ENHANCED PHYSICOCHEMICAL AND FUNCTIONAL PROPERTIES OF PEA (*PISUM SATIVUM*) PROTEIN BY PH-SHIFTING AND ULTRASONICATION COMBINED PROCESS

BY

SHANSHAN JIANG

THESIS

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Master's Committee:

Professor Hao Feng, Adviser
Professor Nicki Jene Engeseth
Research Professor Graciela Wild Padua

ABSTRACT

In recent years, pea protein as a novel food ingredient has drawn increasing attention due to its high nutritional value, hypoallergenic, and low price. As an amphiphilic molecule, protein is known as a natural and bio-safe emulsifier. However, similar to other legume proteins, the low water solubility and poor functional properties of pea protein limit its applications in the food industry. This study was undertaken to investigate the effects of pH-shifting in combination with ultrasonication on the structural and physicochemical properties of pea protein isolate (PPI).

PPI dispersions (30 mg/ml each) were treated with ultrasonication, pH-shifting, and pH-shifting in combination with ultrasound and compared to control (no treatment). Water solubility, particle size, solution turbidity, surface hydrophobicity, free sulfhydryl group content, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the soluble pea protein obtained by the above treatments were determined. The PPI samples (10 mg/ml) treated with pH-shifting at pH 12 in combination with ultrasound (pH12+U5), which had highest solubility, were used to prepare nanoemulsions (0.25% oil) and nanocomplexes loaded with vitamin D3 (VD3). Storage stability, photooxidation protective ability, and morphological structure of the PPI-stabilized nano-systems were examined.

The pH12+U5 treatment increased the solubility of PPI from 8.17% (Control) to 60.83%, and reduced the volume-weighted mean diameters D [4, 3] of the soluble protein aggregates from 206.9 (Control) to 45.2 nm. The surface hydrophobicity of the pH12+U5-treated PPI was significantly higher than that of the native protein, while its free sulfhydryl group content was slightly decreased. Structural rearrangement of the treated PPI was observed in the SDS-PAGE, showing that the alkaline pH-shifting and ultrasonic treatment can disrupt covalent and non-

covalent bonds. Even though there was no significant improvement in the antioxidant activity of the pH12+U5-processed protein compared to the native PPI, it exhibited good radical scavenging ability. After exposure to UV-light (312 nm, 15 W) for 180 minutes, the VD3 retained in the PPI-based nanoemulsion and nanocomplex was 74.22% and 65.37%, respectively, in contrast to 8.71% in the Control, demonstrating a good photooxidation protection ability of the nano-structures. Besides, the D [4, 3] of the droplets in the nanoemulsion and nanocomplex stabilized by the pH12+U5-treated PPI were 113.93 and 88.90 nm, respectively, and both nano-systems exhibited good stability during storage for 30 days.

In summary, the combination of pH-shifting and ultrasonication effectively improved the structural and physicochemical properties of pea protein isolate. The pea protein isolate processed with this new method would be a promising carrier to deliver and protect lipophilic bioactive components in food products, which could lead to foods with improved flavor, nutritional value, and shelf life.

With love to my family and friends

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CHAPTER 1

INTRODUCTION

Pea protein is a relatively new plant protein that has gained increasing interest in both academia and industry in recent years. Due to the excellent profile of essential amino acids, pea protein can be added to processed foods as an animal protein substitute, and is valuable for the development of new food products. Compared to soy protein, pea protein has no genetically modification issues and produces fewer allergic reactions in people. Besides, reports showed that pea protein is a better emulsifier with smaller emulsion droplet sizes compared with that of soy protein (O'Sullivan, Murray, Flynn, & Norton, 2015). Consequently, pea protein has the potential to replace the leading position of soy protein on the global market. However, the limited water solubility and relatively poor functional properties of pea protein hinder its applications in the food industry. A number of modification strategies have been investigated to improve the functional properties of plant protein, including physical (Chen, Yu, Wu, Liu, & Chai, 2012; Li, Zhu, Zhou, & Peng, 2011; Morales, Martínez, Pizones Ruiz-Henestrosa, & Pilosof, 2015), chemical (Franco, 2000; Jiang, Chen, & Xiong, 2009; Liang & Tang, 2013), and biological (Bae, Kim, & Lee, 2012; Ribotta, Colombo, & Rosell, 2012) methods. Since proteins with high molecular weights such as soy and pea proteins have compact structures stabilized by disulfide bonds, hydrophobic integration, and Van der Waals interaction, they are hard to have structural changes. Hence, effective modification methods, especially strategies utilizing the additive or even synergistic effect of multiple treatments are often used to modify those protein molecules. In this study, a combination of pH-shifting, a chemical treatment, with ultrasonication, a physical treatment was proposed and tested to modify the functional properties of pea protein and to make

pea protein mediated nano-structures for the purpose of carrying, protecting, and delivery of hydrophobic compounds.

pH adjustment is an easy but effective method to change protein properties, and the mechanism of such protein modification is well understood. On this basis, Jiang et al. (2009) introduced a pH-shifting method to effectively alter structural and emulsifying properties of soy protein, a treatment during which a protein was first exposed to an acidic and alkaline pH condition followed by neutralization to pH 7. High intensity ultrasound or power ultrasound is an emerging non-thermal technology, which has found application or shown promise in a number of food processing unit operations, such as extraction, homogenization, cutting, microbial and enzyme inactivation, and enhancement of heat and mass transfer (Kentish & Feng, 2014). It was reported that power ultrasound induced conformational rearrangement and improved physical properties of soy protein (Hu et al., 2013). Up to now, only a few studies have been conducted to investigate the characteristics of pea protein, and its functional properties have not been well understood. Moreover, to the best of my knowledge, no study has been reported using the combination of pH-shifting and ultrasonication to enhance pea protein functional properties.

The objective of this study was to explore the impact of pH-shifting and ultrasonication combined treatment on the physicochemical and functional properties of pea protein isolate (PPI). The nano-sized soluble PPI aggregates produced by this method were used to produce nano-structures to carry and protect vitamin D, a photosensitive compound. To optimize the modification methods, a series of pH-shifting processes in combination with/without sonication were applied on pea protein samples. Specifically, water solubility, particle size, turbidity, surface hydrophobicity, free sulfhydryl content, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the soluble PPI obtained by the treatments were determined. In

addition, antioxidant activity, droplet size, storage stability, bioaccessibility, and transmission electron microscopy (TEM) image of nanoemulsion and nanocomplex stabilized by soluble PPI obtained by a pH12-shifting plus ultrasound treatment were investigated. The photostability of vitamin D encapsulated in the nanoemulsion or associated with the nanocomplex was tested by a UV irradiation test.

CHAPTER 2

LITERATURE REVIEW

2.1 FIELD PEAS

Pea is commonly referred to the seed or the pod of *Pisum sativum*, which is known as garden or field pea. Sometimes the word "pea" also describes other plant seeds like chickpea, pigeon pea, and cowpea. Peas are usually the green or yellow cotyledon varieties in the Fabaceae family, which is an annual plant having one year life cycle (Dahl, Foster, & Tyler, 2012). Field pea, as a cool season legume plant, has been cultivated for at least 7,000 years. It has been appeared in human diet as an important component for a very long time. Field peas are not only high in starch and protein, but also have a significant amount of dietary fiber, vitamins and minerals.

2.1.1 World production of peas

As a cool season legume crop, pea is grown on over 25 million hectares annually worldwide (Rubio et al., 2014). These lands are mostly in Canada, United States, Russia, Europe, Australia, and some locations in Asia, i.e., India and China. According to the data of 2009, the total world production of peas was more than ten million metric tonnes (Dahl et al., 2012). Among these, Canada, the leading country in pea production, carries 28% of the total yield, followed by France and Russia holding 14% and 10%, respectively (Roy, Boye, & Simpson, 2010).

2.1.2 Pea protein

2.1.2.1 Introduction of pea protein

Pea protein is a plant protein, which is commonly obtained from field peas (*Pisum sativum*) by wet extraction. It is known that peas are a good source of food protein, and the protein content in peas commonly depends on the plant varieties and growing environment. Similar to other legumes, the protein content in peas is typically 18-30% (Shand, Ya, Pietrasik, & Wanasundara, 2007).

Based on the solubility properties of protein, pulse proteins can usually be classified into albumins, globulins and prolamins (Rubio et al., 2014). Albumins and globulins are the major proteins in peas. The content of albumin and globulin proteins and their composition of amino acids vary for different pea varieties and environmental factors. Albumins are the protein fraction that is able to be solubilized in water. It comprises 15-25% of the total protein, and has molecular masses (MM) ranging from 5,000 to 80,000 Da (Boye, Zare, & Pletch, 2010). Globulins are salt soluble and contain 50-60% of the total protein. It mainly composed of legumin (11S) and vicilin (7S) (Shand et al., 2007). Several studies were conducted on the ratios of legumin/vicilin, and the results are varied with different cultivars (Boye et al., 2010). It is generally known that legumin has higher content of sulphur-containing amino acids such as cysteine and methionine than vicilin. However, comparted to other legumes, pea protein is low in sulphur-containing amino acids.

2.1.2.2 Health benefits of pea protein

Pea protein provides a balanced amino acid profile. As a complete protein which is really rare for a non-animal protein, it contains all the essential amino acid for the daily needs of humans. Since its high quality, pea protein is 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 mainly determined by the amino acid composition. Pea protein has a high content of lysine, as well as leucine, isoleucine and valine, which are known as branched-chain amino acids. It was reported that proteins with high content of branched-chain amino acids have health benefits (Oomah, 2001). These amino acids are helpful to lowering cholesterol, and play important roles in the formation of collagen, bones, skin and tendons. They also support the development and maintenance of a healthy immune system. Pea protein also has an effect on weight maintenance and regulating blood sugar. It has been demonstrated that consuming significant peas in the diet can effectively reduce the incidence of colon cancer, type-2 diabetes, LDL-cholesterol and heart disease (Roy et al., 2010). It has also been reported that the digestibility of pea protein is higher than that of soybean and other pulses (Dahl et al., 2012).

2.1.2.3 Food applications of plant protein

Proteins are one of the essential nutrients that provide amino acids, and they also work as functional ingredients in food products. They are widely used in food products to improve the nutritional properties as well as food texture (Taherian et al., 2011). With consumers' growing demand for nutritional and health food products, vegetable proteins especially legume proteins have gained increased interest in the food industry. Proteins as a natural emulsifier reduce the interfacial tension between oil/water and air/water phase (Taherian et al., 2011), working as an important ingredient in food systems like bread, ice cream, dressings, and milk-like beverages.

As an amphiphilic molecule, pea protein has good emulsifying, foaming, film-forming and gelling properties, which are important functional qualities in food applications. For instance, good water and fat holding capacity makes pulse protein a good ingredient in meat

products. Several studies have worked on incorporating pulse proteins in the formulation of meat products. Addition of pulse flour increased protein content and, at the same time, 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). Another example is adding protein to gluten-free products that are nutritionally unbalanced. Fortified gluten-free food with pulse proteins enhances nutritional value while makes no negative impact to product flavor.

Pea protein is sustainable and environment friendly. Comparted to raising livestock, people can produce about five times more protein if they grow vegetable or grains on the same area of farmland. Therefore, with the increasing demand of dietary protein due to the growth of world population, plant proteins especially legume proteins become much more important. In addition, for economic reasons, it is less expensive to use pea protein in the place of milk protein and soy protein (Kent & Doherty, 2014). As a GMO-free and allergen-free protein, pea protein can be an excellent alternative to dairy products.

2.2 ULTRASOUND

Ultrasound is a kind of mechanical wave having a frequency above the threshold of human hearing (> 20 kHz). It can be divided into two categories based on its frequency and acoustic energy level, i.e. low frequency (20-100 kHz) ultrasound and high frequency (100 kHz-1 MHz) ultrasound (Kentish & Feng, 2014). High frequency (low intensity) ultrasound is usually applied to determine the chemical and physical properties of foods, while low frequency (high intensity) ultrasound or power ultrasound is useful for altering physicochemical properties of food materials, such as solubility, emulsifying properties, and microstructure (Soria & Villamiel, 2010).

2.2.1 Mechanism of ultrasound treatment

The mode of action for an ultrasound treatment is attributed to the acoustic cavitation phenomenon. Acoustic cavitation refers to the formation, growth, and implosion of cavitational bubbles produced when the negative pressure in the rarefaction region of the sound wave (**Figure 2.1**) is greater than the tensile stress of the liquid (Feng & Yang, 2011). Due to violently collapse of the cavitational bubbles, extreme localized physical and chemical activities will be produced by inertial and stable cavitation bubbles. The high shear forces, shock waves, and water jets produced by cavitation bubbles provide physical forces to alter the microstructures or even destruct molecular structures when the acoustic power density (ADP) is high and treatment time is long. The implosion of cavitational bubbles can also produce free radicals in the liquid, which on some occasions can help to enhance a sonochemical reaction. Since acoustic cavitation is produced when an ultrasound wave travels through a liquid medium, such a treatment is often termed as sonication or ultrasonication.

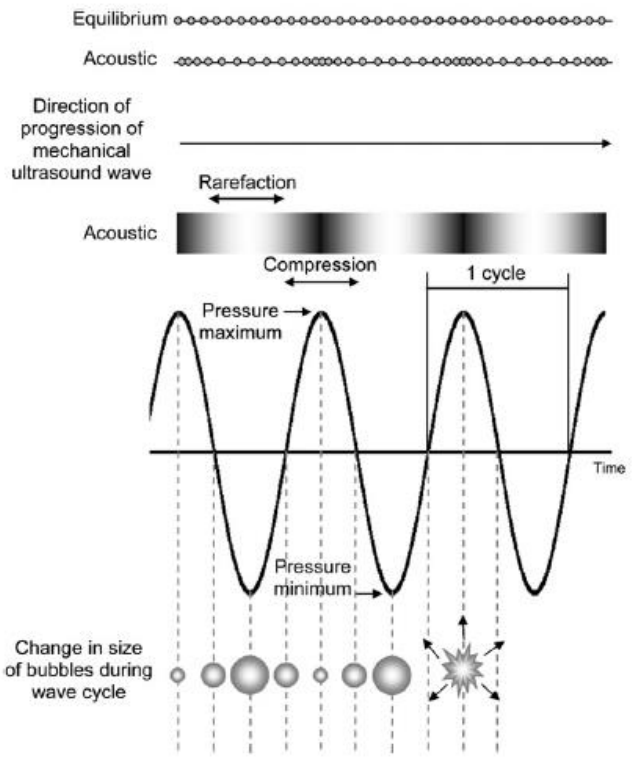


Figure 2.1 Cavitation phenomenon of ultrasonication (Soria & Villamiel, 2010).

2.2.2 Power ultrasound in food applications

Ultrasound as a nonthermal technology has shown promise in a number of food processing unit operations. Compared to traditional thermal processing methods and some nonthermal processing techniques, ultrasound processes are normally simple, effective, energy saving, and low cost. It also has a green image due to the wide adoption of ultrasound in medical diagnostic applications. Power ultrasound is effective on processes such as cutting, extraction, degassing, homogenization, emulsification, microbial and enzyme inactivation, and heat and mass transfer enhancement (Chemat, Zill-e-Huma, & Khan, 2011).

The most successful application of ultrasound that has been used in the food industry is cutting. With advantages such as the ability to produce a visually excellent cut surface, reduced smearing, low product lost, and less deformation, with less tendency to shatter brittle products

and the ability to handle sticky or brittle foods, ultrasonic cutting is becoming increasingly important in the food industry. For instance, Red delicious and golden delicious apples cut with an ultrasonic knife showed a smooth surface appearance, while the surfaces were rough for samples cut without ultrasound. Apples cut with ultrasound had lower PPO activity than the Control (Yildiz & Feng, 2013). Cheddar, mozzarella, and Swiss cheeses cut with ultrasound showed a shiny and smooth surface appearance and lower peroxide values compared to the Control indicating less lipid degradation and hence a better quality (Yildiz, Rababah, & Feng, 2012). Patist and Bates (2011) listed 5 other applications of ultrasound in industrial settings, including defoaming, emulsification, extrusion, extraction, and waste treatment with a payback time of 6 weeks to one year and a benefit of up to $2,000 k$/year.

Power ultrasound was reported to affect the physical and chemical properties of a treated material; hence several studies were conducted to examine the effect of power ultrasound treatment on the functional properties of proteins, mainly in laboratory settings. Power ultrasound processed whey protein showed improved solubility and foaming ability (Jambrak, Mason, Lelas, Herceg, & Herceg, 2008). Hu et al. (2013) applied low-frequency (high-intensity) ultrasonication to soy protein isolate, and concluded that ultrasound treated protein showed better solubility, surface hydrophobicity, and fluid properties compared to the non-treated samples.

2.3 PH-SHIFTING

The term “pH-shifting” was first introduced by Choi and Kim (2005) for increasing the recovery of fish protein from frozen and pelagic fishes. They applied extremely low or high pH to fish muscle protein to increase its solubility in water, then precipitated the soluble protein by adjusting the pH to the isoelectric point. Finally, the recovered protein was adjusted to neutral pH. The work showed that the pH shifting treated fish protein has excellent gel-forming ability.

Jiang et al. (2009) first applied this pH-shifting process to plant proteins (soy protein isolate) and reported good results. This processing can be simply described as adjusting the pH to extremely acid or alkaline for length of time, followed by shifting the pH back to normal.

