o}C. Concentric cylinder geometry was used for the samples. Flow curve analyses were recorded using a steady state flow ramp in the 1-100 s^{-1} range of shear rate. The shear rate was recorded point by point with consecutive 3 min steps of constant shear rate. This time allowed obtaining constant shear rates for each point. The viscosity was then determined for each point and the flow curve was built.

4.2.11 Microstructure

Microstructure of the protein-polysaccharide complexes was analyzed by Environmental Scanning Electron Microscope (ESEM). The protein-polysaccharide complexes were tested under wet mode. The sample was frozen with nitrogen before ESEM analysis. Small amount of frozen sample (a small drop) was gently deposited on an aluminum stub and was placed into vacuum chamber. The analysis was performed in wet mode to inhibit high voltage damages. The samples were examined by environmental scanning electron microscope (ESEM, Philips XL30 ESEM-FEG, FEI Co., Hillsboro, OR., U.S.A.) operated at an accelerating voltage of 5.0 kV.

4.2.12 Protein-polysaccharide complex emulsion preparation

Protein-polysaccharide complex emulsions (oil-in-water emulsions) were prepared by adding 0.25 g of canola oil in 50 mL of protein-polysaccharide complexes. The concentration of oil was 0.5% (w/w). The mixture of oil and protein-polysaccharide complexes was vigorously stirred for 5 min. To achieve fine homogenization, samples were sonicated (5 min) using a VC-750 ultrasound unit (20 kHz, Sonics & Materials, Inc., Newtown, CT, USA) in an ice bath. Flow chart of preparation approach of protein/polysaccharide complex emulsions is shown in Figure 1.

4.2.13 Measurement of lipid oxidation

Lipid hydroperoxide formation during storage of protein-polysaccharide complex emulsions was determined as reported by Min et al. (2003). Protein-polysaccharide complex emulsions (5 mL) were placed in lightly sealed screw-cap test tubes and allowed to oxidize at 37°C in the dark. Lipid hydroperoxides were measured by mixing 0.3 mL of emulsion with 1.5 mL of isooctane/2-propanol (3:1, v/v) by vortexing (10 s, 3 times) and isolation of the organic solvent phase by centrifugation at 1,000g for 2 min. The organic solvent phase (200 μL) was added to 2.8 mL of methanol/1-butanol (2:1, v/v), followed by 15 μL of 3.97 M ammonium thiocyanate and 15 μL of ferrous iron solution (prepared by mixing 0.132 M $BaCl_2$ and 0.144 M $FeSO_4$). After 20 min, the absorbance of the solution was measured at 510 nm. Lipid hydroperoxides of the protein/polysaccharide complex emulsions were measured during 40 days.

4.2.14 Statistics

Three replications for each treatment were used for all measurements, unless otherwise stated. The results were analyzed by analysis of variance using the General Linear Models (PROC

GLM) procedure in SAS (version 9.3, SAS Institute, Inc., Cary, North Carolina, USA). Differences among the mean values were obtained by Fisher's least significant difference (LSD) test at alpha = 0.05.

4.3. Results and discussion

4.3.1 Solubility

The soluble protein content (%) of six types of protein-polysaccharide complexes (SPI/PPI-Modified starch, SPI/PPI-gum arabic and SPI/PPI-Pectin) is shown in Table 4.2. The highest protein solubility was observed in the PPI-modified starch complexes (72.50 %). It had higher solubility compared to the SPI complexes for all types of polysaccharides. For example, the solubility was as 65.20% for the PPI-gum arabic complexes while it was 62.02% for the SPI-gum arabic complexes. Among the polysaccharides, modified starch complexes had a high solubility than the complexes made with to gum arabic and pectin. Solubility is one of the most important functional properties of proteins since it impacts other functional properties (Sikorski 2001). In many protein-based formulations, solubility of the protein is usually required to form an emulsion (Hayakawa & Nakai 1985). Generally, solubility of a protein decreases remarkably at the pH around its isoelectricy point (pI), where the net charge of the protein is about zero and the protein tends to aggregate because of the electrostatic interactions caused by the charge asymmetry of the protein (Mu et al., 2010). A protein-polysaccharide complex, once formed, does not dissociate, regardless of the pH or salt concentration. (Kobayashi et al. 1990). The formation of protein-polysaccharide at pH 4.0 allowed the net positive charge of the proteins become prominent that interacts with negatively charged polysaccharides, and this interaction significantly ($P < 0.05$) increased the solubility in acid pH range (pH 4.0).

4.3.2 Water solubility profile

Extrinsic factors such as pH, temperature and ionic strength effect protein solubility (Bolontrande et al., 2013). The effect of pH value on the solubility of protein-polysaccharide complexes is shown in Figure 4.2. The lowest solubility was observed around pH 4-6. On the other hand, the solubility was increased while the pH moved away from pH 4-6. Compared to complexes at the acidic conditions (pH: 2.0), the complexes at alkaline conditions (pH: 8,10, and 12) showed a higher solubility. The effect of pH on both pea and soy protein solubility was reported to show a u-shaped curve, with high solubility to be on both sides of the isoelectric point, ~pH 4.5 (Lee et al., 2013). It can be seen in Fig. 4.2 that the protein and polysaccharides complexes exhibited a relatively high solubility around isoelectric point than that with protein alone. Moreover, the complexes created by PPI showed higher solubility compared the complexes produced by SPI. The increase in solubility might be related to the denatured state of the proteins contributing to changes in the number of hydrophobic residues, charge, and electrostatic repulsion. Pea protein is mainly composed of 11S legumin and 7S vicilin and has a pI of about 4.3 (Gharsallaoui et al., 2009), whereas, soy protein has a pI around 4.5. Pea protein and soy protein both exhibit a poor solubility at pH around 4-5 (Liu et al., 2010). A significant increased solubility (around 40%) at pH 4-5 was achieved with the protein-polysaccharide complexes produced in this work (Figure 4.2).

4.3.3 Droplet size and turbidity

The droplet sizes of protein-polysaccharide complexes (SPI/PPI-modified starch, SPI/PPI-gum Arabic, and SPI/PPI-pectin) are shown in Table 4.4. The largest droplets were observed in the Control in all protein-polysaccharide complexes (around 750 nm). The protein-polysaccharide

complexes made with proteins treated by ultrasound had significantly smaller droplet sizes (<403 nm). Among the protein/polysaccharides complexes, the PPI and modified starch complexes had the smallest droplet size, which could be related to more dispersible structure of this complex with a relatively high solubility compared the other types of complexes (Table 4.2). Moreover, the droplet size distribution became more homogeneous in the case of PPI and modified starch complexes. When ultrasound was apply to SPI dispersions, a significant reduction in the particle sizes was observed by Jiang et al. (2010). Jambrak et al. (2008) reported that the particle size reduction by the ultrasound increased the surface area in contact between the proteins and the water. It was caused by the strong cavitational forces present in the system during an ultrasound treatment. It was observed that wheat protein isolate-pectin conjugates at pH 12 showed a lower droplet size as compared to those of the non-treated samples (Neirynck et al. 2004).

The turbidity results of protein-polysaccharide complexes are shown in Table 4.4, and the appearance of the protein-polysaccharide complexes are shown in Figure 4.3. The complexes created with modified starch (M-SPIMS and M-PPIMS) had high number concentration (solubility), however since they also had the small particle sizes, their turbidity was among the lowest (0.1525 and 0.1504, respectively) and these samples were relatively more transparent than the other complexes (Fig. 4.3).

4.3.4 Free sulfhydryl content (SH) and disulfide bond (S-S) content

The free sulfhydryl (SH) and disulfide bond (S-S) contents of the protein-polysaccharide complexes (SPI/PPI-modified starch, SPI/PPI-gum arabic, and SPI/PPI-pectin) are shown in Table 4.2. All the protein/polysaccharides complexes had SH and S-S contents significantly higher than the control. The SH and S-S contents of the SPI/PPI-modified starch complexes were significantly

higher than the other protein-polysaccharide complexes. This combination also had higher protein solubility (Table 4.2), showing that an increase in double bonds contributed to increase solubility. Fernandez-Diaz et al. (2000) stated that electric pulse processing resulted in partial unfolding of an ovalbumin protein, thus exposing SH groups to the surface. Since the ultrasonic treatment can partially unfold SPI protein, it could also expose the SH groups to the surface resulting with the increase in the SH content.

4.3.5 Surface hydrophobicity

Surface hydrophobicity (H_0) of the protein-polysaccharide complexes are shown in Table 4.2. The highest surface hydrophobicity was observed for the PPI-Modified starch complexes (2970). Similar to solubility results, the complexes produced by PPI with all three types polysaccharides had higher surface hydrophobicity compared to those made from SPI. For example, the surface hydrophobicity (H_0) was found as 2220 for the PPI-gum arabic complexes while it was 2190 for SPI-gum arabic complexes. The lowest surface hydrophobicity was observed for the control in all types protein-polysaccharide complexes. There is a positive relationship between the solubility (Table 4.2) and surface hydrophobicity. The PPI-modified starch showed highest solubility (69.20%) with highest surface hydrophobicity (2970). The untreated protein-polysaccharide complexes, however, showed the lowest solubility and surface hydrophobicity. The surface hydrophobicity is an index of a protein's capacity for intermolecular interaction, and hence an index of its functionality (Wang et al. 2006**).** Lee et al. (2016) reported that an ultrasound treatment may lead to the exposure of hydrophobic groups initially buried in the interior of the protein molecules, since proteins with high hydrophobicity would have higher EAI values as compared with those with low hydrophobicity. Yin et al. (2008) found that the proteins with high

hydrophobicity had higher emulsifying activity index (EAI) values. The highest hydrophobicity was found for PPI-Modified starch complexes (2970) and similarly the highest EAI was observed for PPI-Modified starch complexes (364.4 m^2 /g - Table 4.4).

4.3.6 Emulsifying properties

Emulsifying activity index (EAI) and emulsifying stability index (ESI) of the protein-polysaccharide complexes are shown in Table 4.4. The highest emulsifying activity index (EAI) was observed for the PPI-modified starch complexes (364.4 m^2/g) while the lowest EAI was observed in the untreated samples for all protein-polysaccharide complexes (from 109.6 to 124.0 m^2/g - Table 4.4). Similarly, the highest emulsifying stability index (ESI) was observed for both the SPI-modified starch (47.0 min) and the PPI-modified starch (46.0 min) complexes while the lowest ESI was observed in untreated samples as 16.0 min for the SPI-pectin complexes and 12.0 min for the PPI-pectin complexes. It was found that the PPI-modified starch complexes had good emulsifying activity and stability indexes in comparison to other protein-polysaccharide complexes. Mu et al. (2010) found that emulsifying activity index of soy protein isolate (SPI)-gum arabic (GA) conjugates at neutral and acidic pH values was significantly increased compared to the protein alone samples. This might be mainly attributed to the difference between the solubility of the samples at those pH values. Similar results were found in other protein-polysaccharide conjugates (Dickinson & Galazka 1991; Kato et al. 1993; Diftis & Kiosseoglou 2003). The solubility of protein is necessary for the film formation since the rapid migration and adsorption on the oil-water interface is critical. A positive correlation between the solubility and emulsifying capacity of proteins has been reported (Felix et al. 1990). Our results related to the solubility and emulsifying capacity are in good agreement with this report. While the highest solubility was

obtained for the PPI-modified starch complexes (Table 4.2), the highest emulsifying properties were also determined in the PPI-modified starch conjugates (Table 4.4).