2.3.1 Mechanism of pH-shifting process

pH-shifting is a chemical method that modifies proteins by controlling acidity and alkalinity. Protein has isoelectric point where it has zero net charge and lowest water solubility and when the pH shifts away from the isoelectric point, protein solubility increases. The solubility of proteins in an aqueous phase is determined by the electrostatic and hydrophobic interactions between protein-protein and protein-water molecules. These interactions mainly depend on the distribution of hydrophilic and hydrophobic amino acid residues of the protein. When more hydrophilic amino acids exist on the protein surface, the electrostatic repulsion is greater than hydrophobic interactions, and the protein is more soluble.

Jiang, Chen, and Xiong (2009) postulated that a pH-shifting process induced partially unfolding of the protein structure due to increased charge repulsion. Because of the shifting of pH in the medium, the protein may carry positive or negative charges which increase electrostatic repulsion and cause the loss of protein side-chain interactions, and therefor affect the solubility and emulsifying ability of proteins. This partially unfolded state of globular proteins has been referred as “molten globule” structure since the protein still maintains a relatively intact structure (Goto, 1989).

2.3.2 Applications of pH-shifting in science and technology

The pH-shifting process was originally aimed at acid or alkaline solubilization applied to improve the recovery of proteins from foods especially animal based materials. In recent years, pH-shifting was tested for using as a simple and cost-effective method to extract proteins (Fu,

Wu, & Li, 2012; Özyurt, Simsek, Karakaya, Aksun, & Yesilsu, 2015), as well as to enhance protein functional properties (Jiang, Xiong, Newman, & Rentfrow, 2012). Alkaline pH shifting (pH 12.0) was reported to significantly increase the solubility of soy protein isolate (Jiang, Xiong, & Chen, 2010).

2.4 EMULSION

An emulsion is a colloidal dispersion consists of oil, emulsifier which can also be called surfactant, and a water phase. Depending on the distribution of oil and water phase, emulsions can be classified into oil-in-water (O/W) emulsion, where oil is the disperse phase and water is the continuous phase; and water-in-oil (W/O) emulsion as verse. Moreover, it is also possible to have oil-in-water-in-oil (O/W/O) and water-in-oil-in-water (W/O/W) emulsions. Emulsion systems are commonly used in the areas of food, medicine, and cosmetics.

2.4.1 Emulsion systems in the food industry

Many food products are recognized as emulsions in our daily life, such as milk and beverages, ice cream, mayonnaise, salad dressings, butter and sausages. Milk is a natural emulsion composed by milk fat dispersed in an aqueous phase. Milk protein functions as an emulsifier that stabilizes the whole system. In contrast, butter is a water-in-oil emulsion generally produced from milk. In recent decades, emulsion-based delivery systems are getting more attention, which can be used to protect and deliver flavors, lipids, and nutraceuticals. In the production of chewing gums, encapsulation technology has been applied to slowly release the flavors during chewing. It is also commonly to utilize emulsion systems in milk beverage to fortify oil-soluble vitamins like Vitamin A and D, as well as omega-3 fatty acids, DHA and EPA.

2.4.2 Methods to create emulsions

An emulsion is a mixture of two or more immiscible phases that is unstable due to flocculation, coalescence, and gravitational separation. The formation of an emulsion system is an energy input process. Based on the energy level, this can be classified as high-energy or low-energy emulsification (McClements & Rao, 2011). High-energy approaches such as high pressure homogenizing, microfluidizer method, and ultrasound, are commonly used in the food industry. These strategies apply extremely intense disruptive forces to the mixture to generate tiny droplets for emulsion formation. Currently, a number of low-energy methods have been developed to produce emulsions as well, including spontaneous emulsification, membrane emulsification, and emulsion phase inversion (EPI) methods.

2.4.3 Nanoemulsion

A nanoemulsion refers to an emulsion that usually has a droplet size between 10 to 100 nanometers. As one application of nanotechnology, nanoemulsions have attracted a lot of attention in recent years. Compared to conventional emulsions, nanoemulsions are usually more stable to gravitational separation and molecular aggregation. Since they have smaller particle size, their products are normally optically clear or only slightly turbid. Besides, nano-sized food materials may have improved its bioavailability through gastrointestinal tract (McClements & Rao, 2011).

CHAPTER 3

MODIFYING THE PHYSICOCHEMICAL PROPERTIES OF PEA PROTEIN BY PH-SHIFTING AND ULTRASONICATION

3.1 INTRODUCTION

Pea is a traditional legume crop that has been used in human diets for thousands of years. The majority of pea production, especially field peas, is coming from Canada. Since the high quality of field peas, the United States had raised the growth area from 149,000 acres in 1993 to about 924,174 acres in 2006, and reached approximately 517,962 metric tons of pea production in 2004 (USA dry pea & lentil council). Pea protein, which comprises approximately 20-27% of the dry weight of pea seeds (Sun & Arntfield, 2012), has attracted much interest in the food industry recently. It is now referred as an alternative of soy protein in food formulas on account of its high nutritional value and comparable functional properties. Importantly, pea protein is inexpensive, hypoallergenic, and with no issues of genetic modification. It is one of the few vegetable proteins that are regarded as complete proteins. Pea protein has a low content of sulfur-containing amino acids but is rich in lysine. Recently, Dahl et al. (2012) reviewed the nutrition and health benefits of dry field peas. They pointed out that hydrolyzed pea protein provides bioactivities and antioxidant activity. Compared to soybean or other pulse proteins, pea protein has high *in vitro* digestibility, producing enhanced intestinal health. Dominika et al. (2011) evaluated the effect of glycosylated pea protein on intestinal microbial activity, and found improved bacteria homeostasis.

The major proteins in pea seeds are storage proteins, including legumin (11S) and vicilin (7S). Legumin (11S) has a hexameric quaternary structure composed by six subunits through

disulfide bonds. Vicilin (7S) comprises approximately 35% of the total protein in peas, and is a trimer composed of three subunits (Liang & Tang, 2013). Koyoro and Powers (1987) found that 11S has better emulsifying ability than 7S, while 7S showed better emulsifying stability due to its low molecular weight. Although pea protein has a better emulsifying capability at neutral pH compared to soy protein (Aluko, Mofolasayo, & Watts, 2009), its applications in the food industry are still limited by the weak functionality (Liang & Tang, 2013). In addition, a lack of knowledge on pea protein properties also imposes restriction on its application as a food ingredient.

In order to overcome the limitations of vegetable proteins, a number of strategies using physical, chemical and biological (Bae et al., 2012; Ribotta et al., 2012) methods to modify protein functional properties have been proposed. Among chemical treatments, pH-induced modification is a traditional and simple method widely used to alter the physicochemical and functional properties of proteins. The effect of pH on proteins has been well understood, and the method has already been applied to modify both animal and vegetable proteins. Jiang et al. (2010) proposed and tested a pH-shifting method to treat soy protein isolate and its globulin fractions (7S and 11S) to enhance the solubility characteristics. Although the pH-shifting treatment can effectively enhance soy protein properties, its solubility is still low between pH 4 to neutral. In addition, when ionic strength is high, pH-shifting treatment has little effect on protein solubility improvement. For this reason, the application of soy protein in low-acid or salty liquid foods is still limited.

Enzymatic modification is a biological method that utilizes enzymes to hydrolyze or crosslink proteins, resulting in changes in their structures (Ribotta et al., 2012). Bae et al. (2012) reported a method using protease to enhance the solubility of soy protein isolate. They found a

dramatic increase in the solubility of soy protein in acidic pH conditions, showing promise for the use of soy protein in acid foods. However, enzymatic reactions require good control of reaction conditions, which sets high requirement for equipment. Usually the price of enzymes is high, another factor making the enzymatic method less attractive to food companies.

Power ultrasound or high intensity ultrasound is an emerging physical method for modifying the structure of proteins. Ultrasound-induced protein modification is often attributed to acoustic cavitation. The high shear and normal forces by micro- and macro-streaming, shock waves, and water jets, help to reduce the size of protein aggregates and alter the molecular structure of protein (Baumann, 2005). Ultrasound has been tested for modifying the functional properties of soy proteins (Chen et al., 2012; Morales et al., 2015). Compared to the chemical and biological methods, ultrasonication as a physical strategy is more acceptable by consumers. In addition, this method is less time and energy consuming. To date, no study has been reported using the combination of ultrasonication and pH-shifting to improve the physicochemical and functional properties of pea proteins. In this study, the effects of ultrasonication in combination with pH-shifting under different pH values on the physico-chemical properties of pea protein isolate (PPI) were examined. Protein water solubility, particle sizes, turbidity, surface hydrophobicity, free sulfhydryl group content, and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of the treated PPI samples were investigated.

3.2 MATERIALS AND METHODS

3.2.1 Materials

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

chemicals were purchased from Bio-Rad (Hercules, CA, USA), Sigma-Aldrich (St. Louis, MO, USA), and Fisher Scientific (Pittsburgh, PA, USA) and were of analytical or higher grade.

3.2.2 pH-shifting and/or ultrasonication processes

pH-shifting treatment was applied to PPI solution as described by Jiang et al. (2014) with some modification. Ultrasound treatment was applied using a VC 750 ultrasonic processor at 20 kHz (Sonics & Materials, Inc., Newtown, CT, USA). An ultrasonic probe (13 mm diameter) was used to deliver acoustic energy into the sample, and the acoustic power density (APD) was controlled at 68.02W/100ml. Heat produced by ultrasonication will increase the temperature which may cause protein denaturation (Kent & Doherty, 2014). In order to avoid overheating, an ice bath was used to cool the samples during ultrasonication.

PPI dispersion (30 mg/ml, pH 7.0) was stirred at room temperature for 30 min, and then adjusted to pH 2, 4, 10, or 12 with 2M NaOH or 2M HCl at room temperature. Immediately, 5 min ultrasonication was applied to the protein dispersion in a beaker (250 ml) that was placed in an ice bath to avoid overheating. Treated protein solution was held at room temperature for 1 hr before adjusting pH back to 7 using 2M NaOH or 2M HCl. Supernatant was collected after centrifuged (Sorvall Instruments RC5C, Rotor GSA code 10, Newton, CT) at 8,610 RPM, under 15°C for 15 min, and stored in a refrigerator (Roper Refrigerator, Whirlpool Corporation) at 4°C before use. Sample treated with 5 min ultrasonication in combination with pH-shifting was labeled as pH2+U5, pH4+U5, pH10+U5, or pH12+U5. The one treated with only pH-shifting or ultrasonic was denoted as pH2, pH4, pH10, pH12, and U5, respectively. The sample with no treatment but only stirred 30 min under room temperature was used as the Control. The flow chart of sample preparation was shown in **Figure 3.1**.

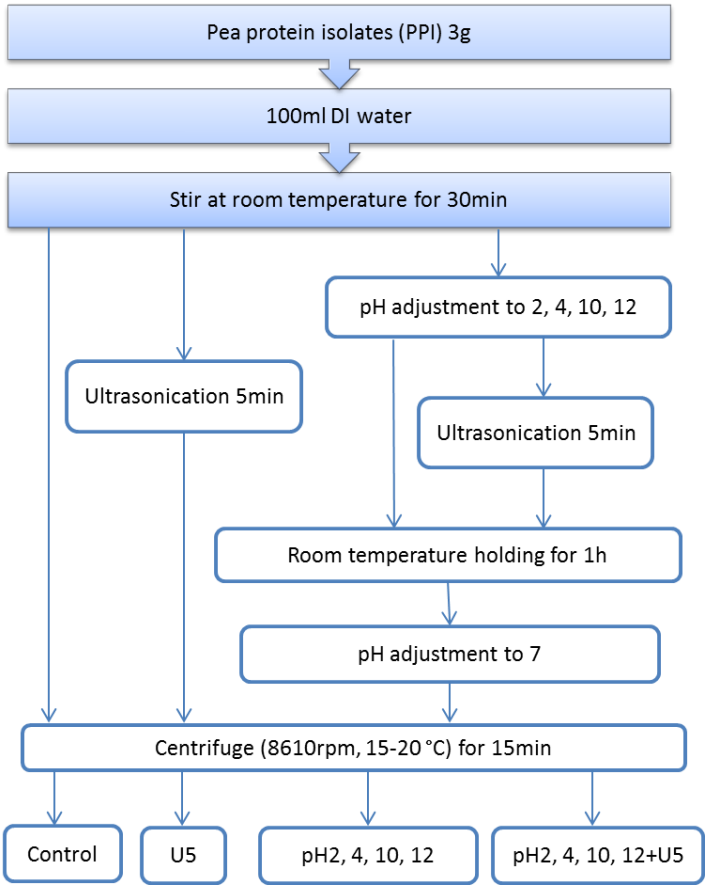


Figure 3.1 Preparation of pea protein isolate samples and treatment conditions.

3.2.3 Soluble protein content and protein solubility

Soluble protein content was determined with a Bio-Rad Protein Assay based on the method described as 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 (Grade 1 Qualitative Filter Paper, circle, 55 mm). Pea protein solution was diluted to an appropriate concentration so the absorbance would fall into the range of standard, followed by adding diluted dye reagent. Soluble protein concentration in PPI solution was measured using a spectrophotometer (Lambda 1050 UV/VIS/NIR Spectrometer, PerkinElmer) 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.

$$\text{Protein solubility (\%)} = \frac{\text{Protein concentration in soluble PPI}}{\text{Initial protein concentration}} \times 100\%$$

3.2.4 Particle size

The volume-weighted mean diameters (D4, 3) of soluble proteins were detected by dynamic light scattering (DLS) using a NICOMP 380 DLS instrument (Santa Barbara, CA, USA). DLS is a technique used to analyze particle size through measuring Brownian motion. There is a relationship between Brownian motion and particle size. The faster the movement is, the smaller the molecular size. Samples were diluted 500-fold with DI water before measurement. The measurements were conducted at 23°C, and the liquid viscosity and index of refraction was set according to water, which was 0.933 and 1.333, respectively.

3.2.5 Turbidity

The turbidity of soluble protein samples 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.

3.2.6 Surface hydrophobicity

Surface hydrophobicity (Ho) was determined according to Kato and Nakai (1980), and Haskard and Li-Chan (1998) with slightly modification. 1-anilino-8-naphthalenesulfonate (ANS) (Sigma-Aldrich, St. Louis, MO, USA) was used as the fluorescence probe. The ANS stock solution (8 mM) was prepared in phosphate buffer (0.01 M, pH7). Five protein concentrations, from 0.04 to 0.2 mg/ml, were also prepared with the same phosphate buffer (0.01 M, pH7). Twenty µl ANS stock solution was added to 4 ml of protein sample solutions, and fluorescence intensity was measured (Synergy^{TM} 2, BioTek Instrument Inc., Winooski, VT, USA) at 340 nm

(excitation) and 440 nm (emission). The initial slope of fluorescence intensity versus protein concentration calculated by linear regression analysis was an indicator of the surface hydrophobicity of proteins.

3.2.7 Free sulfhydryl group (SH) determination

The free sulfhydryl group content of protein samples was analyzed according to the method of Beveridge, Toma, and Nakai (1974) with some modification. 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB), also known as Ellman's reagent (Sigma D8130), was utilized to determine the content of free sulfhydryl group in the samples. Sodium phosphate buffer (0.1 M, pH 8.0) was used to dilute the protein solution to a certain concentration. L-Cysteine hydrochloride was used as a standard. Serial dilutions of cysteine (0.25 to 1.5 mM) in the same sodium phosphate buffer (0.1 M, pH 8.0) were prepared to plot a standard curve. 50 µl of Ellman's reagent solution was added in the mixture of 250 µl of protein sample and 2.5 ml of sodium phosphate buffer. After incubation at room temperature for 15 min, the absorbance was measured at 412 nm using a spectrophotometer (Lambda 1050 UV/VIS/NIR Spectrometer, PerkinElmer, Waltham, MA, USA). The free sulfhydryl group content was expressed as µmol/g protein.

3.2.8 Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted as described by Jiang et al. (2009) with modification. Commercial precast gel, Mini-PROTEAN®TGX™ (12% acrylamide, Bio-Rad 456-1043, Hercules, CA, USA) was used as the resolving gel. Protein samples were mixed with reducing sample buffer (65.8 mM Tris-HCl, 2.1% SDS, 26.3% (w/v) glycerol, 5% 2-mercaptoethanol (BME), 0.01% bromophenol, pH 6.8) or non-reducing sample buffer (without BME) at a ratio of 1:1 (v/v). To prevent the formation of

disulfide artifacts, 1 mM N-ethylmaleimide was added to the non-reducing sample buffer. The mixed samples were denatured at 95°C for 5 min and cooled immediately on ice. Prepared samples were loaded on the gel at 0.1 mg protein in each sample well. Electrophoresis was conducted at 200 V for 40 min. The SDS-PAGE gel was fixed (40% methanol, 10% acetic acid, 20 min) and stained with Coomassie Brilliant Blue (Bio-Safe^{TM} Coomassie G-250 Stain, Bio-Rad, Hercules, CA, USA) overnight and destained in 10% acetic acid for around 20 min. Images of the gels were captured utilizing a Carestream Gel Logic 4000 PRO Imaging system (Caresream Molecular Imaging, Woodbridge, CT, USA).

SDS-PAGE samples were prepared with and without 5% β-mercaptoethanol. For samples without β-mercaptoethanol, 1 mM NEM was added to prevent possible formation of disulfide cross-linkage during sample preparation.

3.2.9 Statistical analysis

All experiments were conducted at least three independent trials. Results were reported as the mean and standard deviation based on independent experiments. The differences were analyzed using ANOVA with SAS program. Significant differences ($P < 0.05$) between means were identified by Tukey HSD all-pairwise multiple comparisons.

3.3 RESULTS AND DISCUSSION

3.3.1 Protein solubility

Protein solubility was regarded as the most practical index of protein functional properties. Enhanced protein properties could be obtained from an increase in protein solubility (Arzeni et al., 2012; Chen et al., 2011; Hu et al., 2013; Tang, Wang, Yang, & Li, 2009). For instance, good solubility is a precondition for the use of a protein as an emulsifier (Damodaran,

1996). It is a key factor when considering the application of proteins to the food industry, especially the beverage industry.

The soluble protein content and water solubility of PPI treaded by sonication, pH-shifting, and pH-shifting + ultrasonication are shown in **Table 3.1**. For the commercial PPI (Control), the soluble protein content was 2.07 mg/ml, with a solubility of 8.17%. This solubility was low compared to that reported in the literature, around 20-40% for commercial PPI (Adebiyi & Aluko, 2011; Barac et al., 2010). The difference may be caused by the preparation method of the PPI used in different research groups. It was reported that salt-extracted protein has a better solubility than that produced by acid precipitation or alkali extraction (Liang & Tang, 2013).

In general, pH-shifting alone did not improve water solubility, except under extreme alkaline conditions (pH12) (**Table 3.1**). There is no significant difference between the control and other pH-shifting (pH2, 4, and 10) treated samples. The pH-shifting near the isoelectric point (pI = 4 ~ 5) even displayed slightly decline in solubility, and it made no difference when ultrasonication was added. This may be due to the impact structure of pea protein near isoelectric point. The solubility of the pH12 treated PPI dramatically increased to 54.94%. The pH12-shifting treatment was at a pH value far away from the isoelectric point of pea protein, and more extensive protein structural changes may occur as postulated by (Jiang, Xiong, & Chen, 2010). Few studies have used pH-shifting to treat pea proteins without ultrasound, and all reported notable increase in SPI solubility for pH12-shifting treated samples (Jiang et al., 2010). It was demonstrated that when exposed proteins to extremely acidic or alkaline pH conditions, increased ionic strength in the medium lead to a partial unfolding of proteins, which is also known as “molten globule (MG)” structure. Protein in this MG state may lose some side-chain interactions and become flexible. The increase of solubility may also be due to the increase of

ionic interactions of charged proteins and water (Jiang, Chen, & Xiong, 2009).

Ultrasonication has contributed to increased pea protein solubility. The PPI treated with ultrasonication alone for 5 minutes had a solubility of 55.80% while that of the untreated (Control) was 8.17%. For the ultrasonication + pH-shifting treatments, an increase in solubility over that of the pH-shifting alone counterparts can be observed, especially under alkaline conditions (pH10, pH12) (**Table 3.1**). Under extremely acidic conditions, the pH2+U5 treated sample doubled PPI solubility compared to the pH-shifting alone sample. Similarly, treatment near the isoelectric point did not yield any improvement in protein solubility. Hu examined the effects of ultrasound on soy protein isolate, and found that treated SPI had improved solubility in deionized water at pH 8.0 (Hu et al., 2013). Hu suggested that ultrasound treatment could disrupt some of the non-covalent interactions of protein, such as hydrogen bonds and hydrophobic interactions, which was similar to denaturation (Hu et al., 2013). The dissociation of native protein complexes into individual subunits was thought to be the driving force for the increased solubility (Jiang, Xiong, & Chen, 2011). The increase in water solubility may be owing to the conformational change and formation of soluble protein aggregates.

Among all, the pH12+U5 obtained the highest solubility (60.83%). After pH12-shifting process, pea protein was in the “molten globule” state, which is more flexible and partially unfolded. This may have allowed the physical forces, such as shear forces and shock waves produced by acoustic cavitation to further alter the structure of PPI resulting in an increase in protein solubility.

Table 3.1 The concentration and solubility of pea protein isolate.

	Concentration (mg/ml)		Solubility (%)	
	Without ultrasound	With ultrasound	Without ultrasound	With ultrasound
Control	2.07±0.11^{d}	14.16±0.56^{b}	8.17±0.43^{h}	55.80±2.22^{f}
pH2	2.44±0.09^{d}	4.64±0.94^{c}	9.63±0.36^{h}	18.27±3.72^{g}
pH4	1.70±0.12^{d}	1.91±0.10^{d}	6.69±0.49^{h}	7.53±0.41^{h}
pH10	2.49±0.05^{d}	14.54±0.16^{ab}	9.80±0.21^{h}	57.28±0.61^{ef}
pH12	13.94±0.43^{b}	15.44±0.19^{a}	54.94±1.69^{f}	60.83±0.75^{e}

^{abcd} Mean ±standard deviation (n=3) of concentration with the same letter are not significantly different ($p < 0.05$)

^{efgh} Mean ± standard deviation (n=3) of solubility with the same letter are not significantly different ($p < 0.05$)

3.3.2 Protein aggregate size and turbidity

The particle size measured by dynamic light scattering (DLS) is a hydrodynamic diameter. The obtained size is the diameter of a sphere having the same translational diffusion coefficient as the particle. The volume weighted mean diameters (nm) of the pea protein samples were shown in **Figure 3.2**. pH-shifting and ultrasonication reduced the sizes of pea protein aggregates. The pH-shifting treatment was effective reducing the PPI aggregate sizes with smaller particle size achieved at pH2. The particle size of ultrasound alone treated PPI was 75.3 nm, which was two times smaller than that of the Control. However, at pH2 and pH10, ultrasound made insignificant changes on protein size. Sonication induced particle size reduction was most significant in the pH12+U5 sample. The protein aggregate size decreased from 206.9 nm (control) to 45.2 nm after pH12+U5 treatment. Ultrasonic treatment was reported to decrease the size of both animal and vegetable proteins (O'Sullivan et al., 2015). It is believed that this

reduction in protein size is owing to the disruption of hydrogen bonding, as well as hydrophobic and electrostatic interactions which are used to maintain protein aggregates through ultrasonic cavitation phenomena and high hydrodynamic shear forces. O'Sullivan et al (2015) examined the stability of ultrasonic treated PPI and SPI by measuring the particle sizes immediately after process and after 7 days. They reported that after ultrasonic treatment, the average protein particle size of PPI dropped from 5,250 nm to 187 nm.

In general, protein aggregates with smaller sizes were more soluble. Smaller protein aggregates may contribute to increased water solubility due to a larger interaction area between protein and water molecules (Jambrak et al., 2008). The high molecular weight and complex structure of proteins made them transfer slowly to the oil-water interface in the aqueous phase. Reduced protein size increased the adsorption rate of protein to the oil-water interface, which may improve the emulsifying ability (O'Sullivan et al., 2015).

The turbidity values of the soluble protein solution of the control, ultrasound, pH-shifting, and pH-shifting + ultrasound treated PPI were shown in **Figure 3.3**. Extreme alkaline pH-shifting (pH12) dramatically raised the turbidity of soluble protein solution from 0.10 (control) to 1.67, which may be caused by the high protein concentration (54.9%) and large particle size (123.1 nm) of the PPI solution. Jiang et al (2010) examined the turbidity of native and pH-shifting treated soy protein isolate (SPI) under different ionic concentrations at different temperatures. They reported that pH-shifting processed SPI had lower turbidity than native SPI (Jiang, Xiong, & Chen, 2010), which was in contrast with our results. The reason for the discrepancy may lie in the fact that Jiang et al. (2010) adjusted the protein content of each sample to a same value (2 mg/ml) before measuring the turbidity, while no adjustment of protein concentration was used in this study for turbidity measurement.

The pH-shifting + ultrasound samples had a higher turbidity compared to those treated by pH-shifting alone, except for the pH-shifting alone treatment at pH4 and pH12, which may be due to the higher soluble protein content in the pH-shifting + ultrasound samples. It was noted that the pH12+U5 treated PPI had a much smaller turbidity (0.13) compared to that (1.67) of the pH12-shifiting alone sample and the former was more transparent than the later. This may be caused by the difference in protein aggregate sizes. The particle size of the pH12+U5 sample was almost three times smaller than that of the pH12-shifting alone. Consequently, the pH12+U5 treated PPI formed smaller protein aggregates in the aqueous phase and displayed a clear protein solution.

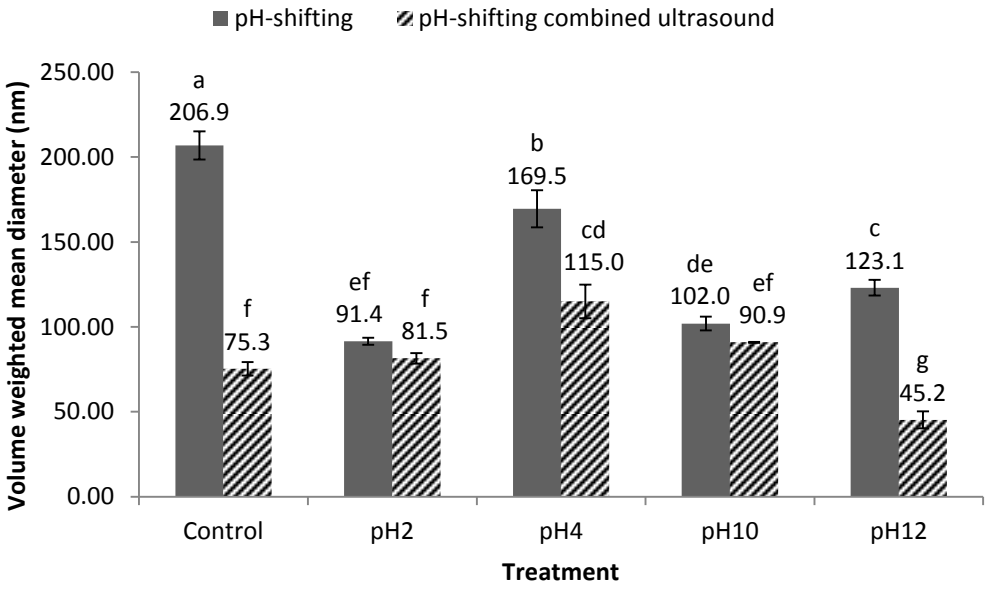


Figure 3.2 The volume weighted mean diameter (nm) of soluble pea protein isolate samples.

[abcdefg] Mean ± standard deviation (n=3) of protein aggregates size with the same letter are not significantly different ($p < 0.05$)

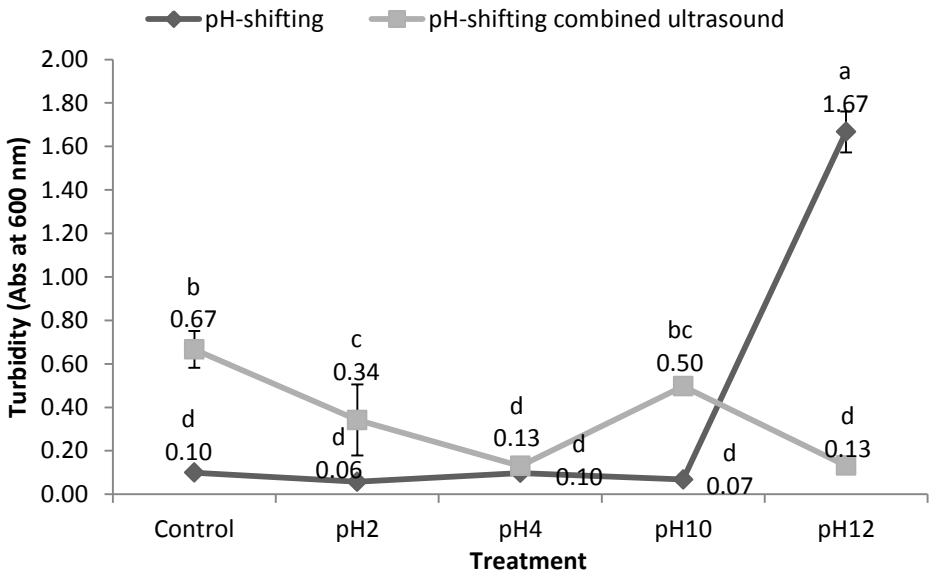


Figure 3.3 Turbidity of soluble pea protein solutions.

[abcd] Mean ± standard deviation (n=3) of turbidity with the same letter are not significantly different ($p < 0.05$)

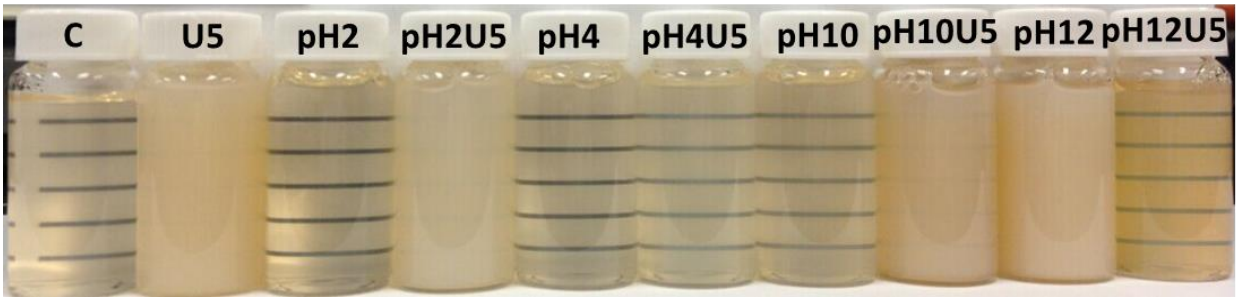


Figure 3.4 Soluble pea protein isolate (PPI) samples treated by different methods.

3.3.3 Surface hydrophobicity

Surface hydrophobicity is an important parameter describing protein functional properties. It was reported to correlate well with protein emulsifying, foaming, and gelation capacities (Nakai, 1983). An increase in protein surface hydrophobicity improved protein emulsifying, and foaming capacities and stabilities (Nakai, 1983). The content of exposed hydrophobic amino acids residues of proteins was used to indicate the protein hydrophobicity. In

this study, 8-Anilinonaphthalene-1-sulfonic acid (ANS) was used as a fluorescent molecular probe. The fluorescent properties of ANS will change as it binds to hydrophobic regions on the protein surface, as ANS fluorescence is intensified in a more hydrophobic environment. Ultrasonication significantly increased protein surface hydrophobicity, while pH-shifting alone treatments did not significantly change the hydrophobicity (**Figure 3.5**). Compared to the Control, the ultrasonic processed pea protein increased its surface hydrophobicity from 25.8 to 55.8. Among all pH-shifting alone treatments, only the extremely alkaline pH-shifting treatment (pH12) increased the protein hydrophobicity to 35.8. Jiang et al. (2014) also reported the enhanced surface hydrophobicity in pea protein treated under extreme alkaline pH conditions. The treatment at pH12 was farther away from the isoelectric region of pea proteins and therefore would cause stronger intramolecular electrostatic repulsions leading to more extensive unfolding and higher protein hydrophobicity (Jiang, Xiong, & Chen, 2011). In addition, the structural changes induced by extreme pH seemed to be difficult to completely reverse upon refolding treatment at pH7 (Jiang, Chen, & Xiong, 2009).

Compared to the pH-shifting alone, the pH-shifting + US treatments significantly enhanced the surface hydrophobicity of PPI, which was in agreement with previous studies where the use of ultrasound resulted in an increase in protein hydrophobicity (Hu et al., 2013). The dissociation of PPI complexes into individual subunits caused by sonication would lead to the exposure of hydrophobic groups occluded in the native agglomerates, thereby contributing to the surface hydrophobicity change (Jiang, Xiong, & Chen, 2011). Alkaline pH-shifting (pH10, and pH12) and extremely acidic pH-shifting (pH2) followed by ultrasonication achieved similar hydrophobicity, that is 57.5, 59.2, and 50.0, respectively. An increase in protein surface hydrophobicity is an indication of the exposure of hydrophobic portion of peptides and non-polar

amino acid side chain groups of amino acid residues (Jiang, Zhu, Liu, & Xiong, 2014). The exposure of hydrophobic side-chain groups which were originally occluded in the interior of the compact pea proteins was an indication of the change of tertiary structure (Jiang, Chen, & Xiong, 2009).

Surface hydrophobicity and solubility are main factors affecting emulsifying activity of a protein (Jiang, Xiong, & Chen, 2011). Good emulsifying and foaming ability depends on the balance between hydrophilic and hydrophobic groups (Nakai, 1983). The pH12+U5 treated PPI exhibited both high solubility and increased surface hydrophobicity, which may be an indication of decreased intermolecular interactions (Hu et al., 2013) and might show better emulsifying capacity and stability.