4.3.7 Rheological measurements

The flow curves (apparent viscosity versus shear rate) of the protein-polysaccharide complexes are presented in Fig. 4.4. The data were collected at 25°C at various shear rates (1–100 s^{-1}). The Power law coefficients (Apparent viscosity (n_{app}), flow behavior indices (n), consistency coefficients (k)) and the regression coefficients (r^2) of the protein-polysaccharide complexes are shown in Table 4.3. It was found that the apparent viscosity of all the protein-polysaccharide complexes (SPI/PPI-modified starch, SPI/PPI-gum Arabic, and SPI/PPI-pectin) was decreased with increased shear rate, which suggests that the suspensions had a shear-thinning or pseudoplastic behavior. In addition, all the protein-polysaccharide complexes had $n<1$, which indicates a pseudo-plastic behavior. The apparent viscosity of the PPI-MS complexes was higher than other complexes that could be attributed to the high solubility and thus high number density of the complexes.

4.3.8 Environmental scanning electron microscopy (ESEM)

ESEM images (500 nm) of the protein-polysaccharide complexes (SPI/PPI-Modified starch, SPI/PPI-Gum arabic and SPI/PPI-Pectin) are shown in Figure 4.5. All the protein-polysaccharide complexes exhibited a spherical morphology with particle diameters closely corresponding to the results obtained by DLS (Table 4.4). The droplets in the complexes made with modified starch for both PPI and SPI had smaller sizes (< 300 nm) compared to the complexes made with other polysaccharides (>300 nm).

4.3.9 Protein-polysaccharide complex emulsions characterization (Droplet size)

Stability of protein-polysaccharide complex emulsions was examined by monitoring the droplet sizes for 40 days, see Figure 4.6. The droplet sizes of the protein-polysaccharide complex emulsions changed from 402 nm to 415 nm during 40 days of storage for the PPI-modified starch complex emulsions while the changes were significantly higher for the SPI-modified starch complex emulsions (458 nm to 471 nm). In general, the protein-polysaccharide complex emulsions created with PPI showed better stability compared to the emulsions created by SPI. Among three polysaccharides (modified starch, gum arabic, and pectin), the modified starch complex emulsions showed more stable droplet sizes (Fig. 4.6). The PPI-MS complex emulsions exhibited good stability compared to others.

4.3.10 Lipid oxidation

Lipid hydroperoxide values of the protein-polysaccharide complex emulsions during 40-day storage are shown in Figure 4.7. In the first 10 days, no lipid oxidation was detected for all the protein-polysaccharide complex emulsions. At days 20, 30, and 40, some lipid hydroperoxide were detected for all types of protein-polysaccharide complex emulsions. However, the lowest lipid oxidation was observed in the PPI-modified starch complex emulsions. It was reported that the free sulfhydryl group of cysteine can inhibit lipid oxidation (Hu et al., 2003). The relatively lower oxidation of the PPI-modified starch complex emulsions can be related to the high free SH groups of the complex (Table 4.2).

4.4. Summary and conclusions

In conclusion, ultrasound treatment disrupts the PPI/SPI aggregates and forms dispersible protein/polysaccharide complexes successfully. The complexes developed with PPI showed better physicochemical (e.g., solubility, surface hydrophobicity, free sulfhydryl and disulfide bond (S-S) content), interfacial (e.g., rheology), and emulsifying (e.g., droplet size, turbidity, emulsion stability and emulsion activity indexes) properties compared to the complexes developed with SPI. In the protein/polysaccharide complex emulsions, the oil droplets were protected by protein-polysaccharide complexes. The study indicates that the PPI can function as a stable wall material to form stable protein-polysaccharide complexes with a modified starch that can be used as a carrier for bioactive compounds.

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4.6 Figures and tables

Table 4.1. Description of samples used in this study

Sample	Protein	Polysaccharide
C-SPIMS	Untreated SPI	Modified Starch
C-PPIMS	Untreated PPI	Modified Starch
M-SPIMS	pH 12-MTS treated SPI	Modified starch
M-PPIMS	pH 12-MTS treated PPI	Modified Starch
C-SPIGA	Untreated SPI	Gum Arabic
C-PPIGA	Untreated PPI	Gum Arabic
M-SPIGA	pH 12-MTS treated SPI	Gum Arabic
M-PPIGA	pH 12-MTS treated PPI	Gum Arabic
C-SPIPE	Untreated SPI	Pectin
C-PPIPE	Untreated PPI	Pectin
M-SPIPE	pH 12-MTS treated SPI	Pectin
M-PPIPE	pH 12-MTS treated PPI	Pectin

Table 4. 2. Physicochemical properties of protein-polysaccharide complexes (SPI/PPI-Modified starch, SPI/PPI-Gum Arabic, SPI/PPI-Pectin)

Treatment	Soluble protein recovery (%)	Free SH (μmol/g)	S-S content (μmol/g)	Surface Hydrophobicity (H_0)
C-SPIMS	9.13 ± 0.02^{d}	7.13 ± 0.14^{bc}	11.86 ± 0.08^{d}	1890 ± 0.6^{c}
C-PPIMS	8.17 ± 0.04^{d}	7.18 ± 0.42^{bc}	11.72 ± 0.12^{d}	1940 ± 0.4^{c}
M-SPIMS	66.40 ± 0.07^{b}	12.66 ± 0.02^{a}	19.86 ± 0.12^{b}	2800 ± 0.1^{a}
M-PPIMS	72.50 ± 0.04^{a}	13.22 ± 0.44^{a}	24.72 ± 0.04^{a}	2970 ± 0.6^{a}
C-SPIGA	7.21 ± 0.02^{d}	6.15 ± 0.28^{c}	9.16 ± 0.14^{d}	1560 ± 0.8^{d}
C-PPIGA	9.12 ± 0.05^{d}	6.88 ± 0.12^{c}	10.33 ± 0.07^{d}	1720 ± 0.5^{cd}
M-SPIGA	62.02 ± 0.48^{bc}	8.06 ± 0.18^{b}	15.26 ± 0.09^{c}	2190 ± 0.1^{b}
M-PPIGA	65.20 ± 0.73^{b}	8.99 ± 0.15^{b}	16.77 ± 0.12^{c}	2220 ± 0.3^{b}
C-SPIPE	7.33 ± 0.01^{d}	6.03 ± 0.22^{c}	8.88 ± 0.21^{d}	1480 ± 0.2^{d}
C-PPIPE	8.24 ± 0.04^{d}	7.11 ± 0.27^{bc}	9.72 ± 0.18^{d}	1640 ± 0.7^{cd}
M-SPIPE	58.32 ± 0.03^{c}	8.33 ± 0.39^{b}	15.33 ± 0.12^{c}	2080 ± 0.3^{bc}
M-PPIPE	59.16 ± 0.03^{c}	9.27 ± 0.12^{b}	16.72 ± 0.12^{c}	2310 ± 0.5^{b}

[a-d] Means within treatments with the same letter in each sample are not significantly different ($p<0.05$) (Three replications) (at pH 4).

Table 4.3. Interfacial properties (apparent viscosity (n_{app}), flow behavior indices (*n*), consistency coefficients (*k*) and regression coefficients) of protein-polysaccharide complexes (SPI/PPI-Modified starch, SPI/PPI-Gum Arabic, SPI/PPI-Pectin)

Treatments	Apparent viscosity (n_{app}) mPas	Consistency coefficients (*k*) mPas	Flow behavior indices (*n*)	Regression coefficients (r^2)
C-SPIMS	319.0 ± 0.4^{c}	$22\ 10^3 \pm 1.4^{a}$	0.324 ± 0.214^{a}	0.993
C-PPIMS	299.0 ± 0.2^{c}	$18\ 10^3 \pm 1.2^{a}$	0.332 ± 0.418^{a}	0.991
M-SPIMS	377.0 ± 0.4^{b}	$15\ 10^3 \pm 1.2^{a}$	0.261 ± 0.422^{a}	0.992
M-PPIMS	403.0 ± 0.4^{a}	$12\ 10^3 \pm 1.1^{a}$	0.255 ± 0.349^{a}	0.997
C-SPIGA	313.0 ± 0.3^{c}	$23\ 10^3 \pm 1.7^{a}$	0.371 ± 0.117^{a}	0.997
C-PPIGA	302.0 ± 0.7^{c}	$19\ 10^3 \pm 1.6^{a}$	0.287 ± 0.125^{a}	0.994
M-SPIGA	368.0 ± 0.4^{b}	$14\ 10^3 \pm 2.2^{a}$	0.265 ± 0.411^{a}	0.996
M-PPIGA	374.0 ± 0.2^{b}	$13\ 10^3 \pm 1.3^{a}$	0.233 ± 0.234^{a}	0.996
C-SPIPE	308.0 ± 0.2^{c}	$25\ 10^3 \pm 1.1^{a}$	0.319 ± 0.288^{a}	0.995
C-PPIPE	305.0 ± 0.5^{c}	$21\ 10^3 \pm 1.4^{a}$	0.355 ± 0.405^{a}	0.993
M-SPIPE	359.0 ± 0.4^{b}	$12\ 10^3 \pm 1.5^{a}$	0.408 ± 0.119^{a}	0.993
M-PPIPE	364.0 ± 0.7^{b}	$10\ 10^3 \pm 1.6^{a}$	0.471 ± 0.231^{a}	0.991

$^{a-c}$ Means within treatments with the same letter in each sample are not significantly different ($p<0.05$) (Three replications).

Table 4.4. Emulsifying properties (emulsion activity, emulsion stability, droplet size and turbidity) of protein-polysaccharide complexes (SPI/PPI-Modified starch, SPI/PPI-Gum Arabic, SPI/PPI-Pectin)

Treatment	EAI (m^2/g)	ESI (min)	Droplet size (nm)	Turbidity(abs at 600 nm)
C-SPIMS	116.0 ± 2^{c}	24.0^{c}	731.6 ± 1^{a}	1.1363 ± 0.06^{a}
C-PPIMS	124.0 ± 2^{c}	22.0^{c}	745.2 ± 3^{a}	1.1348 ± 0.04^{a}
M-SPIMS	328.4 ± 1^{ab}	47.0^{a}	293.7 ± 1^{c}	0.1525 ± 0.02^{c}
M-PPIMS	364.4 ± 1^{a}	46.0^{a}	278.4 ± 1^{c}	0.1504 ± 0.06^{c}
C-SPIGA	109.6 ± 3^{c}	20.0^{c}	755.2 ± 4^{a}	1.1402 ± 0.03^{a}
C-PPIGA	115.6 ± 4^{c}	19.0^{c}	744.8 ± 1^{a}	1.1395 ± 0.06^{a}
M-SPIGA	299.6 ± 2^{b}	34.0^{b}	369.1 ± 2^{b}	0.5511 ± 0.05^{b}
M-PPIGA	307.5 ± 2^{b}	35.0^{b}	388.7 ± 2^{b}	0.5508 ± 0.08^{b}
C-SPIPE	111.3 ± 2^{c}	16.0^{cd}	757.1 ± 3^{a}	1.1421 ± 0.02^{a}
C-PPIPE	119.6 ± 3^{c}	12.0^{d}	754.6 ± 2^{a}	1.1398 ± 0.07^{a}
M-SPIPE	302.6 ± 2^{b}	32.0^{b}	403.4 ± 1^{b}	0.5502 ± 0.08^{b}
M-PPIPE	309.6 ± 1^{b}	33.0^{b}	399.4 ± 1^{b}	0.5499 ± 0.05^{b}

$^{a-d}$ Means within treatments with the same letter in each sample are not significantly different ($p<0.05$) (Three replications).