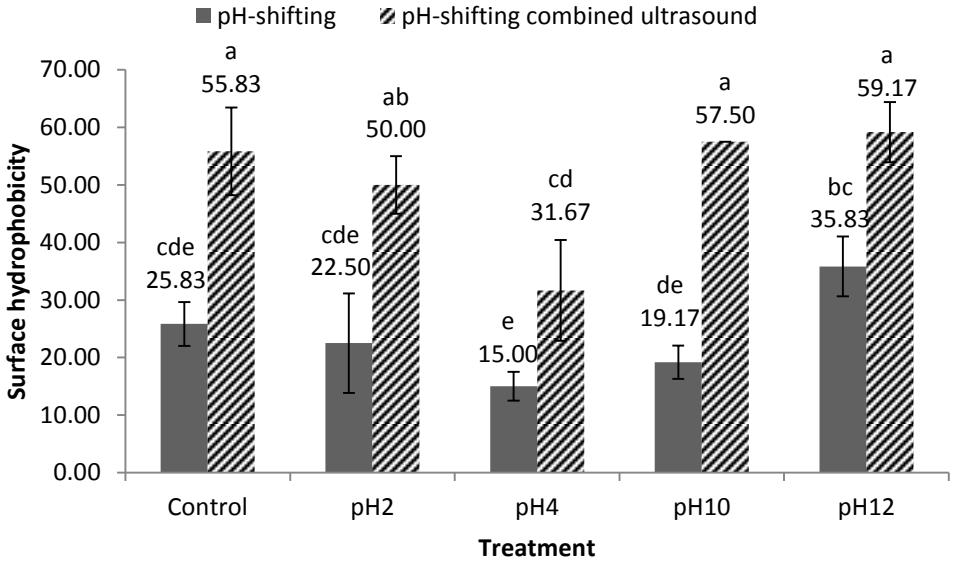


Figure 3.5 Surface hydrophobicity of soluble pea protein treated by pH-shifting alone or in combination with ultrasonication.

[abcde] Mean ± standard deviation (n=3) of surface hydrophobicity with the same letter are not significantly different ($p < 0.05$)

3.3.4 Free sulfhydryl group content

The free sulfhydryl group contents of treated pea protein samples were shown in **Figure 3.6**. Similar to what observed with PPI surface hydrophobicity, the pH-shifting + US treatments had a higher free sulfhydryl group content compared to the pH-shifting alone treatments, except from the pH-shifting at pH 12. The alkaline pH-shifting (pH12) induced changes in free sulfhydryl groups. The free SH content of the pH12 treated PPI increased from 12.6 (control) to 35.5, while there is no significant improvement in the samples of other pH-shifting treatments (pH2, 4, and 10), compared to the control. Higher free sulfhydryl group content indicated 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 appeared to be closely related to conformation changes and protein unfolding, indicating the exposure of SH groups or the breakdown of disulfide bonds.

It has been reported that the free sulfhydryl content of PPI from various cultivars was in the range of 3-70 μmol/g protein (O'Kane, 2005). Ultrasonic treatment increased free sulfhydryl group content of pea protein, except for the pH12-shifting sample. There is no significant difference between the ultrasound only and the pH-shifting + US treatments. The free sulfhydryl content of U5, pH2+U5, pH4+U5, and pH10+U5 processed PPI were 24.9, 25.2, 21.1, and 19.3, respectively. Similarly treatment of soy protein isolate with ultrasonication significantly increased the free sulfhydryl content of SPI (Hu et al., 2013). Legumin in peas contain more sulfur-containing amino acids such as cysteine and methionine, whereas vicilin were enriched in isoleucine, leucine, phenylalanine and lysine (Rubio et al., 2014). Thus the significant increase in free SH content in the treatments may mainly be related to changes in legumin.

Noticeably, the free sulfhydryl group content of PPI treated with the pH12+U5 was very

low. This might be caused when exposed free sulfhydryl groups were oxidized by the hydrogen peroxide generated by acoustic cavitation resulted in the reduction of free SH group content (Gülseren, Gülseren, Güzey, Bruce, & Weiss, 2007). The low SH content after the pH12+U5 treatment may also be attributed to the formation of disulfide bonds via SH/SS interchange reactions which were favored under alkaline pH conditions (Jiang, Xiong, & Chen, 2011). In addition, under alkaline pH conditions, thiol groups tend to be more reactive to form mercaptide ion species (S-) which accelerates SH oxidation (Jiang, Chen, & Xiong, 2009).

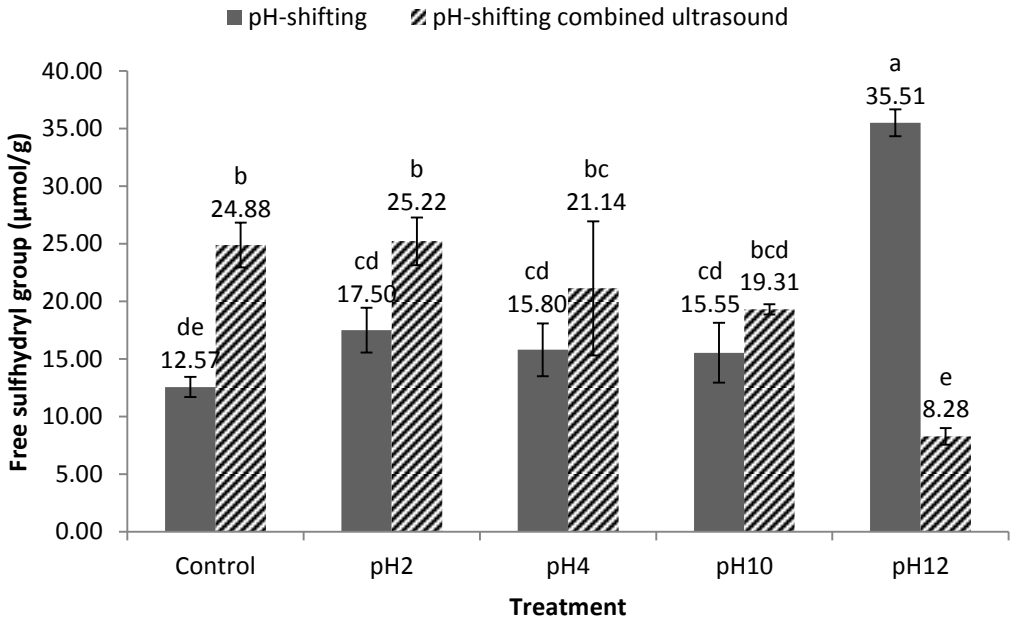


Figure 3.6 The free sulfhydryl group content (µmol/g) of soluble pea protein samples.

[abcde] Mean ± standard deviation (n=3) of sulfhydryl content with the same letter are not significantly different ($p < 0.05$)

3.3.5 SDS-PAGE

Changes of PPI subunits in samples treated by different methods can be visualized by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). β-mercaptoethanol is a

reducing agent used to develop a reducing condition, which can cleave disulfide bonds in proteins (Hu et al., 2013). The electrophoretic patterns of discrepant protein samples under reducing and non-reducing conditions were shown in **Figure 3.7**. A comparison of the gels under reducing and non-reducing conditions made it possible to determine the effect of disulfide bonds involved in the formation of protein aggregates and protein conformation changes.

Bands identification in the electrophoretic patterns was based on the previous studies (Jiang et al., 2014; Mession et al., 2015; Shand et al., 2007). The two major fractions in pea protein isolate are legumin (11S) and vicilin (7S). Pea legumin (11S) is a hexametric protein (330-410 kDa) (Mession et al., 2015). The subunit of legumin (~60 kDa) contains 2-7 cysteine residues (Sun & Arntfield, 2012), and is composited of acidic (38-40 kDa) and basic (19-22 kDa) polypeptides linked by disulfide bonds. Under reducing conditions, legumin subunits (Leg AB) will separate into legumin A (acidic) and legumin B (basic) due to S-S bonds cleavage. Both vicilin and convicilin are trimeric that have molecule weight of 150 kDa and 180-210 kDa, respectively. The molecular weight of vicilin subunit is 48-52 kDa, and it can be dissociated into fragments with low molecular weight (12-16, 20, 25-30, and 30-36 kDa) (Mession et al., 2015). Vicilin (7S) has no cysteine residues, and its subunits are not formed by disulfide-bonded linkage. Usually, convicilin subunit has molecular weight around 70 kDa, and will not be cleaved into small polypeptides as vicilin. The band above 100 kDa might be some polypeptide protein formed during the commercial processing of PPI (Shand et al., 2007).

The electrophoretic patterns of control and treated PPI in non-reducing condition were displayed in **Figure 3.7** (**a-2 and b-2**). It has been demonstrated that the polypeptides which has molecular weight around 90 kDa were lipoxygenases (Shand et al., 2007). The protein profile of pH-shifting alone PPI remained mostly unchanged except at pH 12. The increased intensity of

high molecular weight bands in pH12 revealed the formation of large molecule aggregates of pea protein. Moreover, the densitometric analysis (data not show) showed diminished intensity in legumin AB band compared to untreated PPI. Compared to acidic pH-shifting, the band intensity of legumin AB in alkaline pH treated PPI was lower. Jiang et al. (2010) suggested that alkaline pH-shifting mainly disrupted the native disulfide bonds while acidic pH-shifting may form cross-linking of subunits (Jiang, Xiong, & Chen, 2010).

Ultrasonic treated PPI formed broader bands indicating more hydrophobic regions in the samples compared to non-treated protein, which correlated well with the increased surface hydrophobicity of PPI shown in **Figure 3.5**. In comparison with the control and the pH-shifting alone treatments, the ultrasound alone and pH-shifting + US treatments formed high molecular weight soluble aggregates, that was in agreement with the finding in a previous study on soy protein by Lee et al. (2015). It was obviously that pH12+U5 treated PPI was absent in the legumin AB, while there was no significant enhanced intensity in legumin A and B. It has been indicated that alkaline pH-shifting and power ultrasound disrupted some of the disulfide-bonded complexes in pea protein (Donsì, Donsi`, Senatore, Huang, & Ferrari, 2010). The diminished intensity in legumin AB demonstrated that legumin proteins might be the precursor of soluble protein aggregates. The band of MW ~110 kDa was intense only for the pH10+U5, pH12, pH12+U5, and U5 samples. Compared with the electrophoretic patterns under reducing condition (**Figure 3.7 b-1**), this band should represent legumin protein aggregates formed by S-S bonds.

Under non-reducing conditions, the gel for the control and the pH-shifting under acidic conditions with and without sonication (**Figure 3.7 a-2**) shown similar patterns indicating that at acidic conditions PPI was not broken down to form new bands, a finding similar to that reported

in a previous study (Jiang et al., 2014; O'Sullivan et al., 2015). Since the results of SDS-PAGE for treated and non-treated PPI were similar, it was obvious that under acidic conditions the mainly reason for increased solubility is not due to protein hydrolysis, but the conformational and structural changes.

Under reducing conditions (**Figure 3.7 a-1** and **b-1**), legumin AB complex was dissociated into legumin A and legumin B subunits through S-S bonds disruption. Therefore, legumin AB disappeared in the reducing gels. In addition, the unknown band of MW ~110 kDa which appeared in non-reducing gels (**Figure 3.7 b-2**) for the pH10, pH12, pH12+U5, and U5 samples, was absent as well. The acidic pH-shifting and acidic pH-shifting + US treatments obtained nearly identical electrophoretic patterns with the Control (**Figure 3.7 a-1**), while new bands with low molecular weight can be observed for protein samples treated with alkaline pH-shifting + US (**Figure 3.7 b-1**). These low molecular polypeptides may be produced through the breakdown of S-S bonds by β-mercaptoethanol. Aggregates of large molecules were also observed in the pH12-shifting + US treated PPI under reducing condition, and these aggregates may be formed by non-covalent bonds, such as electrostatic interactions, hydrophobicity interactions, and hydrogen bonding. On the whole, the ultrasound alone, alkaline pH-shifting, and alkaline pH-shifting + US treatments formed large molecular weight soluble aggregates through S-S linkage with increased water solubility. The densitometric analysis applied to evaluate the relative amounts of each polypeptide also confirmed the above results (data not show).

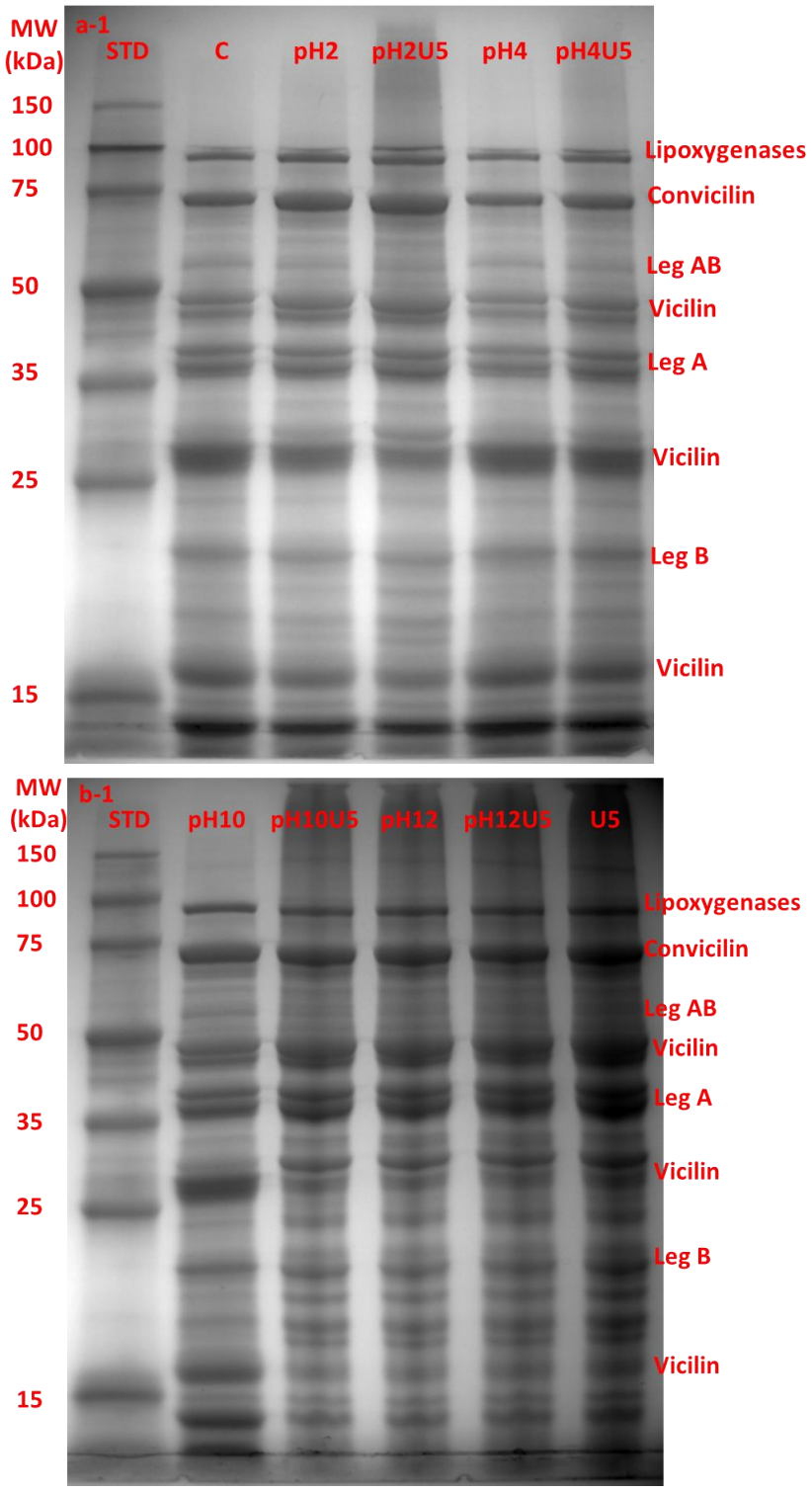
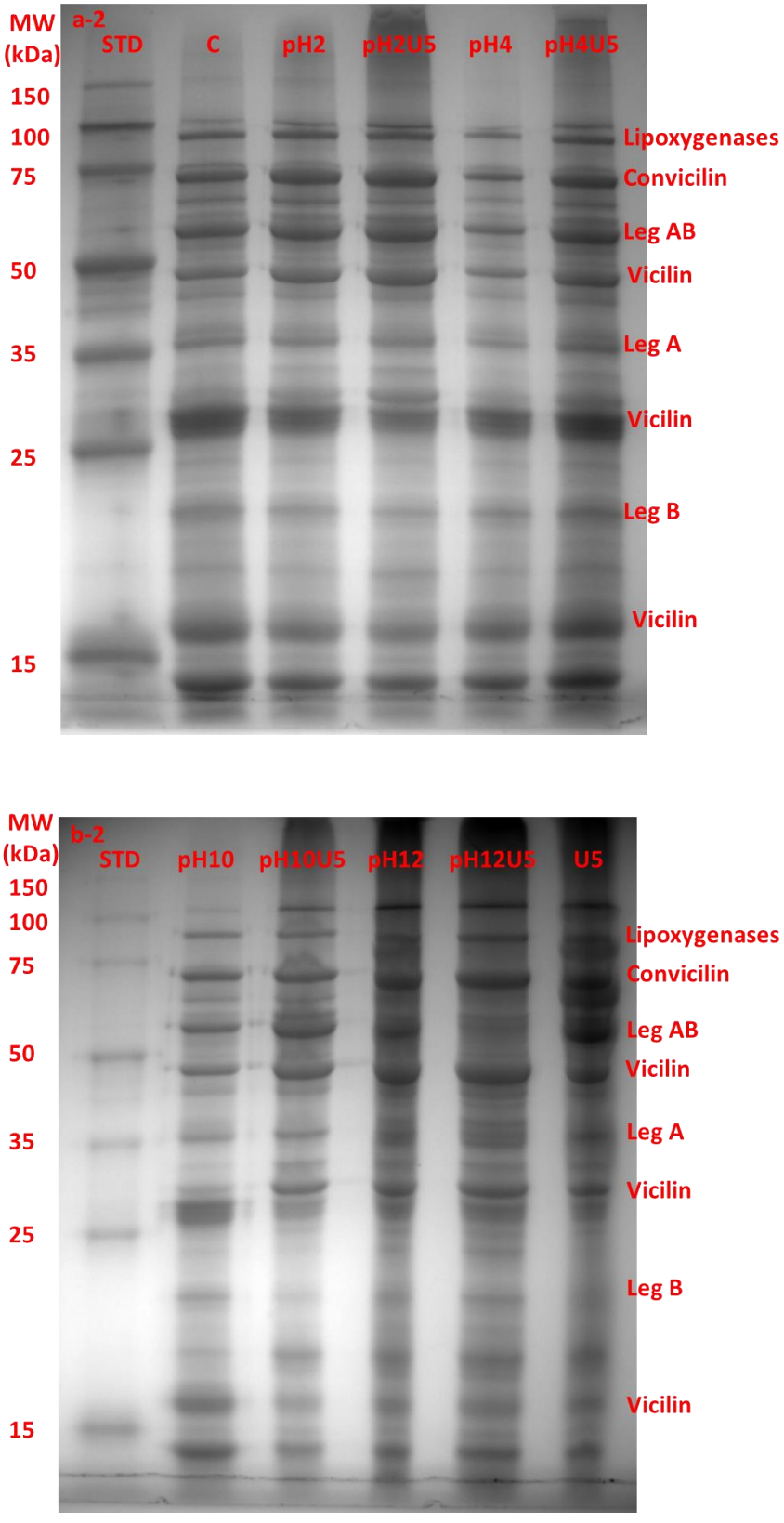


Figure 3.7 Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) patterns of non-treated and treated pea protein samples under reducing (with β-mercaptoethanol) (a-1 and b-1) and non-reducing (a-2 and b-2) conditions.

Figure 3.7 (cont.)



3.4 CONCLUSIONS

It has been revealed that both pH12-shifting and power ultrasound have positive effects on pea protein modification. The pH12-shifting combined with ultrasonic processing successfully improved the solubility of PPI and significantly reduced the sizes of the soluble protein aggregates. The surface hydrophobicity of pH12+U5 treated PPI was enhanced compared to non-treated soluble PPI, and its free sulfhydryl group content was slightly decreased, while alkaline pH-shifting or ultrasound alone processed PPI had increased free sulfhydryl content. Structural modification of treated PPI was observed in the SDS-PAGE patterns, which indicated the disruption of disulfide bonds and non-covalent bonds through alkaline pH-shifting and ultrasonication. This outcome showed the suitability of using extremely alkaline pH-shifting combined with ultrasonication to modify pea protein for extended applications of vegetable protein in the food industry.

CHAPTER 4

ENHANCED FUNCTIONAL PROPERTIES OF PEA PROTEIN AND PROTECTION OF VITAMIN D IN PEA PROTEIN NANOEMULSION

4.1 INTRODUCTION

Encapsulation as a tool to protect bioactive compounds has attracted growing attention from both academia and industry in the recent years. Among the processes to form encapsulation, oil-in-water emulsion is considered as an ideal model for bioactive compound encapsulation. It provides protection to the encapsulated components from harsh environment and contact with other ingredients in food systems (Donsì et al., 2010). Especially for lipophilic molecules, oil-in-water emulsion can increase the solubility of those compounds in an aqueous phase. In addition, the bioavailability of lipophilic bioactive compounds is enhanced due to increased interactions with enzymes and reduced transport resistances through intestine walls (Donsì et al., 2010). Nanoemulsions, which represents an emulsion system with nano-sized (<100 nm) droplets, have a number of advantages in comparison with conventional emulsions (Donsì et al., 2010). Due to the fine particle size of nanoemulsions, it is relatively stable and less likely to form particle aggregates. The low turbidity of nanoemulsions is suitable for usage in beverages and water which need to be clear (McClements & Rao, 2011). Moreover, the nanometric particle size in emulsion may accelerate absorption rate and improve bio-accessibility of the encapsulated bioactive components (Donsì et al., 2010).

Cholecalciferol (vitamin D3) is a fat-soluble vitamin, having a close relationship with calcium absorption and skeletal diseases (Holick, 2007; Tang, Eslick, Nowson, Smith, & Bensoussan, 2007). The human body can produce a small amount of VD3 when exposed to

sunlight, while the main intake of vitamin D comes from foods or dietary supplements (Nik, Corredig, & Wright, 2011). Vitamin D3 is sensitive to environmental factors and easy to degrade. Like other bioactive compounds, vitamin D3 needs to be protected from harsh environmental factors and other food ingredients during food manufacturing, storage, and transportation (Donsì, Annunziata, Vincensi, & Ferrari, 2012). Encapsulation is an effective way to restrict the exposure of vitamin D3 to adverse environmental stresses (Diarrassouba et al., 2015). A number of systems were tested to encapsulate vitamin D3 and showed good protection results, including polylactic acid nanoparticles (Almouazen, Bourgeois, Jordheim, Fessi, & Briançon, 2013), zein-carboxymethyl chitosan nanoparticles (Luo, Teng, & Wang, 2012), whey protein nanoparticles (Abbasi, Emam-Djomeh, Mousavi, & Davoodi, 2014), and soy protein-based emulsions or particles (Nik et al., 2011; Teng, Luo, & Wang, 2013).

An emulsifier is a key factor in the formation of emulsions. Most food grade artificial surfactants show excellent emulsifying ability, but there are much fewer natural emulsifiers having emulsifying capability comparable to their synthetic counterparts. Pea protein, as an amphiphilic macromolecule, contains both polar and nonpolar regions and thus has the potential to be used to stabilize emulsion systems. Besides, pea protein, which has high nutritional value and valuable health benefits, can be used as animal protein substitute to decrease cholesterol and fat content in food products (Donsì et al., 2010). As a non-genetically modified plant, pea protein has a clean label, which is now popular in the food industry. However, native pea protein shows low water solubility and poor functional properties under neutral pH (pH 7.0), which restricts its applications (Liang & Tang, 2013). Both pH-shifting and ultrasonication can alter the protein structure and enhance the amphiphilicity of protein polypeptides (Hu et al., 2013; Jiang et al., 2014). As described in previous research in **Chapter 3**, extreme alkaline pH-shifting + US

treatment can significantly improve the solubility and other functional properties of pea protein, thus the modified nano-sized PPI aggregates may function as a promising emulsifying agent for VD3-loaded emulsion.

Few studies were performed on nanoemulsion delivery systems based on pea protein. Donsì et al. (2012) examined the antimicrobial activity of three kinds of essential oils encapsulated in nanoemulsions stabilized by different emulsifiers, including pea protein. The pea protein based nanoemulsion system showed limited bactericidal activity compared to other emulsifiers (Donsì et al., 2012). Jiang et al. (2014) studied the oxidative stability of pea protein based oil-in-water emulsion, and reported an improved antioxidant activity and emulsion stability of alkaline pH treated pea protein.

In this study, nano-sized soluble pea protein aggregates were utilized to prepare vitamin D3 loaded nanoemulsions and nanocomplexes. The antioxidant activity and the protective effect of the nano-structures on VD3 under UV-light exposure were analyzed. To examine stability of the nanosystems, droplet size of each emulsion sample was detected over a period of 30 days of 4°C storage. Morphological structures of the PPI-stabilized nanoemulsion and nanocomplex were observed using transmission electron microscope. In addition, an *in vitro* digestion of modified PPI prepared nanoemulsions and nanocomplexes was performed to determine the digestion efficiency.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Pea protein isolate (PPI, NUTRALYS® S85F, 85% pea protein based on dry basis) was provided by Roquette (Geneva, IL, USA). These pea proteins were extracted using wet-process from dry yellow peas, and were stored in refrigerator (Roper Refrigerator, Whirlpool

Corporation) at 4°C before use. All of the other reagents and chemicals purchased from Bio-Rad (Hercules, CA, USA), Sigma-Aldrich (St. Louis, MO, USA), and Fisher Scientific (Pittsburgh, PA, USA) were of analytical or higher grade.

pH-shifting and ultrasonication were performed as described in Section **3.2.2**. Extreme alkaline plus ultrasonication (pH12+US) treatment was applied to treat pea protein isolate, labeled as pH12+U5. The protein sample treated with only alkaline pH-shifting or ultrasonication was named as pH12, and U5, respectively. Control represented native protein with no treatment but only stirred 30 minutes under room temperature.

4.2.2 Preparation of nanoemulsion and nanocomplex

Vitamin D3 stock solution was prepared by dissolving cholecalciferol (C9756 Sigma) in commercial canola oil (Wesson pure canola oil, ConAgra Foods, Inc.) at a concentration of around 1.04% (w/w). Meanwhile, ethanol was also used to dissolve VD3 to prepare VD3-ethanol stock solution (26.92 mg/ml) for nanocomplexes.

Oil-in-water (O/W) emulsions were prepared with canola oil (containing VD3) and soluble pea protein solution (10 mg/ml). The concentration of oil was 0.25% (w/w). High intensity ultrasound was used to generate the PPI-based nanoemulsions. The mixture of oil (containing VD3) and soluble protein was stirred strongly for 5 min and then sonicated for 5 min. During ultrasonication, samples were placed in an ice bath to avoid increasing temperature. Nanocomplexes were prepared by adding 50 μl VD3-ethanol stock solution in 50 ml pea protein solution (10 mg/ml) with agitation and then stirring violently for 10 min. Control represented the sample by dissolving 50 μl VD3-ethanol stock solutions in 50 ml DI water followed by 10 min stirring. The flow chart of nanoemulsion and nanocomplex preparation was shown in **Figure 4.1**. All Vitamin D3 containing chemicals and samples were prepared in a dark room with UV

reduced lighting and further protected with aluminum foil wrapping. The samples were stored in a refrigerator at 4°C before use.

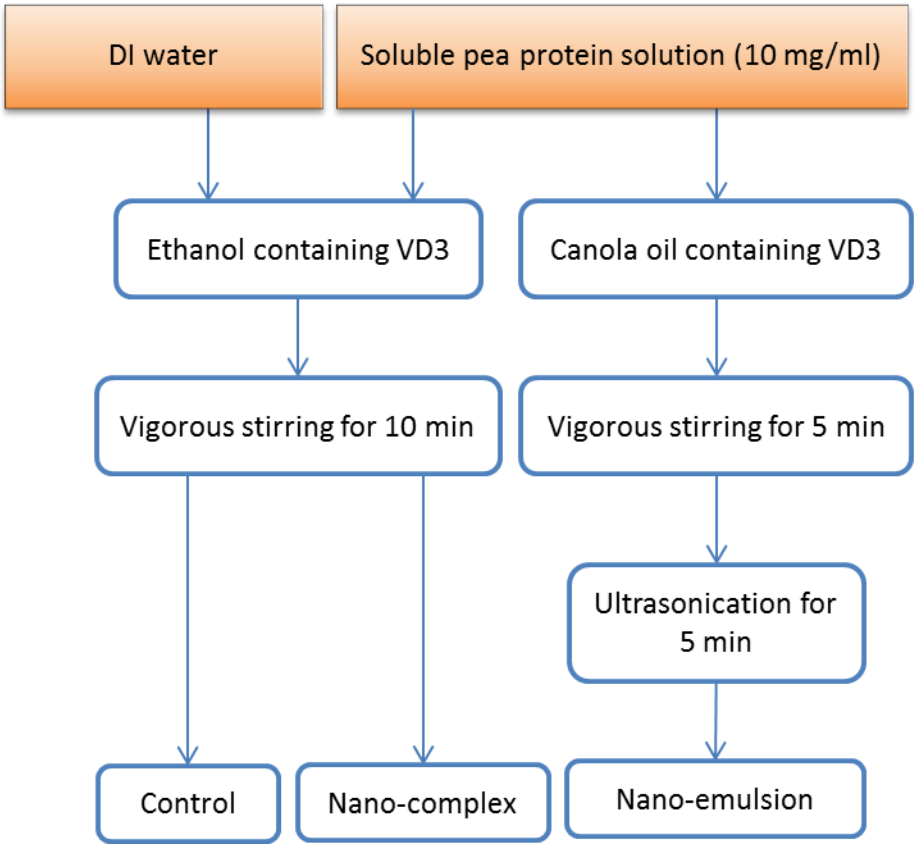


Figure 4.1 Procedure for preparation of vitamin D3 enriched nanoemulsions and nanocomplexes.

4.2.3 Storage stability of nanoemulsion and nanocomplex

The stability of PPI based nanoemulsions and nanocomplexes was examined by measuring their particle size changes over 30 days stored at 4°C. The volume-weighted mean diameters (D4, 3) of nanoemulsions and nanocomplexes were measured by dynamic light scattering (DLS) using a NICOMP 380 DLS instrument (Santa Barbara, CA, USA). Samples were diluted 500-fold with DI water before measurement. The measurement temperature was 23°C, and the liquid viscosity and index of refraction were set according to water, which was

0.933 and 1.333, respectively.

4.2.4 UV stability of Vitamin D3 in nanoemulsion and nanocomplex

Five ml soluble pea protein stabilized nanoemulsions and nanocomplexes were placed into polystyrene petri dishes (60 mm × 15 mm, Fisher, Pittsburgh, PA, USA). UV light was generated by an ultraviolet transilluminator (FisherBiotech™ Ultraviolet Transilluminator, model FBTIV-614, Fisher Scientific, Pittsburgh, PA). Samples were exposed to UV light (312 nm, 15 W) for up to 180 min. 100 µl of each sample was collected after 10, 30, 60, 90, 120, and 180 min exposure. The weight of each petri dish was also measured at each time point before and after sample collected. Sample containing only vitamin D3 in DI water was used as the control in this experiment.

Methanol was used to extract vitamin D from the UV-treated samples. 900 µl of 100% filtered methanol was mixed with 100 µl irradiated sample. Sample was placed in an ultrasound water bath for 30s to assist VD3 extraction. After centrifuge (Sorvall ST 16R centrifuge, Thermo Scientific, Waltham, MA, USA) at 14,000 rpm at 4°C for 10 min, the supernatant was filtered with 0.20 µm nylon filters (Chromafil PP/PTFE disposable filter O-20/15 MS, Macherey-Nagel, Bethlehem, PA, USA). The vitamin D3 content was determined using reversed phase HPLC with UV detection at 265 nm (Waters Alliance e2695 HPLC system with Waters 2489 UV/Visible Detector, Milford, MA). A C18 column (5 µm 250 × 4.6 mm, ODS-2 Hypersil, Thermo Scientific, Waltham, MA, USA) was used to separate VD3 and solvent. Mobile phase was 100% methanol, and the flow rate was 1 ml/min.

4.2.5 DPPH radical scavenging activity

The antioxidant activity assay was conducted based on the method described by Li et al. (2008) with slightly modification. 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich Co,

St. Louis, MO) was used as a free radical. DPPH stock solution (10 mM) was prepared by dissolving DPPH in 100% methanol. Two ml of each pea protein sample (1 mg/ml) were added into 2 ml of 0.1 mM DPPH solution. Blank control was prepared by mixing 2 ml DI water with 2 ml of 0.1 mM DPPH solution. The absorbance of each sample solution was read at 0, 5, 10, 15, 20, 25, 30, and 40 min using a spectrophotometer (Lambda 1050 UV/VIS/NIR Spectrometer, PerkinElmer) under the wavelength of 517 nm. The antioxidant activity was expressed by the percentage of remaining DPPH through following equation:

$$Remaining\ DPPH\ (\%) = \frac{DPPH\ concentration\ at\ each\ time\ point}{DPPH\ concentration\ at\ 0\ min} \times 100\%$$

4.2.6 Encapsulation efficiency

The encapsulation efficiency of pH12+U5 treated PPI based nanoemulsion was determined according to Luo, Teng, and Wang (2012) with modification. Samples were first dried using a freeze drier (FreeZone 6 Liter Console Freeze Dry System, Labconco, Kansas City, MO, USA). Lyophilized samples were stored in a refrigerator (Roper Refrigerator, Whirlpool Corporation) at 4°C before use. Ten milligrams of lyophilized nanoparticles were flushed with 1 ml of ethyl acetate for three times. Whatman #1 filter paper (Grade 1 Qualitative Filter Paper, circle, 55 mm) was used to separate washed nanoparticles and filtrates. The ethyl acetate elutes and washed nanoparticles were dried using a water bath nitrogen blowing concentrator (N-Evap Nitrogen Evaporator, Organomation, Berlin, MA, USA). The free vitamin D3 in ethyl acetate elutes was dissolved in 1 ml methanol. The washed and dried nanoparticles were mixed with 5ml methanol for 30s by a vortex shaker (Fisher Vortex Genie 2 12-812, Fisher Scientific, Pittsburgh, PA, USA), followed by extracted using ultrasound water bath for 30s. After centrifuge at 14,000 rpm at 4°C for 10 min, supernatant was filtered with 0.20 um nylon filters and measured vitamin D3 content through HPLC as addressed above (**4.2.4**). To minimize VD3 degradation, all

experiments were conducted under non-UV lighting.