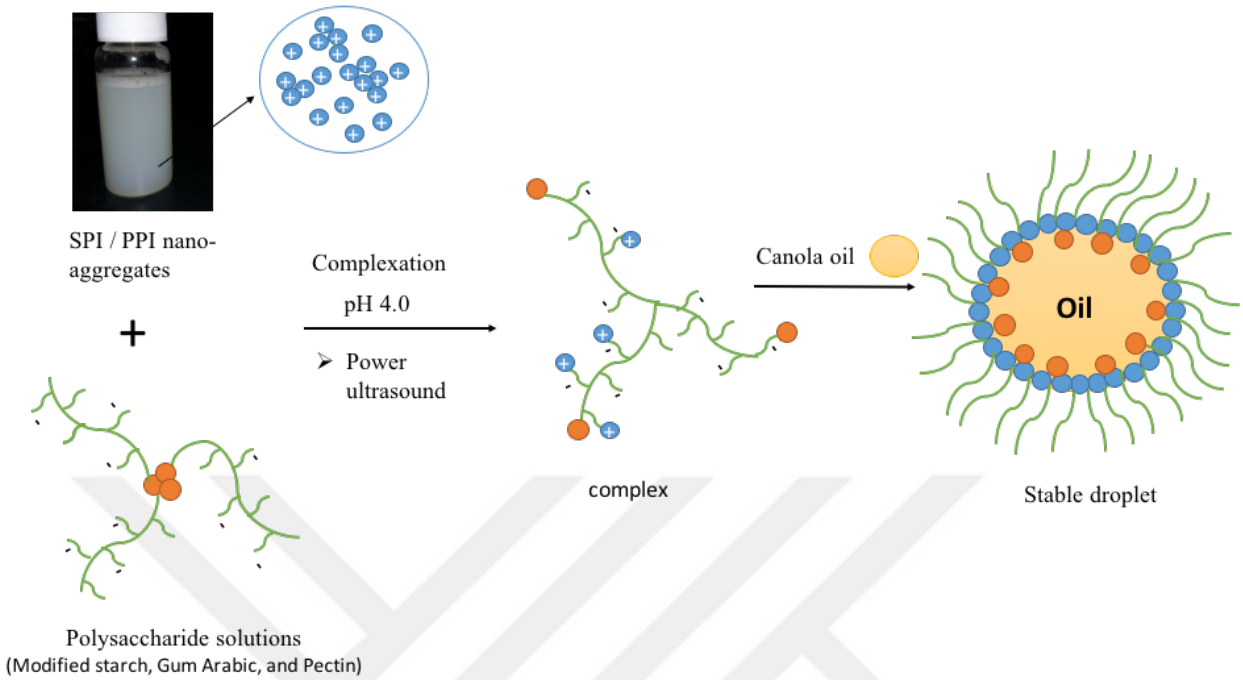

Fig. 4.1. Flow chart of preparation approach of protein/polysaccharide complex emulsions

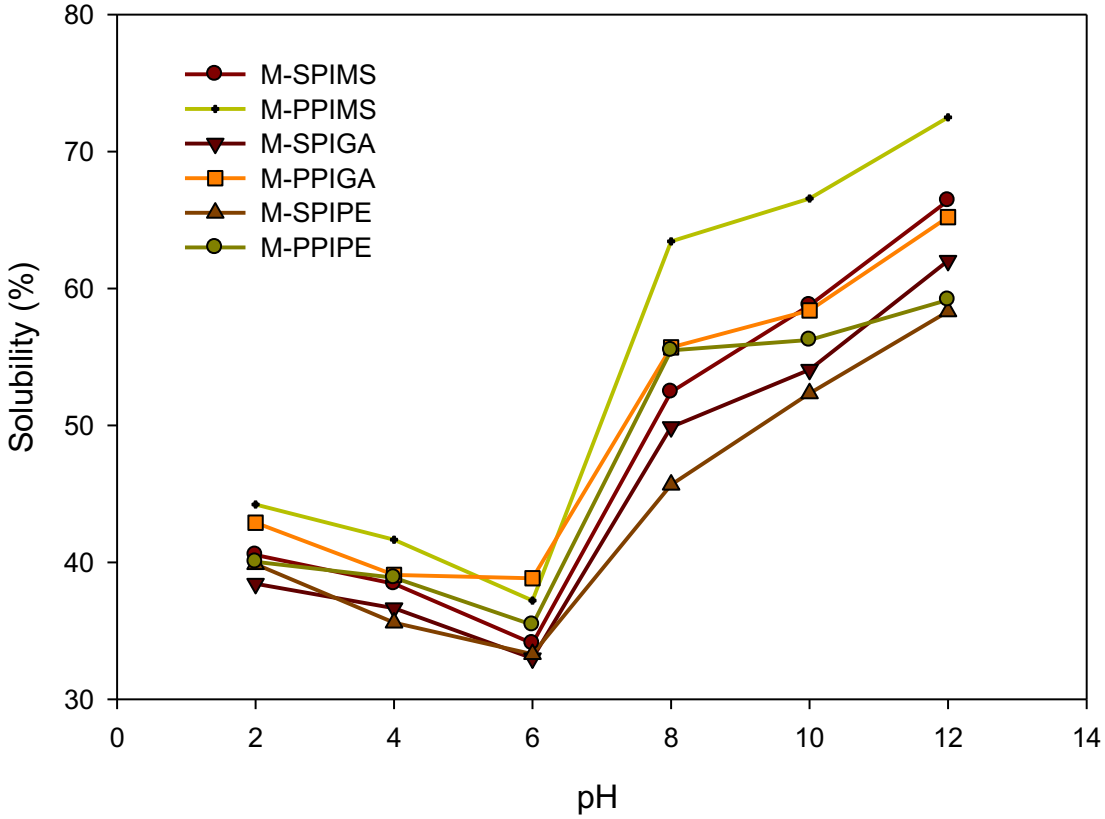

Fig. 4.2. The solubility profile of protein-polysaccharide complexes
*The control: 7-9% solubility. Compared to the protein alone treatment (Fig. 3.2.), the protein and polysaccharides complexes especially PPI/SPI-modified starch complexes exhibited a relatively high solubility around isoelectric point.

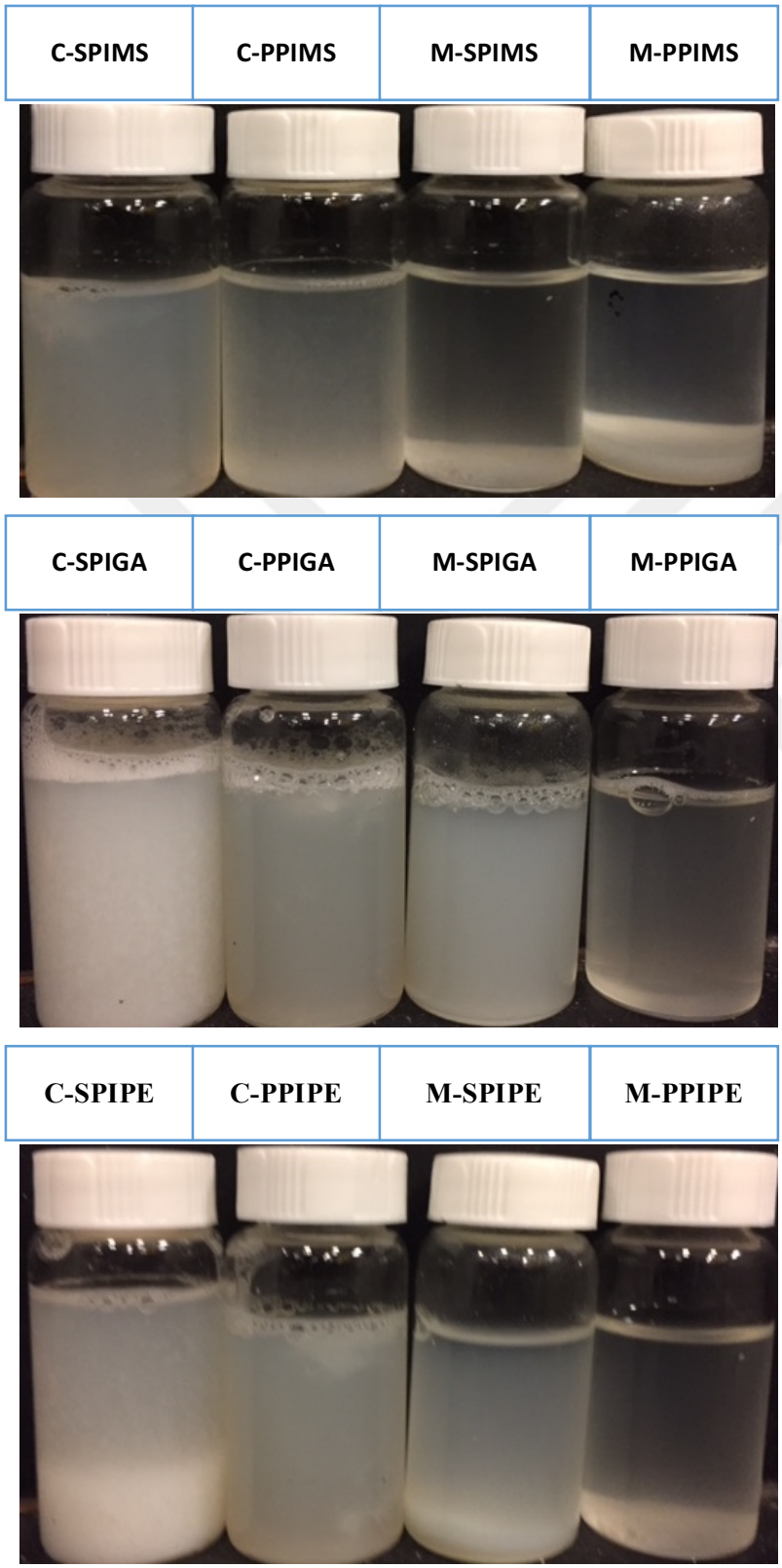

Fig. 4.3. The appearance of protein-polysaccharide complexes

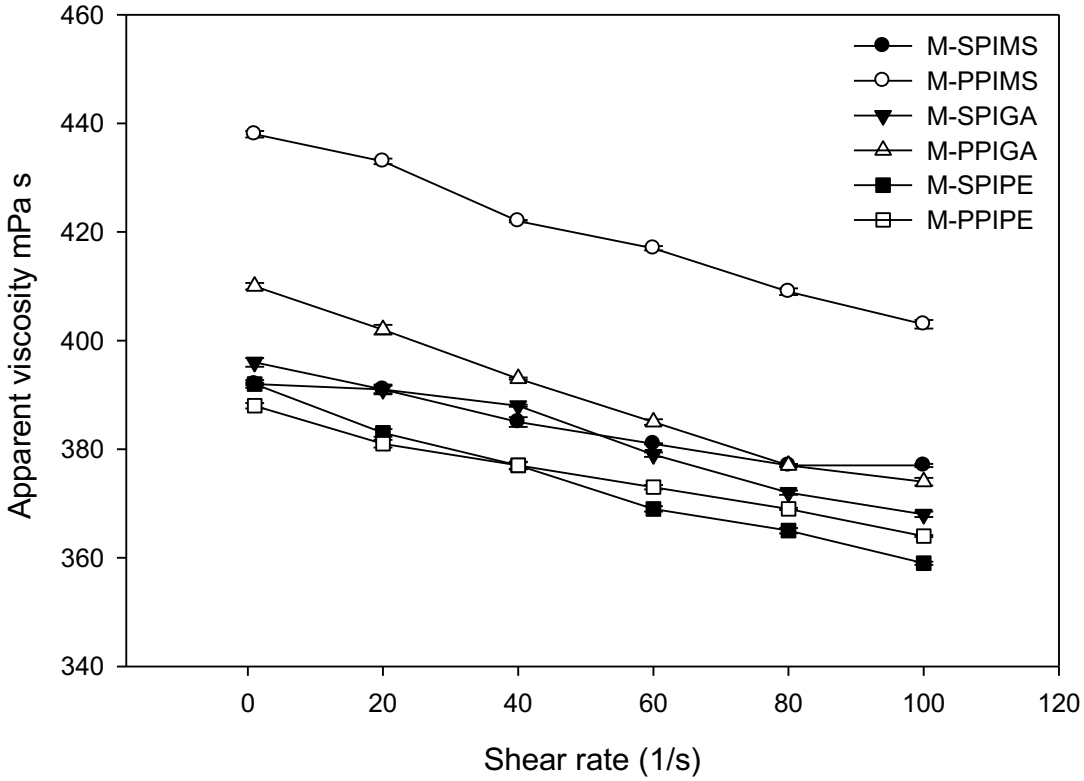

Fig. 4.4. The flow curves (apparent viscosity versus shear rate) of protein/polysaccharide complexes