The encapsulation efficiency (EE) and loading capacity (LC) were determined using the following equations:

$$EE(\%) = \frac{\text{Encapsulated VD3 amount}}{\text{Total VD3 amount}} \times 100\%$$

$$LC(\%) = \frac{\text{Encapsulated VD3 amount}}{\text{Nanoparticles weight}} \times 100\%$$

4.2.7 In vitro digestion

An *in vitro* digestion procedure was used to mimic the fed state of human GI tract, following that reported by Garrett et al. (1999) with a slight modification. Enzyme A was prepared by dissolving pepsin (P7125, Sigma) in 0.1 M HCl to achieve a final concentration of 4 g/l. Enzyme B contained pancreatin (2 g/l) (P1750, Sigma) and bile extract (12 g/l) (B8631, Sigma) in 0.1 M NaHCO3. The pancreatin mixture with bile salts is necessary for the formation of bile salt micelles (Hedrén, 2002). As a response to the intake of a meal, bile is secreted into the duodenum, and in the fed state, the mean bile salt concentrations in human duodenal and jejunal fluids are between 8 mM and 12 mM (Hur, Lim, Decker, & McClements, 2011).

Canola oil was added to the nanoemulsion, nanocomplex, and the control samples to adjust the total oil content to 3.0%. Gastric environment was created through mixing up 5 ml of test sample with 27 ml of saline (0.9% NaCl) and 2 ml of enzyme A, and adjusted pH to 2.0 using 2 M HCl. Samples were incubated at 37°C with orbital shaking (13 mm diameter) at 95 rpm for 1h in an incubator shaker (New Brunswick incubator shaker I24R, Eppendorf, Enfield, CT, USA). The gastric digestion was stopped by adjusting pH to 5.3 using a 0.9 M sodium bicarbonate solution. Intestinal period was simulated by adding 9 ml enzyme B and adjusted pH to 7.5 by adding a few drops of 2 M NaOH. For intestinal digestion, samples were incubated at

37 °C with orbital shaking at 95 rpm for 2 hours. After the whole in-vitro digestion, the supernatant was collected by centrifugation at 5,000 g for 20 min.

The vitamin D3 contained in micelles after *in vitro* digestion was extracted by methanol. 500 µl of methanol was added to 500 µl of digestive solution and properly mixed for 10s. The sample was then sonicated in an ultrasonic bath for 30s for VD3 extraction. The supernatant was collected and filtered with 0.20 µm nylon filters after centrifugation at 14,000 rpm at 4 °C for 10 min. Vitamin D3 content was determined using HPLC as described above (**4.2.4**).

4.2.8 TEM

Morphological structures of the PPI-stabilized nanoemulsions and nanocomplexes were observed by transmission electron microscope (TEM). The staining protocol was followed as described by Garewal et al. (2013) with slight modification. Filtered uranyl acetate (2.0 %) was used as a negative stain which can interact with proteins and lipids to enhance the contrast. First, the grids (Carbon-stabilized formvar coated grids, Ted Pella, Tustin, CA) were placed on the drops of samples for 1 min (bright side up). Then the grids were rinsed in water for three times and dried. After rinsing with uranyl acetate twice, the grids were put on a drop of uranyl acetate for 3 min. After drying, the grids can be analyzed immediately. 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.

4.2.9 Statistical analysis

All experiments were conducted in at least three independent trials. Results were reported as the mean and standard deviation based on independent experiments. The differences were analyzed using ANOVA by SAS. Significant differences ($P < 0.05$) between means were identified by Tukey HSD all-pairwise multiple comparisons.

4.3 RESULTS AND DISCUSSION

4.3.1 Storage stability of nanoemulsion and nanocomplex

The storage stability of the pH12, U5, pH12+U5-treated PPI stabilized nanoemulsions (NE), and pH12+U5-treated PPI based nanocomplexes (NC) during storage at 4°C for 30 days was examined. The droplet sizes of the nanoemulsion and nanocomplex at day 0, 1, 2, 5, 7, 14, 30 were measured and the stability over storage time was shown in **Figure 4.2**. After ultrasonication, modified PPI formed a soy milk-like emulsion, while the nanocomplex was still transparent, similar to the soluble PPI solution. The volume weighted mean diameter (nm) of the pH12 NE, U5 NE, pH12+U5 NE and pH12+U5 NC measured immediately after preparation was 122.0, 134.7, 113.9, and 88.9, respectively. Pea protein is a novel type of vegetable protein, and therefore few studies have been focused on its emulsifying ability. Donsì et al. (2010) applied high pressure homogenization (HPH) to form pea protein-based oil in water (O/W) nanoemulsions, with an average droplet size less than 200 nm.

pH-shifting + US treated PPI formed the emulsion with the smallest droplet size (113.9 nm). Large surface area due to small particle size of processed PPI improved its emulsifying capability (Jiang, Zhu, Liu, & Xiong, 2014). Compared to the nanoemulsions, nanocomplex showed smaller droplet size, which might be due to the incomplete encapsulation structure, as well as the usage of ethanol instead of canola oil. Further studies need to be conducted to understand the structure of PPI-based nanocomplex.

It can be seen in **Figure 4.2** that both the nanoemulsion and nanocomplex showed good stability during 30 days of storage. After 30 days storage, the droplet sizes of pH12 NE, U5 NE, pH12+U5 NE and pH12+U5 NC were 96.2, 87.9, 72.8, and 62.9 nm, respectively. Creaming, sedimentation, flocculation and coalescence are the four reasons affect the stability of emulsions.

Creaming and sedimentation are formed due to gravitational separation, while the other two are the types of droplet aggregation. Therefore, the methods to enhance emulsion stability should be focused on decreasing the density difference between droplets and continuous phase, and altering the surface structure of emulsifiers. The good stability of PPI-based nanoemulsions might be attributed to the structural change of modified pea protein. Extreme pH-shifting + US would change the protein secondary and tertiary structure, therefore enhance the emulsifying ability. The study of Jiang et al. (2014) reported that extreme alkaline pH-shifting (pH 12.0) treated pea protein developed better interfacial distribution ability around fat droplets, which may due to increased amphiphilicity of protein and improved structural flexibility. Protein amphiphilicity is the balance between hydrophile and lipophile. The exposure of protein side chains due to extreme pH conditions or sonication could not be reversed through structure refolding. Thus, the increased surface hydrophobicity improved structural flexibility and limited protein aggregation at the interface, leading to increased emulsifying activity (Jiang, Xiong, & Chen, 2011). In addition, high shear forces and agitation introduced by acoustic cavitation also have similar effects on protein. In addition, the small droplet sizes of emulsions are also beneficial to emulsion stability. It was found that fine droplet could achieve good stability (Donsì et al., 2010). Small emulsion droplet sizes decrease the chances of particle aggregation, reducing the chances of sedimentation of particle aggregates.

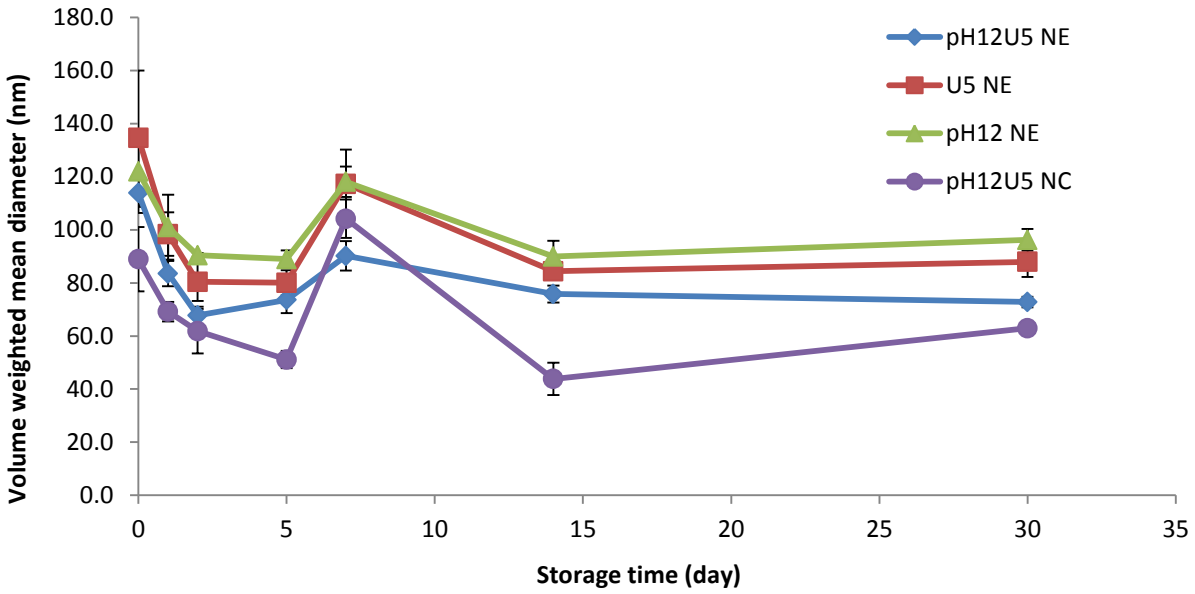


Figure 4.2 The volume weighted mean diameter (nm) of the droplet size of different treated PPI-based nanoemulsions or nanocomplex. U5 NE, nanoemulsion stabilized by ultrasound treated PPI; pH12 NE, nanoemulsion stabilized by pH12-shifting treated PPI; pH12+U5 NE, nanoemulsion stabilized by pH12-shifting combined ultrasound treated PPI; pH12+U5 NC, nanocomplex stabilized by pH12+US treated PPI.

4.3.2 UV stability of Vitamin D3

The stability of vitamin D3 encapsulated in the nanoemulsion and nanocomplex was examined by exposing the vitamin D3 loaded nano-structures to UV light for 180 minutes. The remaining vitamin D3 in the control, pH-shifting + US treated PPI-based nanoemulsion and nanocomplex as a function of UV exposure time was presented in **Figure 4.3**. The control represented the sample consisting only of DI water and VD3.

After 180 minutes UV radiation, there was only 8.71% vitamin D3 left in the control, and the VD3 degradation rate was fast, especially in the first 90 minutes. In contrast, vitamin D3 in PPI-stabilized nanoemulsion and nanocomplex showed significantly lower degradation rate, and

the remaining VD3 content in nanoemulsion and nanocomplex were 74.22% and 65.37% after 180 minutes of UV exposure, showing excellent protection provided by the pea protein-based nanoemulsions and nanocomplexes.

Vitamin D is a fat soluble vitamin, and is very sensitive to environmental factors, such as light, oxygen, and heat. Photooxidation of vitamin D3 is one of the major problems in the food industry for vitamin fortification (Luo et al., 2012). Encapsulation of Vitamin D3 is an effective strategy to protect VD3 isomerization or oxidation from adverse environment factors. A number of studies using proteins to protect vitamin D against degradation have been reported, including using soybean β-conglycinin nanoparticles (Levinson, Israeli Lev, & Livney, 2014), β-lactoglobulin-based coagulum (Diarrassouba et al., 2015), zein-carboxymethyl chitosan nanoparticles (Luo et al., 2012), and whey protein isolate nanoparticles (Abbasi et al., 2014).

The protective ability of pH12+U5 processed PPI-based nanoemulsions and nanocomplexes might be due to the barrier structure that protein formed against UV irradiation. Jiang et al. (2014) also revealed that alkaline pH-shifting induced nonpolar amino acid residue exposure, enhancing the hydrophobic interaction between pea protein and oil droplets, resulting in more stable emulsions, which providing good protection against adverse environment factors, such as UV light, oxygen and other chemicals. However, nanocomplexes prepared only through simple agitation might not form perfect encapsulation and provided less sufficient protection against UV light. The aromatic side chains and double bonds in proteins might absorb UV light and hence protect the photochemical degradation of VD3 (Diarrassouba et al., 2015; Luo et al., 2012).

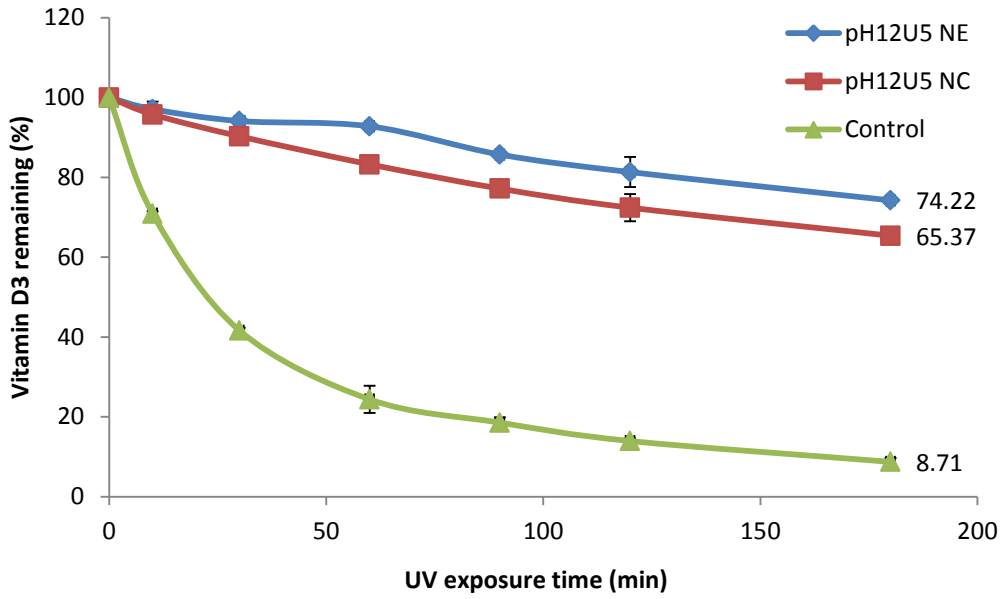


Figure 4.3 UV radiation stability of vitamin D3 in nanoemulsion, nanocomplex, and control. Control, vitamin D3 in DI water; pH12+U5 NE, nanoemulsion stabilized by pH12-shifting combined ultrasonic treated PPI; pH12+U5 NC, nanocomplex stabilized by pH12-shifting combined ultrasonic treated PPI.

4.3.3 Antioxidant activity of PPI

The antioxidant activity of the PPI expressed by DPPH radical scavenging ability is shown in **Figure 4.4**. Low DPPH content remaining in the sample solution indicated a higher antioxidant activity. After 40 minutes incubation in the dark, 84.49% of DPPH was retained in the pH12-shifting treated PPI, having the lowest antioxidant ability compared to other samples. The control, ultrasonic treated, and pH12+U5 processed PPI reduced the concentration of DPPH to 69.81, 65.00, and 59.60% after 40 minutes of incubation, respectively.

Pea protein contains 40.6% hydrophobic amino acids. It was stated that hydrophobic and aromatic amino acids have strong radical scavenging and metal-chelating activities (Jiang, Zhu, Liu, & Xiong, 2014). After pH adjusting and ultrasonication, the hydrophobic and aromatic

amino acid side chains may get exposed, which would improve the antioxidant activity of pea protein.

The antioxidant activity of pea protein has not been fully investigated. A few researchers analyzed the antioxidant properties of pea protein hydrolysate (Humiski & Aluko, 2007; Pownall, Udenigwe, & Aluko, 2010; Pownall, Udenigwe, & Aluko, 2011), which showed similar DPPH radical scavenging activity as modified PPI in this case. Recently, Jiang et al. (2014) examined the antioxidant activity of alkaline pH-shifting treated pea protein, and demonstrated that the alkaline pH treated PPI showed 60% greater antiradical activity than the non-treated PPI. In contrast, the pH12-shifting treated PPI in this study expressed decreased DPPH radical scavenging activity compared to the control, while there was no significant difference between ultrasonic and pH-shifting + US treated PPI and the control. This discrepancy may be due to the different preparation methods of PPI. The control samples in this test were the soluble part of commercial PPI, while in the study of Jiang et al. (2014), the whole PPI was used as the native pea protein.

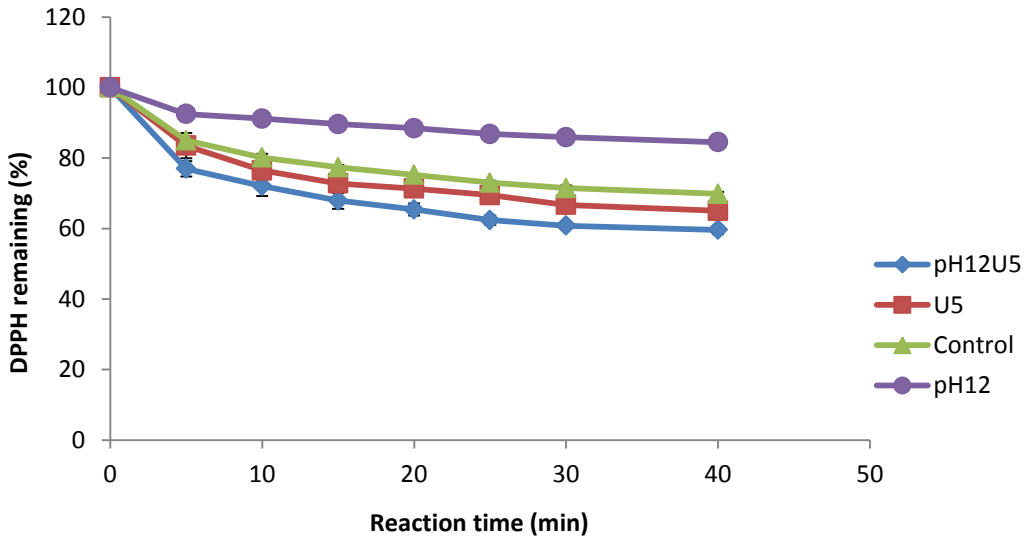


Figure 4.4 The DPPH scavenging activity of treated soluble PPI. Control, non-treated PPI; U5,

PPI treated with 5 minutes of ultrasonic; pH12, PPI treated pH12-shifting; pH12+U5, PPI treated with pH12-shifting combined ultrasonic.

4.3.4 Encapsulation efficiency

The encapsulation efficiency (EE) was determined using freeze-dried nanoparticles from the pH12-shifting + US treated PPI. The vitamin D3 loaded nanoparticles stabilized by alkaline pH-shifting + US treated PPI showed good EE, as high as 93.2 ± 2.1%. Meanwhile, the loading capacity of the PPI-based nanoparticles was 1.5 ± 0.2 μg/mg pea protein. The EE was comparable to that from the nano-structures used to encapsulate VD3 reported in the literature (Diarrassouba et al., 2015; Luo et al., 2012; Teng et al., 2013). Teng et al. (2013) reported that the VD-loaded soy protein isolate (SPI) nanoparticles (162.4±6.7 nm) had an EE of around 50.19%, while the carboxymethyl chitosan-soy protein nanoparticles (243.1±12.4 nm) achieved encapsulation efficiency of up to 96.75%. The EE of VD3 entrapped zein nanoparticle (120.2±2.2 nm) was around 52.2%, and after coating with carboxymethyl chitosan (109.5±11.3 nm), the EE was raised to 87.9% (Luo et al., 2012).

4.3.5 In vitro digestion

Lipophilic bioactive compounds like vitamin D are absorbed through micelles formed in the small intestine (Levinson et al., 2014). The absorption of vitamin D relies on oil digestion, and is assisted by bile secretion. In this study, the large intestinal tract was not taken into account, since *in vivo* food digestion and absorption of compounds mainly takes place in the small intestine. In addition, VD3 will be degraded due to the extreme acidic environment during gastric digestion. Consequently, simulated gastric and intestinal environments were applied in the *in vitro* digestion test. The hypothesis was that the modified pea protein nano-structures protect VD from degradation in gastric period, and has no adverse impact on intestinal digestion.

The effect of different PPI-VD3 structures on the micellization of vitamin D3 through *in vitro* digestion was depicted in **Figure 4.5**. The recovery of vitamin D3 from the PPI stabilized nanoemulsion and nanocomplex after three hours of *in vitro* digestion was 62.9 ± 11.1% and 39.7 ± 1.3%, respectively. On the contrary, there was 24.4 ± 2.7% recovered from the control, which was consisted of VD3 dispersed in DI water. The recovery of vitamin D3 from each sample, which is the fraction solubilized within the mixed micelle phase after lipid digestion, is defined as the bioaccessibility of VD3 (Yang & McClements, 2013). The PPI protect samples had high bioaccessibility of VD3 compared to the control. In general, unprotected VD3 in the control would almost degrade during digestion (Diarrassouba et al., 2015). However, the oil content adjustment before *in vitro* digestion might increase VD3 ingestion bioavailability in the control.

The enhanced bioaccessibility of VD3 in PPI stabilized nanoemulsions and nanocomplexes might be attributed to the protection of modified pea protein during gastric digestion. The protein fractions in the nanoemulsion and nanocomplex were slowly hydrolyzed in the presence of pepsin in the simulated gastric period, while the remaining peptide segments may retain the ability to protect VD3. The nanocomplex exhibited lower recovery of VD3 after *in vitro* digestion compared with the nanoemulsion, which may be linked to the fact that the VD3 was less protected as no capsules were formed in the nanocomplex. In addition, the reduced electrostatic repulsion under acidic and ionic conditions in stomach, along with the damage of the interfacial layer due to protein hydrolysis would lead to droplet flocculation (Nik et al., 2011), which minimized the gastric release of VD3. Another possible mechanism might be related to the buffering capacity of pea protein, which may provide protection against the acid degradation of VD3 in gastric environment. In addition, it was demonstrated that the characteristics of emulsion droplets, including particle size and interfacial properties, would influence lipolysis of oil

droplets, further altering the micellization of bioactive molecules (Nik et al., 2011). Thus, the fine droplet size of PPI-stabilized nanoemulsion in this study might enhance VD3 micellization.

In vitro release of encapsulated vitamin D from various kinds of vegetable protein based emulsions and particles has been investigated, with emulsions formed by soy protein (Nik et al., 2011; Teng et al., 2013) and zein (Luo et al., 2012). Recently, the characterization of *in vitro* gastric digestion of pea protein was analyzed with a focus on the gastrointestinal satiety responses (Overduin, Guérin-Deremaux, Wils, & Lambers, 2015). No relevant study was reported on using pea protein-stabilized nanoemulsions to protect vitamin D.

The work of Nik et al. (2011), in the presence of pancreatic lipase, the release of vitamin D3 from soy protein-stabilized emulsion after 2 hours of in vitro duodenal digestion was 86.9%. In this research, the recovery of VD3 from PPI-stabilized nanoemulsion in the aqueous phase after in vitro digestion was 62.9 ± 11.1%, which was lower than theVD3-loaded SPI emulsion used by Nik et al. (2011). It is noted that, in the study of Nik et al. (2011), extra lipase and colipase were added in the formula of simulated duodenal and bile fluids, which had positive effects on lipophilic molecule digestion. Moreover, the surface structural differences between soy protein and pea protein might also impact lipolysis and release of VD3.

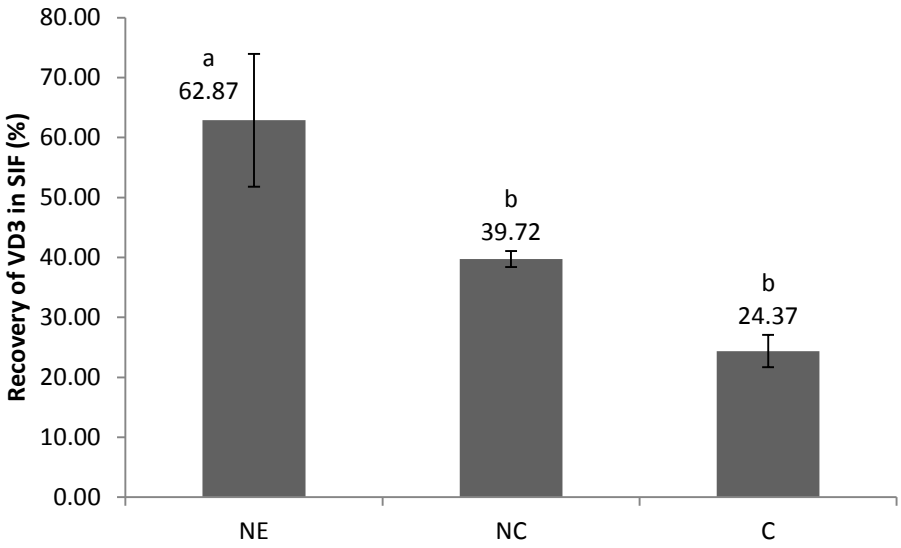


Figure 4.5 Recovery of viatmin D3 in micelles through *in vitro* digestion. NE, nano-emulsion stabilized by pH12+U5 treated PPI; NC, nano-complex synthesized by pH12+U5 treated PPI and VD3 containing canola oil; C, control consisting DI water and VD3.

[ab] Mean ± standard deviation (n=3) of VD3 recovery with the same letter are not significantly different ($p < 0.05$)

4.3.6 TEM

Morphological structures of PPI-stabilized nanoemulsions and nanocomplexes were shown in **Figure 4.6**. Since negative staining process was applied, the dark areas in each image indicated protein fractions that were fixed and coated with a layer of stain (Garewal et al., 2013). The round-shape of light color areas entrapped in protein was the oil droplets. As shown in **Figure 4.6 A**, tiny oil droplets with spherical shape and smooth surface were formed inside of pH12-shifting treated pea protein. The oil droplets with irregular shape were assembled around PPI as well. In **Figure 4.6 A,** the pH12-shifting treated PPI still held compact protein structure. Similar status can be found in ultrasonic treated PPI stabilized nanoemulsion (**Figure 4.6 B**). Round-shape oil droplets were perfectly encapsulated in the PPI-based wall material, and the

particle size of capsule was smaller than the nanoemulsion stabilized by pH12-shifting treated PPI. The ultrasonic processed pea protein had tight structure as well. In contrast, the pH12-shifting + US modified PPI expressed loose protein structure (**Figure 4.6 D**), which confirmed the partial unfolding of PPI induced by alkaline pH-shifting and ultrasonication. As a consequence, there was broader space within the protein peptide chains, which might be able to absorb a large quantity of lipophilic components.

High speed agitation, instead of ultrasonication, was applied in nanocomplex preparation. Therefore, incomplete nanoparticles were formed (**Figure 4.6 C**), which led to exposure of VD3 to harsh environment, including UV light, acidic or alkaline pH, oxygen, and high temperature. The TEM image of the structure of the pH12+U5 modified PPI-stabilized nanocomplexes demonstrated the less sufficient protection of vitamin D3 during UV-light exposure and *in vitro* digestion.

In the pH12+U5 modified PPI-stabilized nanoemulsion (**Figure 4.6 D**), a majority of the tiny particles were clumped and adhered to the protein fractions in the solution. As shown in **Figure 4.6 D**, the tiny and spherical droplets with nanoscale size were observed in the emulsion. These droplets maintained sphere structure consisting of modified PPI as interfacial layer and oil droplets with VD3.

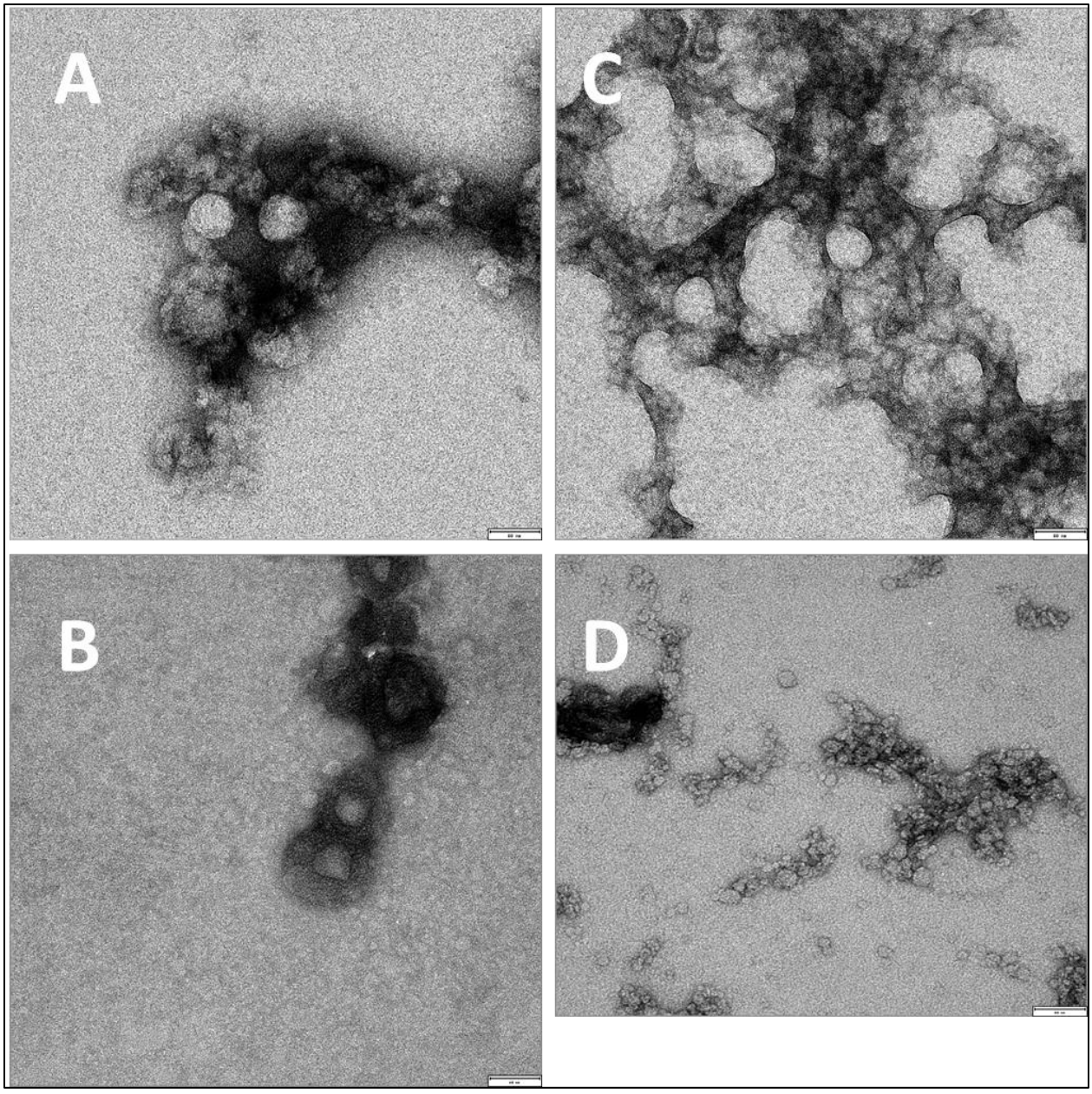


Figure 4.6 Transmission electron microscopy (TEM) images of PPI-stabilized nanoemulsion or nanocomplex: (A) nanoemulsion stabilized by pH12-shifting treated PPI; (B) nanoemulsion stabilized by ultrasonic treated PPI; (C) nanocomplex stabilized by pH12-shifting + US treated PPI; (D) nanoemulsion stabilized by pH12-shifting + US treated PPI. The bar in each image represents 60 nm.

4.4 CONCLUSIONS

In conclusion, extreme alkaline pH-shifting + US (pH12+U5) treatment modified the structure and functional properties of pea protein isolate. The partial unfolding induced by pH-shifting and ultrasonication led to the exposure of amino acid side chains usually embedded inside of the compact protein structure and resulted in improved emulsifying ability of PPI. Besides protecting the encapsulated vitamin D3 from UV light irradiation, the pH12+U5 modified PPI based nanoemulsion also improved the *in vitro* digestion ability. Therefore, the pH12+U5 processed PPI can be a promising food grade delivery system, providing protection to lipophilic bioactive components. Moreover, the water-soluble modified pea protein can be applied to aqueous based food products to increase the accessibility of encapsulated fat-soluble nutraceuticals.

CHAPTER 5

OVERALL CONCLUSIONS AND FUTURE WORK

The current study investigated the effect of pH-shifting and ultrasonication on the structural and physicochemical properties of pea protein isolate (PPI). pH shifting at different conditions (pH2, 4, 10, 12) in combination with/without ultrasonication was explored. In addition, the functional properties and protective effect of the nanoemulsions and nanocomplexes prepared by nano-sized PPI obtained by pH-shifting at pH 12 in combination with ultrasonication (pH12+US) were studied, including storage stability, UV stability, *in vitro* bioaccessibility, and micro-imaging with transmission electron microscopy (TEM).

The pH12-shifting and ultrasonication treatment alone and their combinations enhanced the physicochemical properties of pea protein, and the pH12+US process was the most effective among all the treatments. Specifically, water solubility of the PPI was dramatically improved by the pH12+US process, and the particle size of the PPI aggregates in solution was significantly reduced. The pH12+US process resulted in structural rearrangements of pea protein, as shown by an increase in surface hydrophobicity, changes in free sulfhydryl content, and differences in SDS-PAGE patterns. Although there was no significant enhancement in the antioxidant activity, the pH12+US-processed PPI exhibited good radical scavenging and UV protection ability. Besides, the nanoemulsion stabilized by the pH12+US PPI had good storage stability during 30 days at 4°C. The bioavailability of vitamin D3 in nanoemulsion from the pH12+US-treated PPI was enhanced. Therefore, the pH12+US-processed PPI mediated nano-systems could become a promising carrier to deliver and protect lipophilic bioactive compounds such as vitamin D in processed foods.

To better understand the changes of pea protein induced by pH-shifting and ultrasonication treatments, studies should be conducted to analysis the conformational and structural transformation of treated pea protein. Specifically, circular dichroism can be applied to determine the conformational changes in protein secondary structure. Moreover, the morphological structures of treated-PPI stabilized nano-systems can be observed through other technologies, such as scanning electron microscope (SEM), Raman confocal imaging microscope, and so on. Future studies comparing the emulsifying capacity of PPI treated with this method with other emulsifiers and artificial surfactants widely used in food manufacturing can also be conducted.

For future application in the food industry, the functional properties of the treated PPI or nano-systems in dry powder forms should be explored. If the dried powder of the soluble PPI or nano-systems has a good re-dissolving capacity and still maintains improved functional properties, its further usage in food products can be in a dry form, which can be used in both solid foods (i.e. bakery and snacks) and liquid foods after reconstitution (beverages). Future studies can also look into testing in environments mimicking human gastrointestinal tract. Thus, *in vivo* studies can be carried out to examine the bioactivity of delivered nutrients in real GI tract.