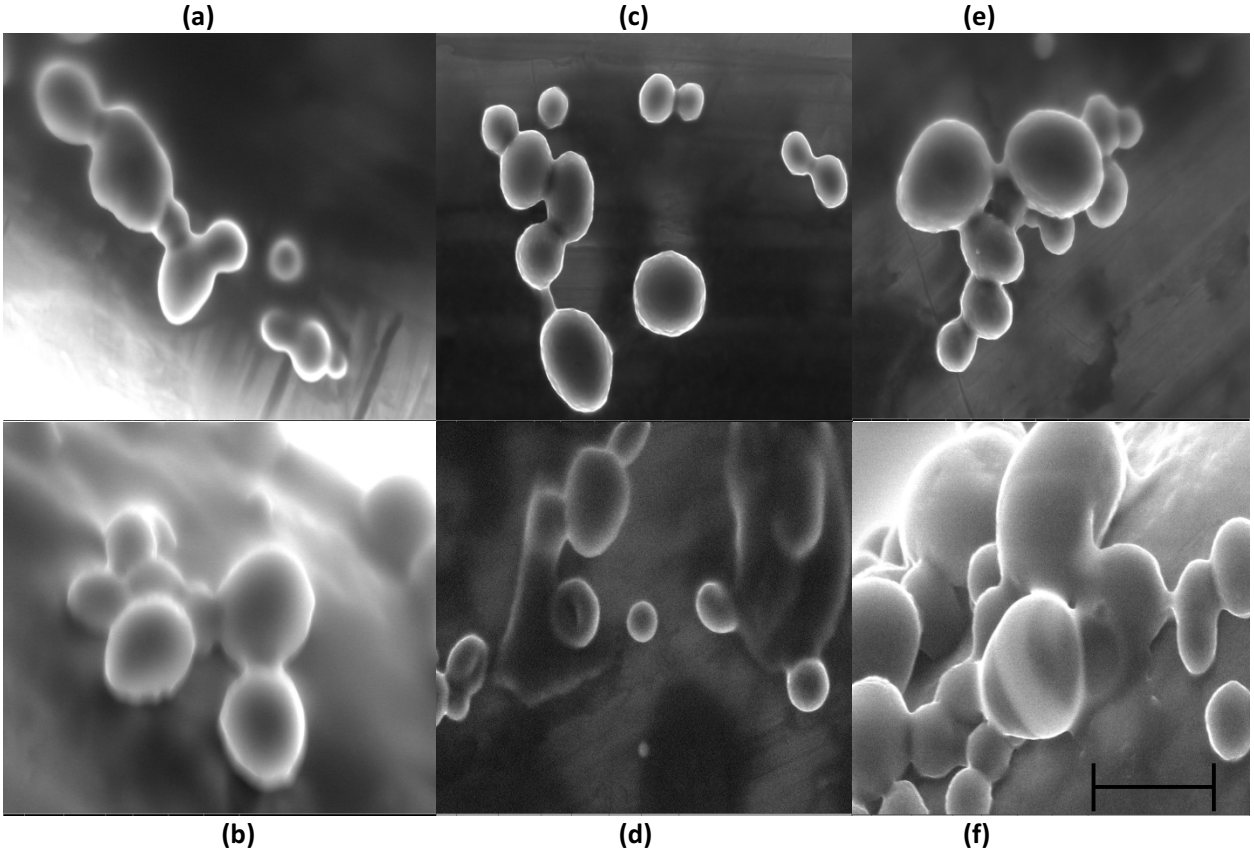

Fig. 4.5. The ESEM images of protein-polysaccharide complexes (a) M-SPIPE; (b) M-PPIPE; (c) M-SPIGA; (d) M-PPIGA; (e) M-SPIMS; (f) M-PPIMS.

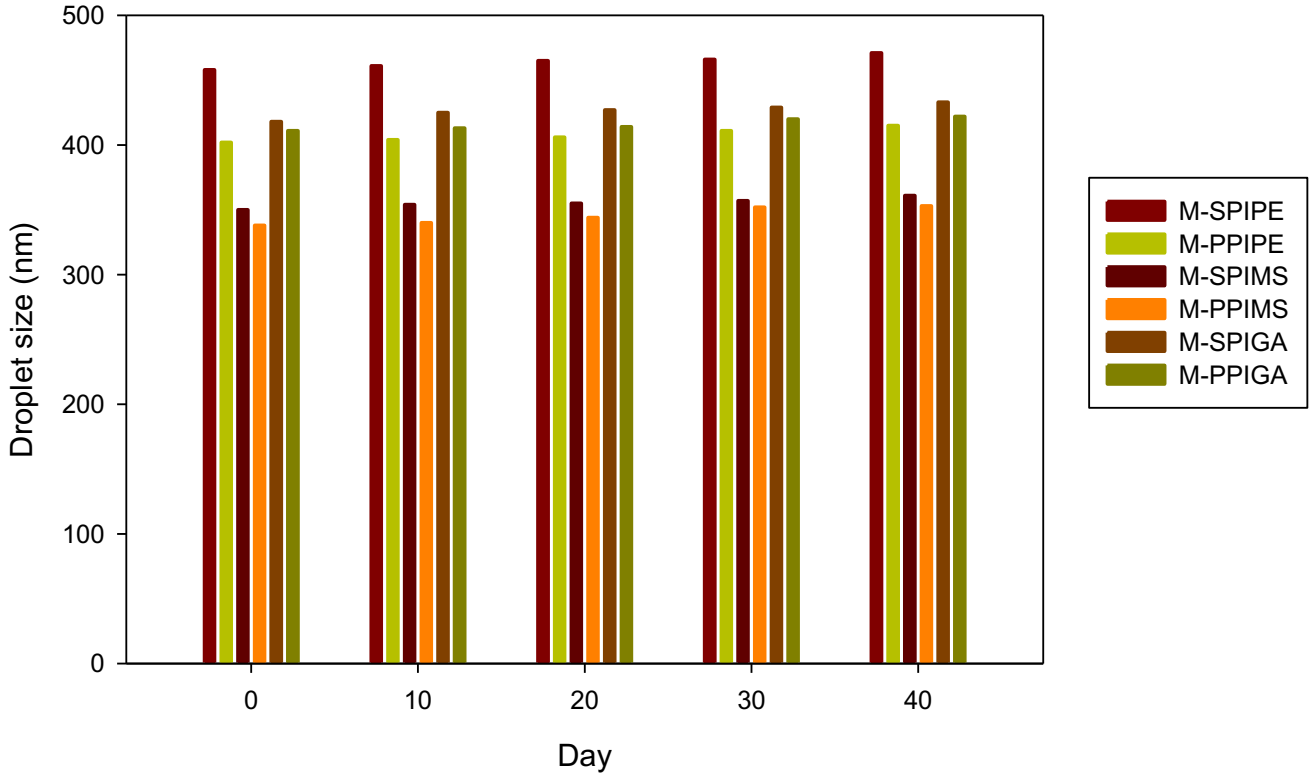

Fig. 4.6. Droplet Size of protein-polysaccharide complex emulsions with 40 days of storage at 4^{o}C

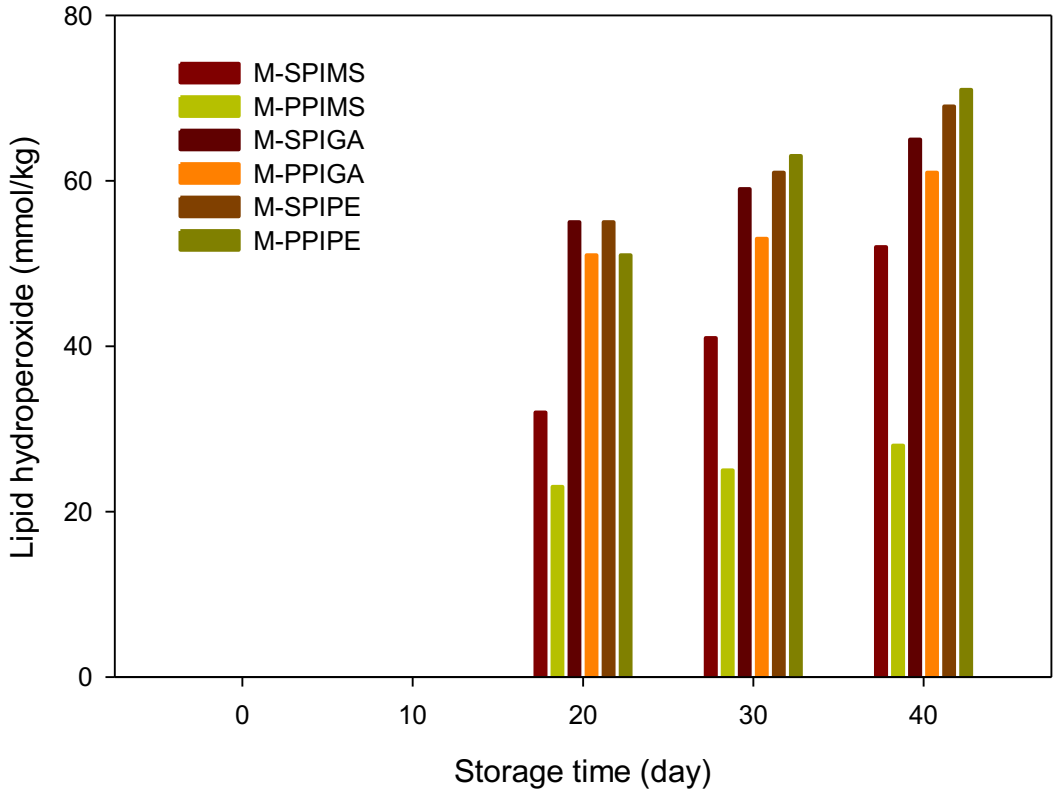

Fig. 4.7. Lipid hydroperoxide values of protein-polysaccharide complex emulsions with 40 days of storage at 4°C

CHAPTER 5

ENCAPSULATION OF DOCOSAHEXAENOIC ACID (DHA) WITH MICROENCAPSULATION USING FOUR WALL MATERIALS INCLUDING PEA PROTEIN-MODIFIED STARCH COMPLEX

5.1. Introduction

Docosahexaenoic acid (DHA, 22 carbons and 6 double bonds) is a polyunsaturated fatty acid (Shahidi, 2008). DHA has positive effects on the prevention and treatment of various diseases. It is a fundamental compound for a healthy nutrition and have many beneficial effects, including neurological benefits (Dyall and Titus, 2008), anti-depressive effects and cardiovascular benefits (Bays, 2008 and Wang et al., 2006).

A major challenge associated with the application of DHA in functional foods is the oxidation during the processing. Oxidation decreases the nutritional quality of the lipid and produces off flavor and aroma compounds because of the disruption of lipid hydroperoxides (Gotoh, Watanabe et al. 2006). The susceptibility of the fatty acids to oxidation is mostly related to their structure. For example, while comparing with the saturated lipids, polyunsaturated lipids are much more susceptible to oxidation because of their high content of bis-alliylic methylene groups (Kulas, Olsen et al. 2006). Oleic acid which contains a single double bond proceeds 10 times faster oxidation than its saturated counterpart, called as stearic acid. In similar way, linoleic acid with 2 double bonds proceeds 100 times faster, and linolenic acid with 3 double bonds proceeds almost 200 times faster (Pokorny, Yanishlieva et al. 2001).