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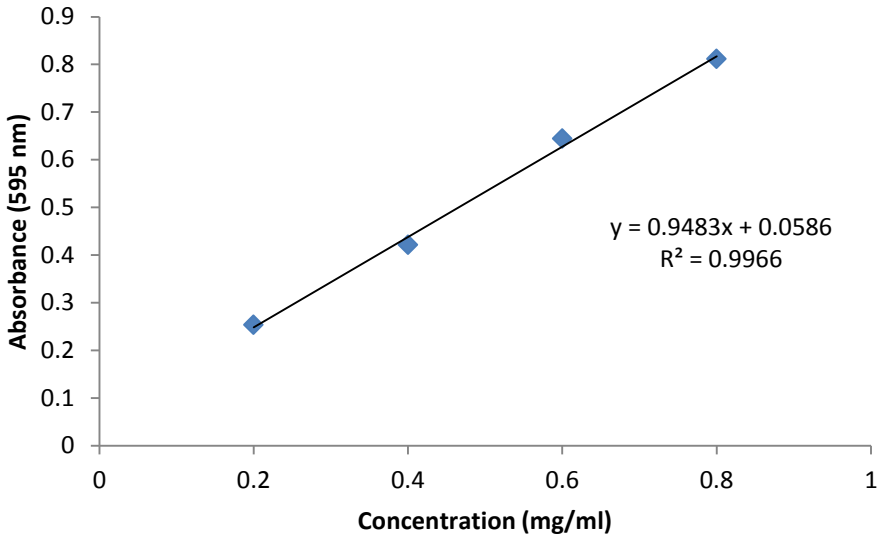
APPENDIX A

Standard curve for protein concentration assay

(Bovine serum albumin (BSA) as standard)

Concentration (mg/ml)	Absorbance (595 nm)
0.2	0.2537
0.4	0.4215
0.6	0.6441
0.8	0.8117

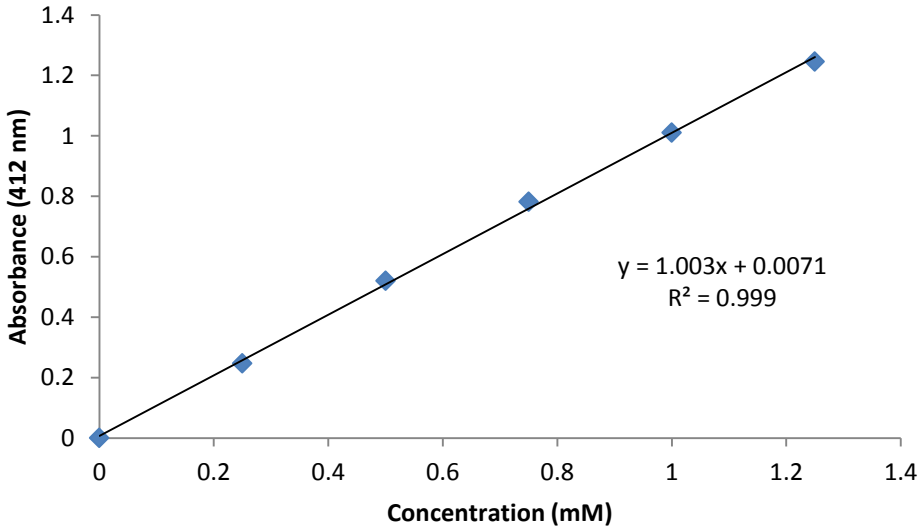


APPENDIX B

Standard curve for free sulfhydryl group content assay

(L-Cysteine hydrochloride as standard)

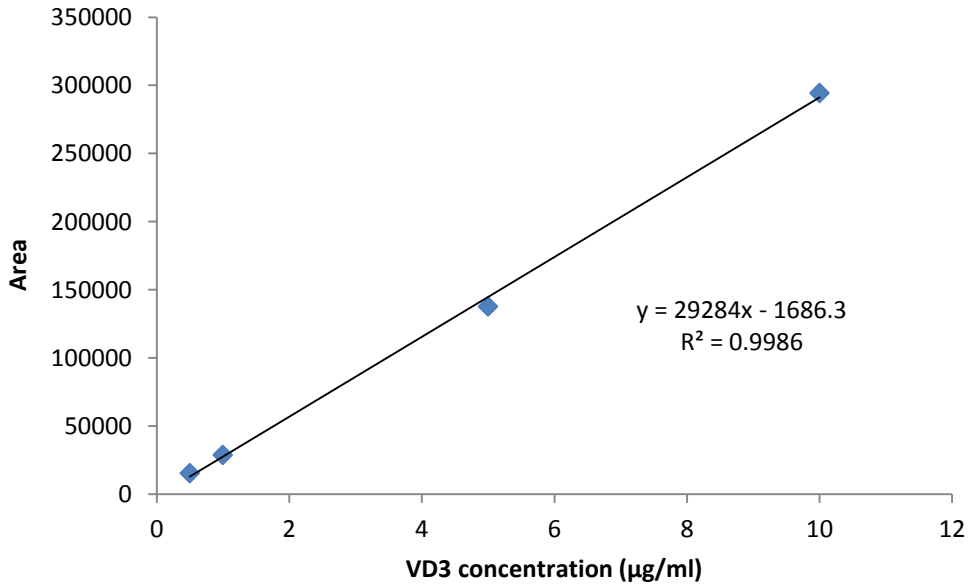
Concentration (mM)	Absorbance (412 nm)
1.50	1.367
1.25	1.245
1.00	1.010
0.75	0.782
0.50	0.520
0.25	0.247
0	0



APPENDIX C

Standard curve for vitamin D3 content determination

VD3 concentration (µg/ml)	Area of peak
0.5	15437
1	28885
5	137704
10	294419



APPENDIX D

pH profile of soluble PPI

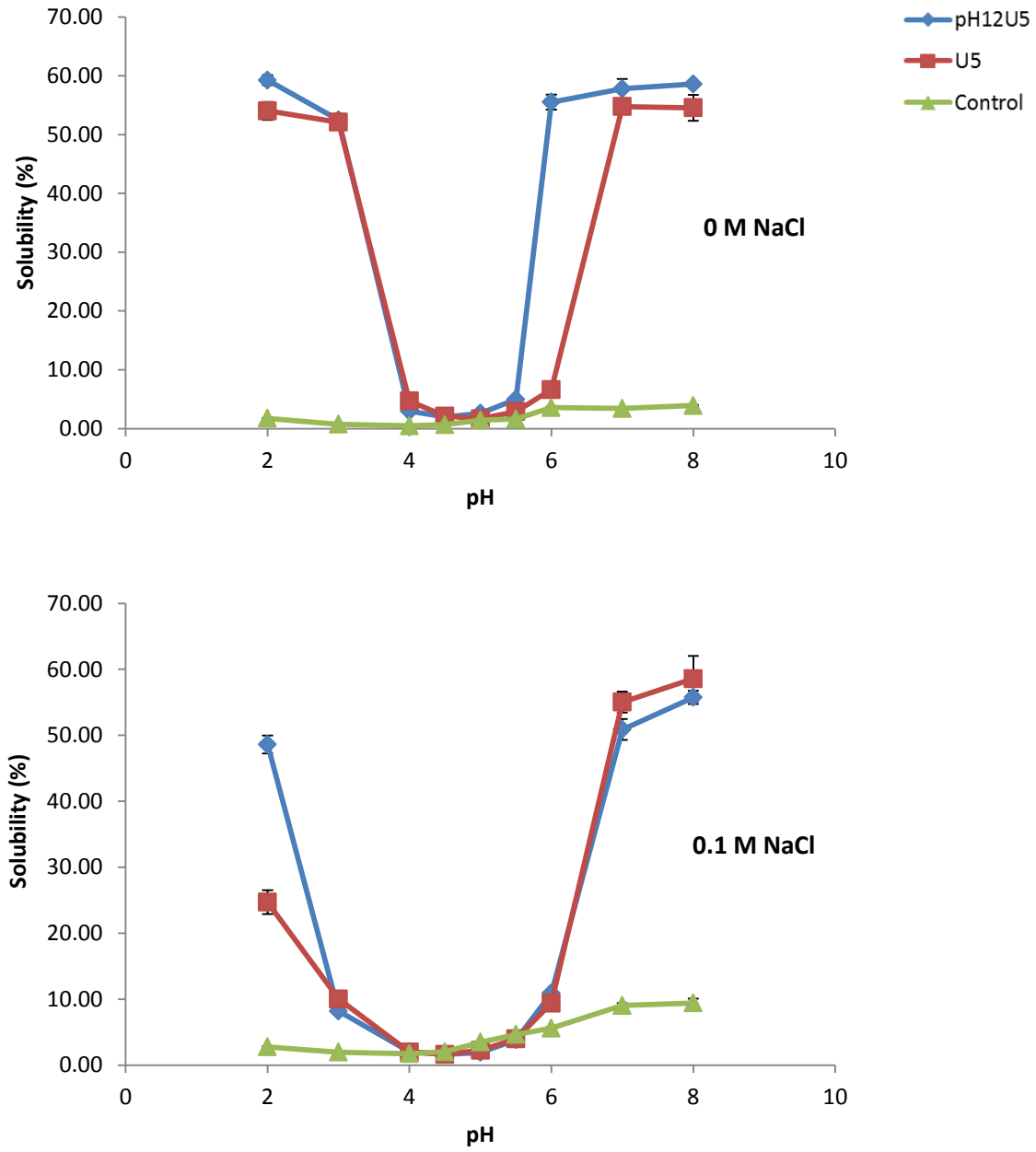
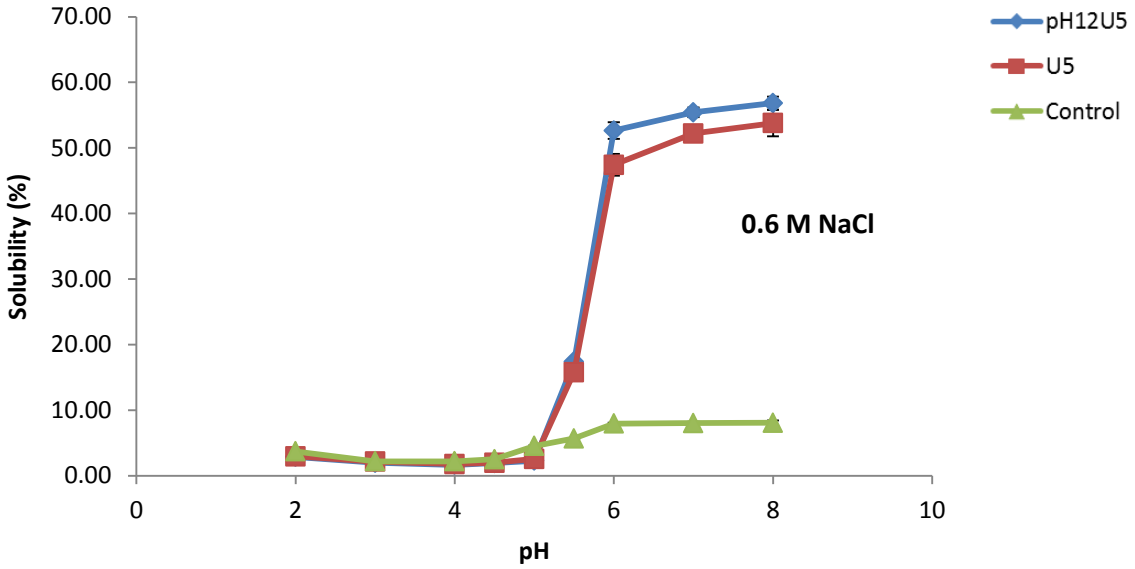


Figure D.1 pH-dependent protein solubility profiles of native and treated pea protein in different salt solutions (0, 0.1, and 0.6 M NaCl).

Appendix D (cont.)

Figure D.1 (cont.)



APPENDIX E

Images of equipment

Figure E.1 VC 750 ultrasonic processor (20 kHz) and ultrasonic probe (13 mm diameter).

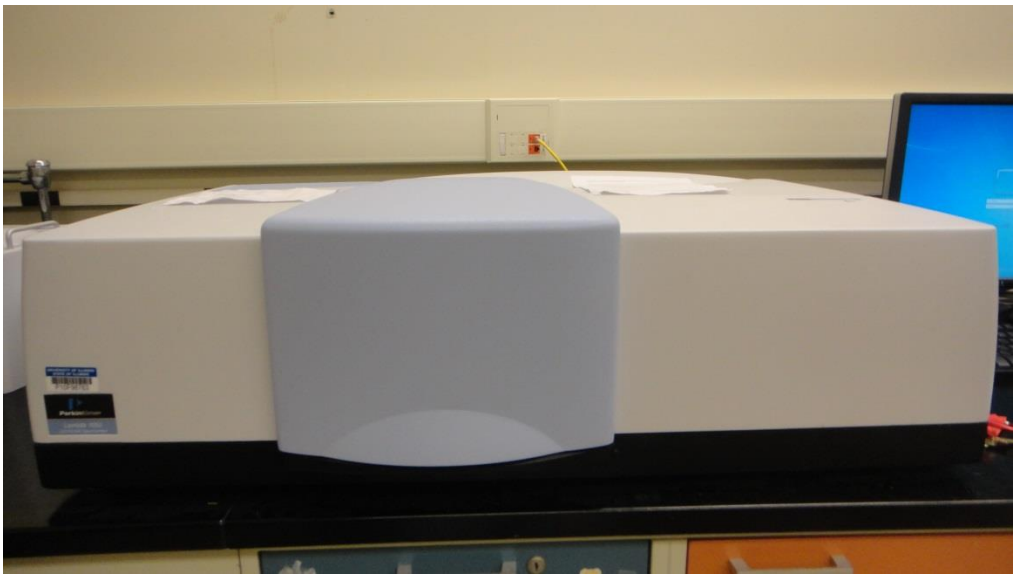


Figure E.2 Spectrophotometer (Lambda 1050 UV/VIS/NIR Spectrometer, PerkinElmer).

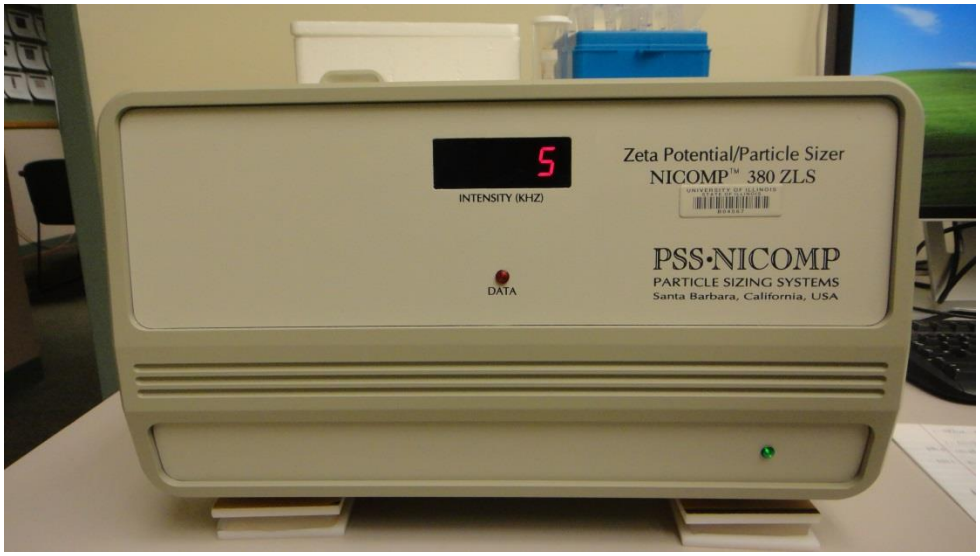


Figure E.3 NICOMP 380 ZLS Particle Sizer.

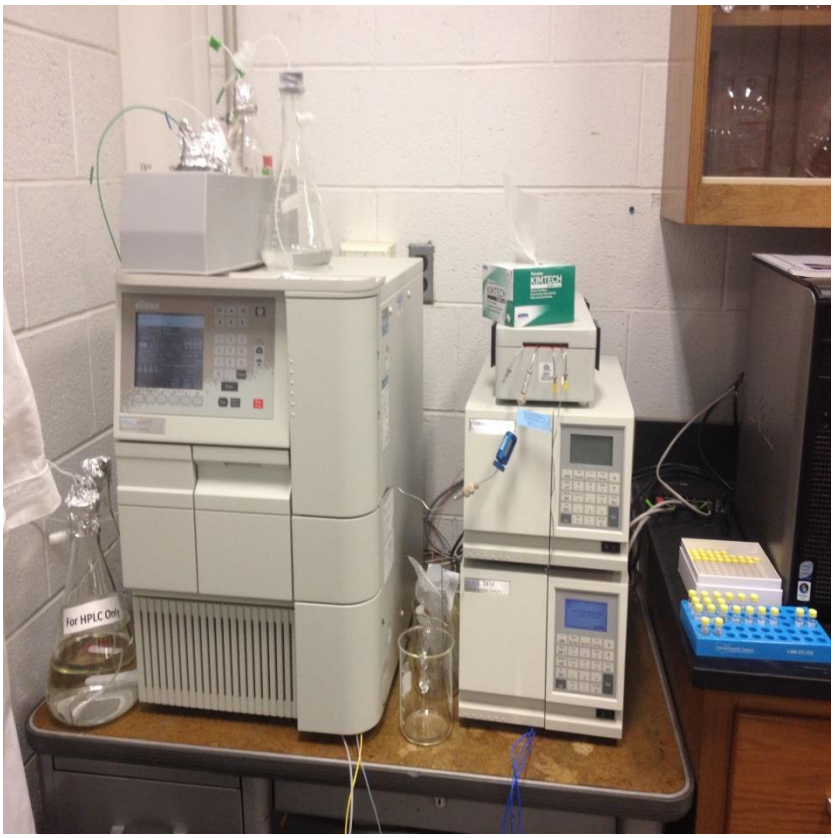


Figure E.4 Waters Alliance e2695 HPLC system with Waters 2489 UV/Visible Detector.

Figure E.5 FisherBiotech™ Ultraviolet Transilluminator (model FBTIV-614).

Figure E.6 Transmission Electron Microscope (TEM).