One commonly used method to minimize oxidation and off-odor production is encapsulation. Encapsulation is a process that creates a functional barrier between the core and wall material to prevent chemical and physical reactions and maintain the biological, functional, and physicochemical properties of the core material (Gharsallaoui et al., 2007; Calvo and others 2011). Encapsulation has the ability to improve the oxidative stability, shelf-life, and biological activity of oils. Moreover, it might also be useful to manage the volatility and release properties of essential oils. Encapsulation of essential oils has been investigated using various methods such as emulsification, spray-drying, freeze-drying, coacervation, and melt-extrusion. Selection of suitable encapsulation technique and wall material depends on the end use of the product and the processing conditions (Let, Jacobsen et al. 2007). According to Nesterenko et al. (2012), no single wall material is able to present all the desired properties thereby a combination of polysaccharides and proteins is often studied to develop microcapsules. The aim of this study was to develop protein/polysaccharides complexes for encapsulation of DHA, utilizing plant proteins nano-aggregates produced by the pH-shifting and mano-thermo-sonication combined method. Four wall materials were tested, including pea protein isolate (PPI), PPI - modified starch (PPI-MS) complex, Tween 20, and SDS. The physicochemical characteristics, oxidative stability, and release properties of the capsules were evaluated.

5.2. Materials and methods

5.2.1 Chemicals

Pea protein isolate (PPI, NUTRALYS® S85F, 85% pea protein based on dry basis) was provided by Roquette (Geneva, IL, USA), and produced using a wet extraction process from dry yellow peas. The PPI was stored in a refrigerator at 4°C before use. Modified starch

(hydroxypropylated starch) and DHA were purchased from Sigma Chemicals Co. (St. Louis, MO, USA), and DHA in a solid form was stored at -20°C. Tween 20 and Sodium dodecyl sulfate (SDS) were obtained from the Fisher Chemical Co. (Fair Lawn, NJ).

5.2.2 Preparation of soluble PPI and PPI-MS complexes as wall materials

For soluble PPI, three grams of PPI were dissolved in 100 mL deionized (DI) water and stirred at room temperature for 30 min to get the protein dispersions. The PPI dispersions were adjusted to pH 12 with 2M sodium hydroxide (NaOH) and treated by MTS (50°C, 200 kPa and 60 s), followed by adjusting the pH back to pH 7 with 2M hydrochloric acid (HCl). The neutralized protein dispersions were centrifuged (1,200 g and 20°C) for 15 min and the supernatants were collected as soluble pea proteins for further experiments.

For preparation of PPI-modified starch complexes**,** six grams of polysaccharide (modified starch) was dissolved in 100 mL deionized (DI) water and stirred at room temperature for an hour. The soluble PPI produced with pH 12-MTS was mixed with polysaccharide solutions. After 3 hours stirring at room temperature, the protein/polysaccharide mixtures were sonicated (5 min) using a VC-750 ultrasound unit (20 kHz, Sonics & Materials, Inc., Newtown, CT, USA) in an ice bath and the pH of the mixtures was adjusted to 4.0 to obtain protein/polysaccharide complexes.

5.2.3 Preparation of DHA microemulsions

Canola oil (2.5 mL) containing 2.5 mg DHA was added to 50 mL of PPI, PPI-modified starch complexes, Tween 20, and SDS solutions (10 mg/mL) (Table 5.1 shows the percentage of wall and core materials used in the study to achieve 1:4 core-to-wall ratio), and vigorously stirred 5 min. To achieve fine homogenization, samples were sonicated (5 min) using a VC-750

ultrasound generator (20 kHz) in an ice bath (Sonics & Materials, Inc., Newtown, CT, USA). The microemulsions containing DHA were centrifuged (1,200 g and 20°C) for 15 min and the supernatants were collected for further experiments.

5.2.4 Droplet size of DHA microemulsions

Volume-weighted mean diameters (D4, 3) of the DHA microemulsions and freeze-dried capsules were detected by dynamic light scattering (DLS) using a NICOMP 380 DLS instrument. Samples were diluted 500-fold with DI water before measurement. The measurements were conducted at 25°C. Freeze-dried powders of 0.5 g were dispersed in 4 mL water and stirred at 500 rpm for 5 min, and the droplet size distributions of the samples were measured.

5.2.5 Microstructure of DHA microemulsions

Microstructure of the DHA microemulsions and freeze dried DHA capsules was analyzed by Environmental Scanning Electron Microscope (ESEM). The sample was frozen with nitrogen before ESEM analysis. Small amount of frozen sample (a small drop) was gently deposited on an aluminum stub and was placed into vacuum chamber. The analysis was performed in a wet mode to inhibit high voltage damages.

5.2.6 Freeze drying

Emulsions containing DHA as prepared in section 2.3 were placed in aluminum plates and frozen at -70 ^{0}C for 24 h. The frozen samples were transferred to a lab-scale freeze drier (LABCONCO, FreeZone 6 Liter Console Freeze Dry System, USA) for removal of solvent (water). The samples were dried for 40 h in the freeze dryer. At the end of the drying, the freeze-

dried powders were packed in a screw capped tube, sealed in aluminum bags, and stored at refrigeration temperature (4 ± 1°C). A flow chart for preparation of freeze-dried powder is shown in Figure 5.1.

5.2.7 Microcapsule characterization

5.2.7.1 Moisture content and water activity of encapsulated DHA

Moisture content of the freeze-dried DHA capsules was determined gravimetrically (Bae and Lee 2008). 0.5 g of the dried samples were placed in an aluminum pan and dried in a hot air oven at 105°C for about 12 h. The experiment was carried out in duplicate samples and average values were taken. The moisture content was calculated by the following equation:

$$\text{Moisture } (\%) = \frac{(W_0 - W_1)}{(W_0)} \times 100 \tag{5.1}$$

where, W_0 is the weight of original dry status of encapsulated sample, and W_1 is the weight of sample after oven treatment.

Water activity of freeze-dried DHA capsules was determined using an AquaLab CX-2 water activity meter (Decagon Devices, Inc., Pullman, WA).

5.2.7.2 Color analysis of encapsulated DHA

Color measurements of the freeze-dried DHA capsules were performed with a Minolta Chroma Meter CR-300 (Minolta Camera Co. Ltd., Osaka, Japan). The color meter was calibrated with a white standard plate. The color of the capsules was expressed as the L* a* b* values (average of two values), where +L* denotes whiteness, −L* denotes blackness, +a* redness, −a*

greenness, + b* yellowness, and −b* blueness.

5.2.7.3 Microcapsule surface and total oil content

Microcapsule surface oil was determined based on the method of Liu et al. (2010). In short, 1 g of freeze-dried DHA capsule was dispersed in 30 ml of hexane, and followed by vigorous shaking for 30 s. The solvent was filtered through Whatman #1 filter paper into a beaker, and the beaker plus solvent was placed in a fume hood overnight in order to facilitate solvent evaporation. Microcapsule surface oil was then determined gravimetrically, after heating the beaker at 105^0C for 30 min to remove any residual solvent.

Total oil content of the freeze-dried DHA capsules was determined following the method of Klinkesorn et al., (2005) with some modifications. In brief, 4 ml of water were added to 1 g of the freeze-dried DHA capsules, followed by mixing for 2 min at 300 rpm. The resulting solution was then mixed with 25 ml of hexane/isopropanol (3:1 v/v), stirred at 300 rpm for 15 min, and centrifuged (1,500g) for 2 min. The clear organic phase was collected and the aqueous phase was re-extracted with the above mentioned solvent mixture. The organic phases were pooled and filtered through anhydrous Na_2SO_4, and the solvent was allowed to evaporate overnight in a fume hood. Total oil content was determined gravimetrically, after heating at 105^0C for 30 min.

5.2.7.4 Encapsulation efficiency

Following the surface and total oil determination, the encapsulation efficiency (EE) was calculated from the quantitative determinations as follows (Anwar & Kunz, 2011):

$$\mathrm{EE} = \frac{\text{Total oil} - \text{Surface oil}}{\text{Total oil}} \text{x}100 \qquad (5.2)$$

5.2.7.5 Oxidative stability

Oxidative stability of free oil (i.e., control) and dried powders was characterized during storage at room temperature (20-23^{0}C) for 30 days using the peroxide value. Oxidative testing was carried out every 5 days till 30 days. Canola oil containing DHA extraction from the freeze-dried capsules followed the same procedure as previously outlined for the total oil determination. Extracted canola oil containing DHA (0.2 g) was weighed into a 250 mL Erlenmeyer flask, and followed by the addition of 30 mL of 3:2 acetic acid/chloroform (v/v) solution and 0.5 mL of saturated potassium iodide (KI). After vigorous shaking for a min, 30 mL of water was added to this mixture. Then, a 0.5 mL aliquot of 1% (w/ v) starch indicator was added to the mixture, and the resulting solution was titrated by using 0.001 N sodium thiosulfate ($Na_2S_2O_3$) until the purple color disappeared. PV was calculated as:

$$\mathrm{PV} = \frac{(\mathrm{S}-\mathrm{B})\mathrm{X\ N\ x\ 1000}}{\mathrm{W}} \tag{5.3}$$

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

5.2.7.6 Release behavior of freeze-dried canola oil containing DHA microcapsules triggered by gastrointestinal environments (pectin and pancreatin)

In vitro model that triggered by gastrointestinal environments (effects of pepsin and both pepsin-pancreatin enzymes) based on the method of Burgar et al. (2009) was applied to evaluate the release behavior of freeze-dried canola oil containing DHA microcapsules. In brief, simulated gastric fluid (SGF) was prepared by dissolving 2.0 g of NaCl and 7.0 ml of 36% HCl in 900 ml of water. After the addition of 3.2 g of pepsin (from Sigma Chemicals Co., Catolog number: R2283),

the solution pH was adjusted to 1.2 with 0.1 M HCl and the final volume was made up to 1000 ml with water. Simulated intestinal fluid (SIF) was prepared by dissolving 6.8 g of K_2HPO_4 in 800 ml of water. To this solution was added 77 ml of 0.2 M NaOH and 100.0 g of pancreatin and the solution was stirred overnight at 4 ^{0}C. Solution pH was adjusted to 6.8 with 1 M NaOH or 1 M HCl and the final volume was made up to 1000 ml with water.

Two grams of freeze-dried DHA capsule samples were mixed with 20 ml of SGF and incubated for 2 h at 37^{0}C and 100 rpm in a water bath. Released oil containing DHA was extracted using hexane and then determined gravimetrically. For exposure to SGF and SIF in sequence, 2 g freeze-dried DHA capsule sample was mixed with 20 ml of SGF and incubated under same conditions for 2 h. Sample pH was adjusted to 6.8 using 1 M NaOH, followed by addition of 20 ml of SIF and the sample was incubated under the same conditions for 3 h. The amount of canola oil containing DHA released from the freeze-dried capsules was determined by gravimetric analysis.

5.2.8 Statistics

Three replications for each treatment were used for all measurements, unless otherwise stated. The results were analyzed by analysis of variance using the General Linear Models (PROC GLM) procedure in SAS (version 9.3, SAS Institute, Inc., Cary, North Carolina, USA). Differences among the mean values were obtained by Fisher's least significant difference (LSD) test at alpha = 0.05.

5.3. Results and discussion

5.3.1 Moisture content and water activity of encapsulated DHA powders

Average moisture contents of the encapsulated DHA powders are given in Table 5.2. The PM-DHA and T-DHA had a moisture content of 2.03% and 2.03%, respectively, lower than the moisture contents of other freeze-dried DHA capsules. The shelf-life of encapsulated powders are influenced by moisture content and temperature (Quispe-Condori et al. 2011). Lim et al. (2012) reported that moisture content was a factor inducing off flavors in the oil due to lipid oxidation. The distribution of oil in the solid matrix of capsules and accessibility of oxygen related to moisture content also impact the oxidation (Velasco et al. 2003). In our study, since the moisture content of PM-DHA and T-DHA was relatively lower compared to other DHA microcapsules, these microcapsules also showed less lipid oxidation.

Water activity of a powder is another indicator of the powder stability and flow ability. Water activity of all the DHA microcapsules was in the range of 0.05 to 0.08, with no significant difference among the water activity values (Table 5.2). The moisture content of the freeze-dried microcapsules ranged between 2.03% and 2.18% ($p > 0.05$), and their water activity varied from 0.05 to 0.08 ($p > 0.05$). These values are below the maximum moisture (4−5%) and water activity (0.3) specifications for dried powders used in the food industry (Klaypradit & Huang, 2008). Changes in wall materials did not have a significant effect on freeze-dried capsules' moisture content and water activity ($p > 0.05$).

5.3.2 Hunter color values of freeze-dried DHA microcapsules

The Hunter L (lightness), a (redness), and b (yellowness) color values of the freeze-dried microcapsules containing DHA are presented in Table 5.3. The P-DHA and PM-DHA

microcapsules were significantly less yellow in color, as shown by relatively low *L* values (82.3 and 83.2), and relatively high *a* values (1.2 and 1.5) and *b* values (11.2 and 11.8) of the microcapsules. Overall, the DHA-microcapsules produced with surfactants (Tween 20 and SDS) had significantly higher L values than the P-DHA and PM-DHA microcapsules (Table 5.3), whereas the *a* values of P-DHA and PM-DHA microcapsules were significantly higher than those of the DHA microcapsules with surfactants. The darker color of the P-DHA and PM-DHA microcapsules may be explained by the presence of pigments within the proteins used in isolate preparation (Bamdad et al., 2006).

5.3.3 Surface oil and encapsulation efficiency of DHA microcapsules

In microcapsules, some oil that remains on the surface is referred as surface oil (Bao et al., 2011). The presence of oil on the microcapsule surface has a negative impact on oxidative stability, and causes rancidity and reduces the shelf life. Decreasing surface oil is important for lipid microencapsulation since it oxidizes significantly faster than the encapsulated oil (Pegg & Shahidi, 2007). The surface oil content of the microcapsules is shown in Table 5.2. It was observed that the surface oil content of the microcapsules ranged from 1.15% to 2.04% depending on the wall materials. The lowest surface oil and highest encapsulation efficiency was observed for PM-DHA with values of 1.15% for surface oil and 92.2% for encapsulation efficiency. This indicated that the canola oil containing DHA was successfully covered by the wall material (i.e., PM-DHA) whereas the other wall materials were less effective in retaining oil inside the capsules. The SDS-DHA sample had the highest surface oil (2.05%) while the others are all below 2%.

Encapsulation efficiency (EE) of the freeze-dried DHA capsules is also shown in Table 5.2. It can be seen that the encapsulation efficiency of the PM-DHA capsules was significantly higher

than the other DHA microcapsules. This indicates that the PPI-MS complex covers the core material (DHA) better than the other wall materials. Better encapsulation efficiency of the PM-DHA is possibly due to a higher amount of entrapped DHA near the surface of the microcapsules.

5.3.4 Droplet size of the DHA microemulsions

The mean droplet diameters of the DHA microemulsions before and after freeze-drying are shown in Fig. 5.2. The volume-weighted mean diameters (D 4,3) of the DHA microemulsions before freeze drying ranged from 388 nm to 421 nm, and after freeze drying it was 366 nm to 410 nm. Before freeze drying and after freeze drying, the lowest droplet size was observed for PM-DHA samples as 388 nm and 366 nm, respectively. Smaller oil droplet sizes were reported to be linked to less microcapsule surface oil, increased oil encapsulation efficiency, and decreased lipid oxidation rates (Lee & Ying, 2008). Our results were in agreement with their observations as the PM-DHA microcapsules having smaller droplet sizes also showed a higher encapsulation efficiency, less oxidation and microcapsule surface oil.

5.3.5 Oxidative stability of encapsulated DHA

DHA (6 double bonds) is highly susceptible to oxidation. One of the main goals in this study was to develop new protein and polysaccharide complex microcapsules to delay DHA oxidation. The peroxide value (PV) of free and microencapsulated canola oil containing DHA was monitored over a 30-d period and is are presented in Fig. 5.3. The PV results for the encapsulated DHA and that of free oil was significantly different after storage for 10 days 10 ($p < 0.05$) (Fig. 5.3). The PV of the free oil steadily increased to 6.05 meq/kg at day 15, 7.02 meq/kg at day 20, and 8.35 meg/kg at day 25, and finally to 10.72 meq/kg at day 30. After 5 days, a slight increase

in the PV values for microencapsulated DHA were observed for all types of wall materials. However, these changes were not found to be significant ($p > 0.05$). These results clearly show that the protein and polysaccharide based microencapsulation employed in this study provides a good protection of the DHA against oxidation during a 30-d storage. Changes in wall materials did not have a significant effect on DHA oxidation ($p > 0.05$). A large number of studies have used encapsulation to protect core materials. For example; Grattard et al. (2002) encapsulated flaxseed oil in a maltodextrin–lecithin–xanthan gum matrix, followed by freeze-drying, and this material was reported to be effective protecting the entrapped oil against oxidation. Notably, in this study, the PV values of the microencapsulated DHA during a 30-day storage were below the industry target of <5 meq/kg (Karaca et al., 2013).

5.3.6 ESEM images of DHA microemulsions and microcapsules

ESEM morphology of both the DHA microemulsions and microcapsules is shown in Figure 5.4 (DHA microemulsions) and Figure 5.5 (DHA microcapsules). The ESEM images of the microemulsions made with different wall materials are different. Relatively large particles can be observed in the SDS-DHA and P-DHA samples, in agreement with the DLS analysis (Fig.2) (around 400 nm), while the particles in the PM-DHA and T-TDA samples are smaller. A spherical shape can be observed for almost all the particles in the ESEM images.

On the other hand, the DHA microcapsules exhibited cakelike structures with rough and porous surfaces. More pores on the surface of the dried aggregates were observed for P-DHA and SDS-DHA microcapsules. The pores in the capsules might be created during ice crystal formation in the freezing step before freeze drying, which sublimes during FD process leaving empty spaces in the samples (Anandharamakrishnan et al. 2010).

5.3.7 Release profile from the DHA microcapsules

In humans, the first step of fat digestion takes place in the stomach. The gastric lipase is secreted in the gastric juice hydrolyses under acidic conditions of 10-60% of dietary triacylglycerols. Then fat digestion is completed in the small intestine where absorption of lipolytic products occurs (Pasquier et al., 1996). The in vitro release behavior of the canola oil containing DHA microencapsulated by the wall materials was investigated by employing both pepsin and pancreatin hydrolytic enzymes. The release of the canola oil containing DHA under SGF (pepsin) conditions ranged from 33.5% to 37.9%, with the highest value observed for the PM-DHA microcapsules. The release of the canola oil containing DHA under the combined SGF-SIF (pepsin-pancreatin) conditions was significantly higher (86.5–91.3%) than that observed for SGF (pepsin) only (Table 5.4). This is probably caused by the combined effect of the hydrolytic enzymes (pancreatin and pepsin) and acidic pH (1.2) on hydrolysis of protein and carbohydrate oligomer of the capsule wall that resulted in a change in capsule structure (e.g., large pore formation) with enhanced release. The release behavior of canola oil containing DHA had a limitation since the measurement was just based on the release under the conditions (pectin and pancreatin enzymes).

5.4. Summary and conclusions

In conclusion, vegetable proteins are more sustainable sources compared to the other emulsifiers such as Tween 20. These emulsifiers might have some potential issues, or are limited in concentration to add to foods, regulations, etc. PPI-MS as a natural polymeric wall material exhibited similar or better encapsulation efficiency and desirable level of peroxide value compared to the synthetic surfactants (Tween 20 and SDS). The utilization of proteins/polysaccharides

complexes allowed the incorporation of specific properties of each polymer to further improve their emulsion stability and produce microcapsules with better oxidative stability of bioactive compound, i.e. DHA. Encapsulation of DHA by protein/polysaccharide complexes was proven to maintain and improve the biological and functional characteristics making it a useful tool for development of healthy food products fortified with omega fatty acids.

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5.6 Figures and tables

Table 5.1. Description of samples used in this study

Sample	Wall material	Core material	%oil containing DHA	%PPI	%MS	%Tween 20	%SDS	%Total solid	Core: Wall
P-DHA	Pea protein isolate	DHA	2.5	10	-	-	-	12.5	1:4
T-DHA	Tween 20			-	-	10	-		
PM-DHA	Pea protein isolate-Modified Starch			5	5	-	-		
SDS-DHA	SDS			-	-	-	10		

Table 5.2. Characteristics of DHA microcapsules

Treatment	Moisture content (%)	Water activity (a_w)	E.E (%)	Surface oil (%)
P-DHA	2.180 ± 0.42^{a}	0.06 ± 0.01^{a}	87.6 ± 4.3^{b}	1.78 ± 0.03^{b}
T-DHA	2.033 ± 0.02^{a}	0.07 ± 0.02^{a}	87.9 ± 2.2^{b}	1.71 ± 0.07^{b}
PM-DHA	2.027 ± 0.20^{a}	0.05 ± 0.01^{a}	92.2 ± 4.0^{a}	1.15 ± 0.11^{c}
SDS-DHA	2.139 ± 0.18^{a}	0.08 ± 0.01^{a}	84.1 ± 3.9^{c}	2.04 ± 0.13^{a}

$^{a-c}$Means within treatments with the same letter in each sample are not significantly different ($p<0.05$).

Table 5.3. Hunter color values of freeze-dried DHA microcapsules

Hunter Color Values	L	a	b
P-DHA	82.3±0.4[b]	1.5±0.2[a]	11.2±0.2[a]
T-DHA	86.9±0.1[a]	-0.4±0.4[b]	9.7±0.1[b]
PM-DHA	83.2±0.1[b]	1.2±0.1[a]	11.8±0.1[a]
SDS-DHA	86.7±0.1[a]	-0.5±0.2[b]	9.4±0.7[b]

[a-b] Means within treatments with the same letter in each sample are not significantly different ($p<0.05$).

Table 5.4. Release behavior of freeze-dried canola oil containing DHA microcapsules triggered by gastrointestinal environments

Sample	SGF (%)	SGF and SFI (%)
P-DHA	35.7±0.5[b]	88.7±0.5[b]
T-DHA	35.2±0.3[b]	89.2±0.4[b]
PM-DHA	37.1±0.7[a]	91.3±0.8[a]
SDS-DHA	33.5±0.2[c]	86.9±0.5[c]

[a-c] Means within treatments with the same letter in each sample are not significantly different ($p<0.05$).

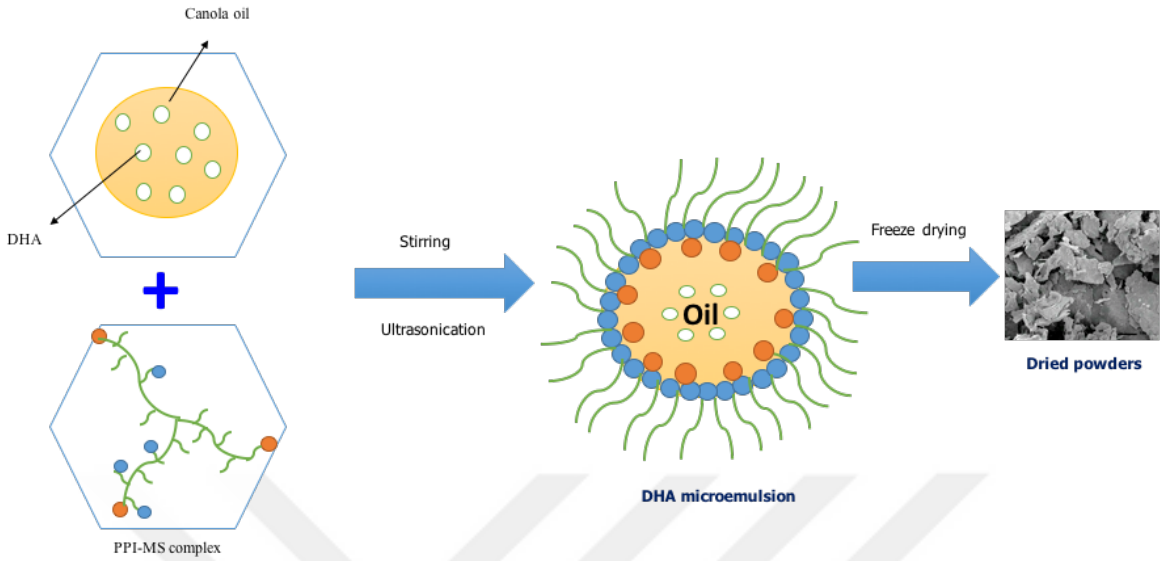


Fig. 5.1. A flow chart for preparation of freeze-dried powder

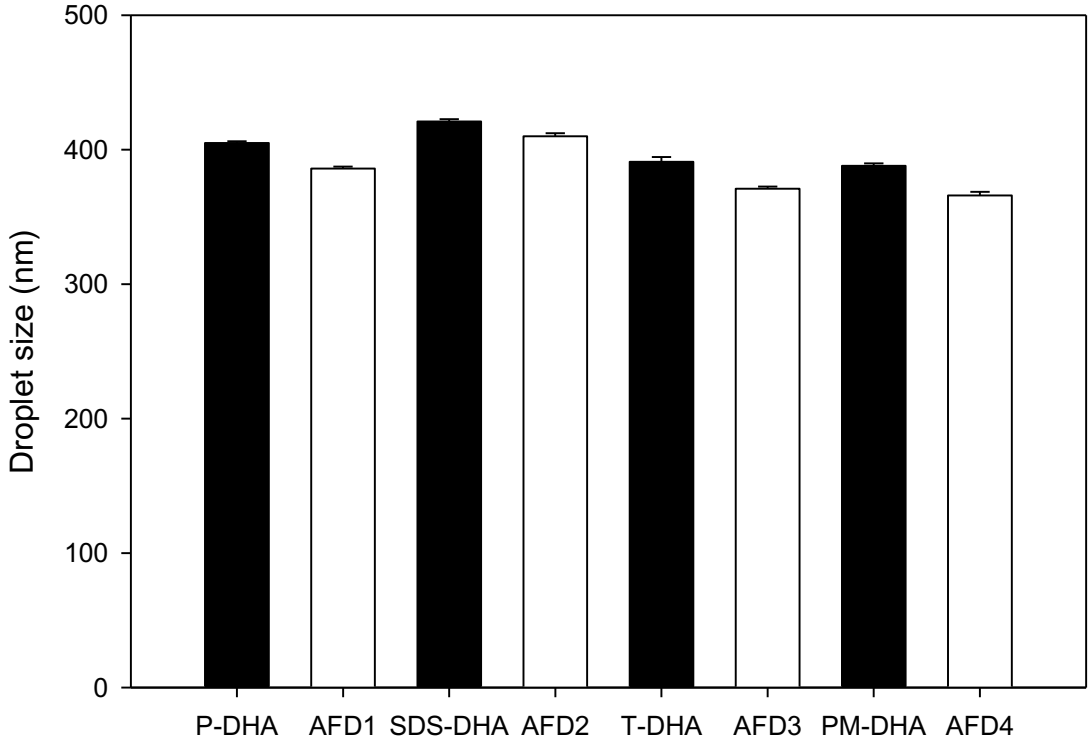


Fig. 5.2. Droplet size of DHA microemulsions and microcapsules
*AFD1: after freeze drying of P-DHA microemulsions; AFD2: after freeze drying of SDS-DHA microemulsions; AFD3: after freeze drying of T-DHA microemulsions; AFD4: after freeze drying of PM-DHA microemulsions.

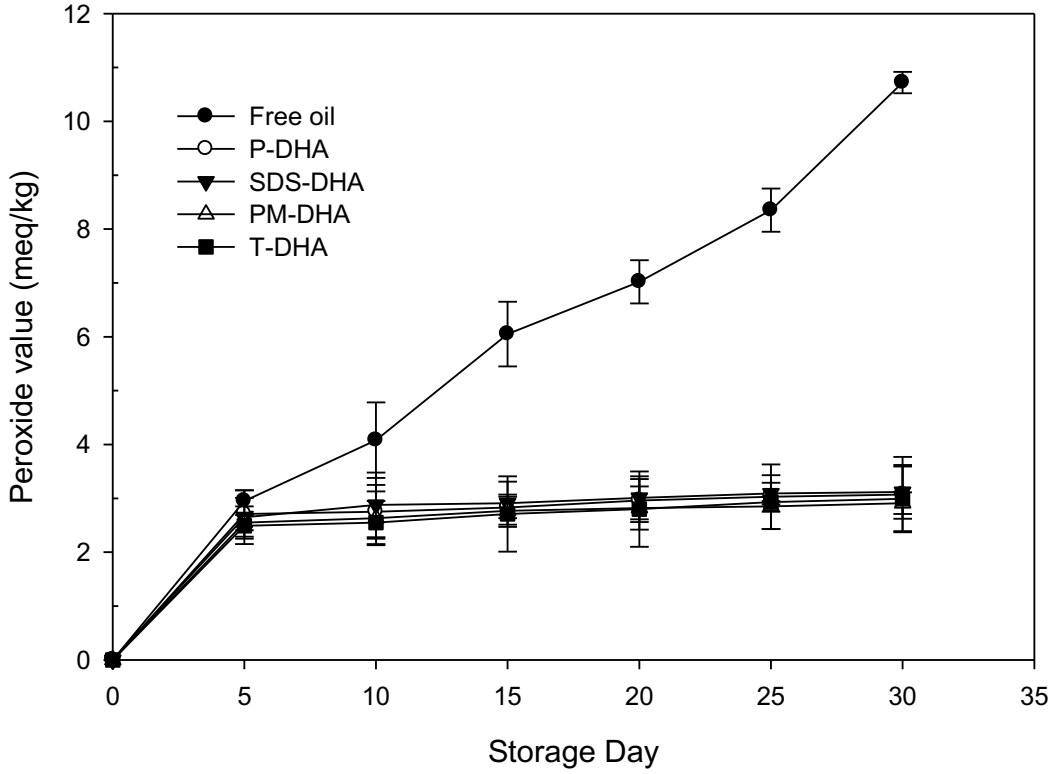


Fig. 5.3. Peroxide values of DHA microcapsules during 30 days (20-23 ^{0}C)

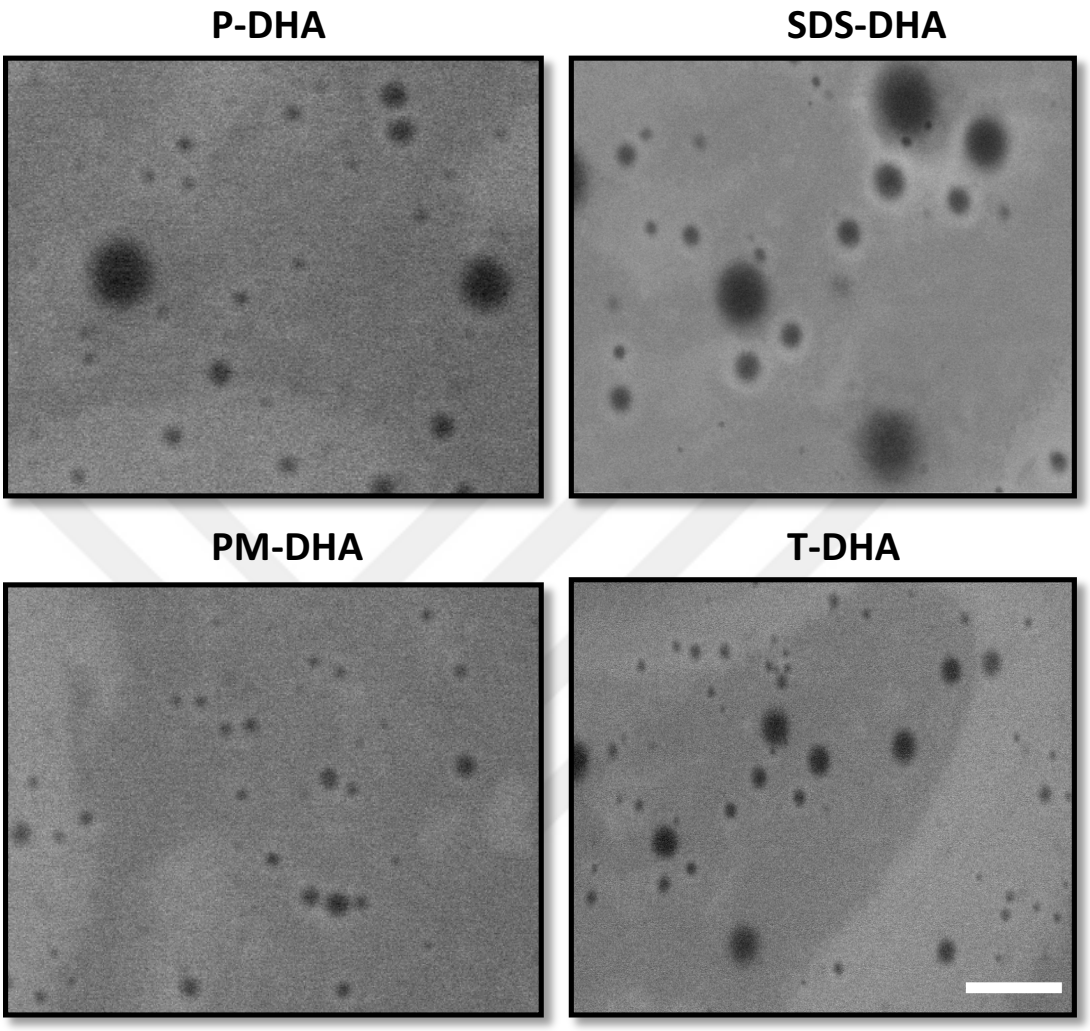


Fig. 5.4. The ESEM images of DHA microemulsions

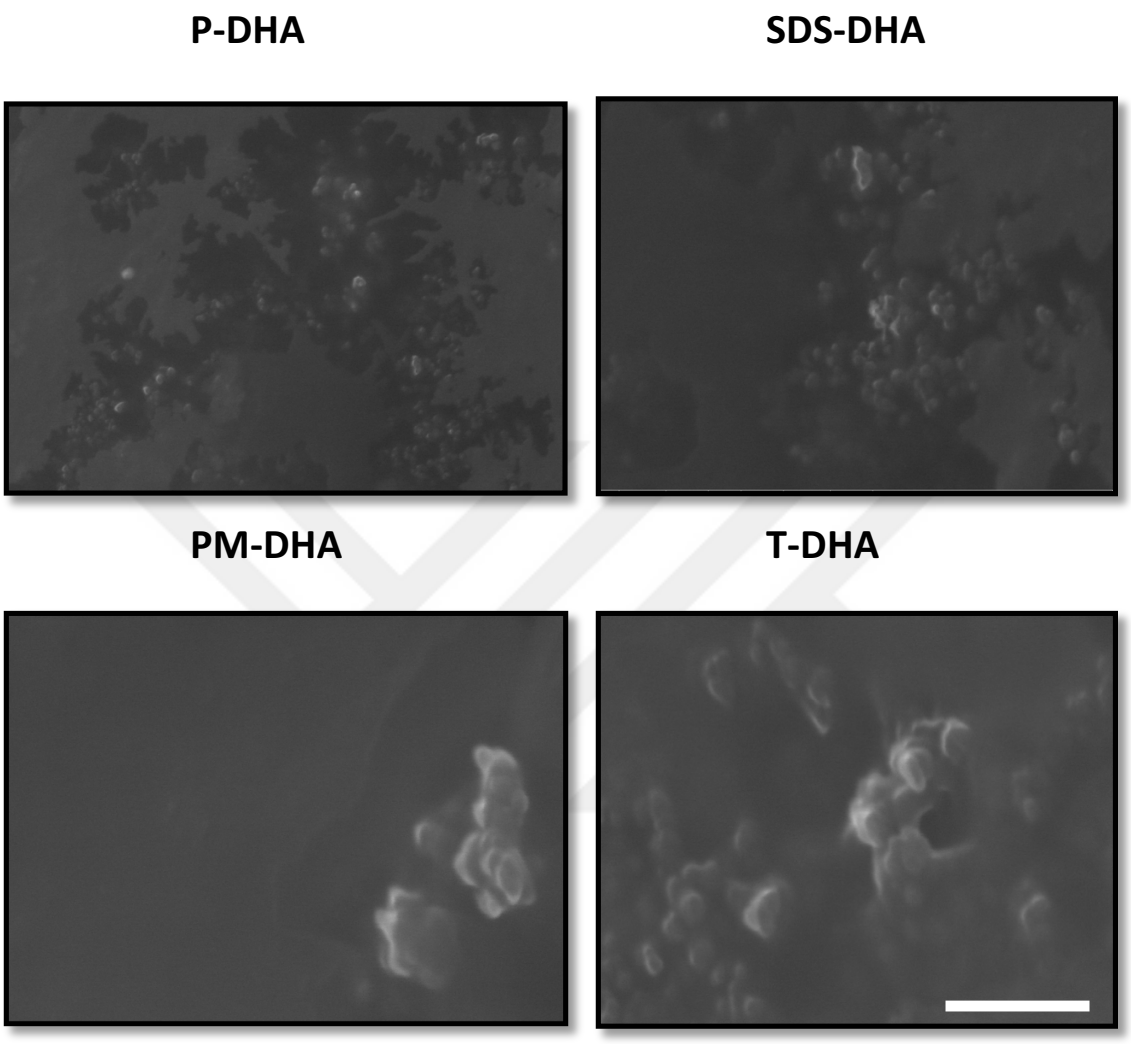


Fig. 5.5. The ESEM images of DHA microcapsules

CHAPTER 6

CONCLUSIONS AND SUGGESTIONS FOR FUTURE RESEARCH

The overall goal of this research was to explore the use of MTS and pH-shifting combined treatment to modify the functional properties of soy and pea proteins for developing protein-mediated nanocarriers to protect and deliver docosahexaenoic acid (DHA). Specifically, a MTS and pH shifting combined method for the production of nano-sized soy protein isolate (SPI) aggregates and nano-emulsions was developed, and their physicochemical, interfacial, and emulsifying properties were investigated. Then, protein-polysaccharide complexes using pH 12-MTS treated soluble proteins (SPI and PPI) and polysaccharides (Modified Starch, Gum Arabic, and Pectin) were created, and their physicochemical, interfacial, and emulsifying properties were investigated. Finally, docosahexaenoic acid (DHA) was encapsulated using pea protein isolate (PPI), pea protein isolate-modified starch (PPI-MS) complex, Tween 20, and SDS as wall materials and the encapsulation properties, physical properties, release properties, and storage stability of the freeze-dried powders were investigated.

In the first stage of this research, the outcome demonstrated that: (1) The MTS and pH-shifting combined treatment significantly improved the functional properties of SPI; (2) Soy protein-stabilized nanoemulsions from the SPI nano-aggregates (<100 nm) exhibited good stability; (3) The MTS and pH-shifting combined treatment is an effective method for producing SPI nano-aggregates that can be used as wall material for the encapsulation of hydrophobic bioactive compounds.

In the second stage of this research, the findings indicated that: (1) The PPI/SPI and polysaccharide complexes significantly improved their water solubility near isoelectric point (pI); (2) The complexes developed with PPI exhibited better physicochemical and emulsifying properties than those developed with SPI.

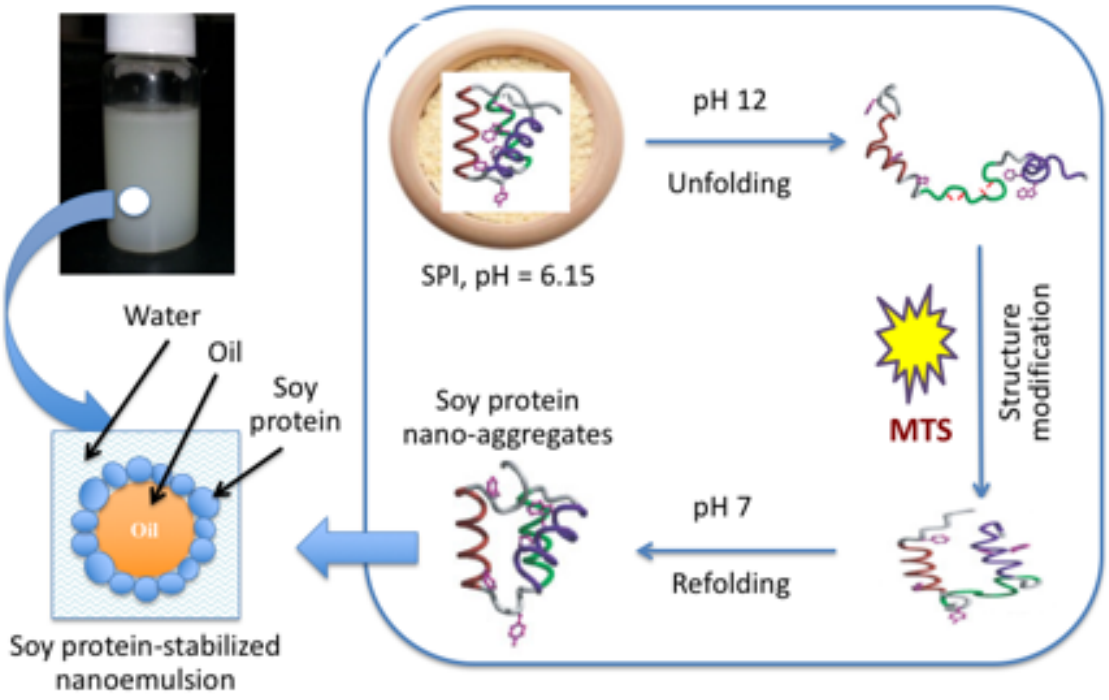
In the third stage of this research, findings showed that: (1) The PPI-modified starch complex provided good protection of DHA against oxidation; (2) The PPI-modified starch complex as a wall material exhibited similar encapsulation efficiency comparable to the synthetic surfactants.

In summary, the MTS and pH-shifting combined treatment effectively modified the functional properties of soy and pea protein that can be used to construct protein-mediated nanocarriers to protect and deliver bioactive compounds.

For future studies, the bioaccessibility of canola oil containing DHA can be measured. In addition, the freeze-dried DHA powders can be re-dispersed in liquid at different pH, and the stability of the regenerated dispersion and its ability to protect DHA can be investigated. Finally, the freeze-dried DHA powders can be applied to different food products such as beverages, cereals, bakeries, and margarines to create functional foods. The stability of the bioactives in such foods and its consumer acceptance can be investigated.

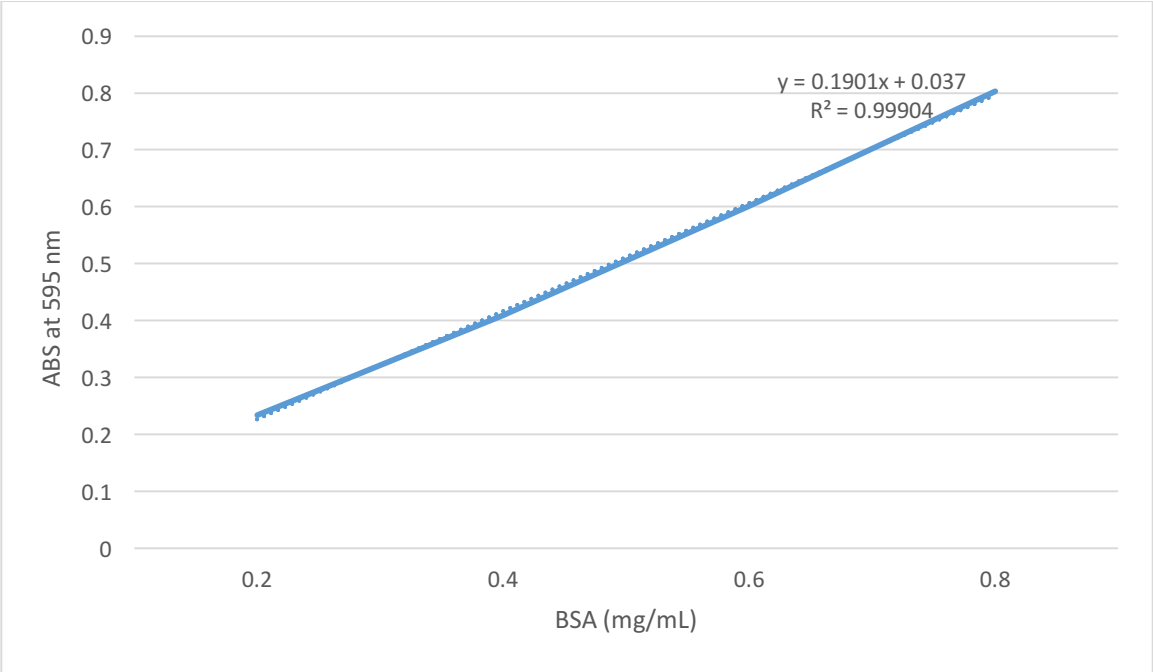
APPENDIX A

GRAPHICAL ABSTRACT OF SOY PROTEIN NANO-AGGREGATES AND SOY PROTEIN STABILIZED NANOEMULSION



APPENDIX B

PROTEIN ANALYSIS OF SUPERNATANT (ABSORBANCE VS. BSA CURVE)



APPENDIX C

PREPARATION OF SIMULATED GASTRIC FLUID (SGF) AND SIMULATED INTESTINAL FLUID (SIF) FOR IN VITRO RELEASE PROFILE OF CANOLA OIL CONTAINING DHA

Simulated gastric fluid (SGF)

Simulated gastric fluid (SGF) was prepared by dissolving 2.0 g of NaCl and 7.0 ml of 36% HCl in 900 ml of water. After the addition of 3.2 g of pepsin, the solution pH was adjusted to 1.2 with 0.1 M HCl and the final volume was made up to 1000 ml with water.

Simulated intestinal fluid (SIF)

Simulated intestinal fluid (SIF) was prepared by dissolving 6.8 g of K_2HPO_4 in 800 ml of water. To this solution was added 77 ml of 0.2 M NaOH and 100.0 g of pancreatin and the solution was stirred overnight at 4 ^{0}C. Solution pH was adjusted to 6.8 with 1 M NaOH or 1 M HCl and the final volume was made up to 1000 ml with water.