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THERMAL, INTERFACIAL, AND APPLICATION PROPERTIES OF PEA PROTEIN MODIFIED WITH HIGH INTENSITY ULTRASOUND

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THERMAL, INTERFACIAL, AND APPLICATION PROPERTIES OF PEA PROTEIN MODIFIED WITH HIGH INTENSITY ULTRASOUND

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
College of Agriculture, Food and Environment
at the University of Kentucky

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ABSTRACT OF THESIS

THERMAL, INTERFACIAL, AND APPLICATION PROPERTIES OF PEA PROTEIN MODIFIED WITH HIGH INTENSITY ULTRASOUND

The overall objective of the study was to investigate different food ingredient conditions and ultrasound treatment on pea protein in terms of surface morphology and thermal characteristics. The motivation of this work was based on previous studies focusing on non-chemical physical modifications of plant proteins and the increasing demand for functional alternative proteins.

Ultrasonication time and amplitude, pH, protein concentration, and salt concentration all influenced the thermal and interfacial properties of pea protein. Ultrasound treatment altered the quaternary and tertiary structure of the storage protein and disrupted non-covalent bonds. The structural altercations and a reduction in particle size led to improved functionality.

For foams generated at pH 5.0 with 4% (w/v) ultrasound treated protein, the foams had acceptable capacity and stability even when high levels of sugar (5% sucrose) and salt (0.6 M) were incorporated. An acceptable angel food cake simulation can be achieved by replacing egg white with ultrasound treated pea protein. Color and loaf height were different, but similar texture profiles were achieved.

Ultrasound treatment significant improved the emulsifying capacity (up to 1.4 fold), emulsion stability, and creaming index compared to control samples (no ultrasound) over two weeks. The ultrasound treated emulsion yielded lower TBARS values, likely due to the change in exposed protein reactive groups.

These findings demonstrate that ultrasound processing is an effective nonchemical method to change the structural and physiochemical properties of pea protein. Pea protein processed with this method might allow for the functionality in a bakery, dressings, or beverage products, which is appealing to many consumers and manufacturers.

KEYWORDS: pea protein, foaming, thermal properties, angel food cake, emulsion

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THERMAL, INTERFACIAL, AND APPLICATION PROPERTIES OF PEA PROTEIN MODIFIED WITH HIGH INTENSITY ULTRASOUND

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CHAPTER 1.

Introduction and Thesis Objectives

Peas are seeds from the *Pisum sativum,* which is a species of legume high in carbohydrates, protein, and fiber, and low in lipids. Pea protein has become an important functional and nutritional ingredient in the food and beverage industry as a novel or alternative protein source to traditional proteins such as dairy, soy, egg, and wheat proteins (Wang, Hatcher, Tyler, Toews, & Gawalko, 2013). This shift is driven by a desire for ingredient flexibility, moral preferences, allergies, and genetic modification concerns. Plant proteins are underutilized ingredients and would benefit from research of functional and structural modifications (Adebiyi & Aluko, 2011).

Pea protein has many of the same properties that have made soy protein the dominant plant protein for decades. Compared to soy protein, pea protein is more resistant to genetic modification and has a lower allergen potential (De Graaf, Harmsen, Vereijken, & Mönikes, 2001). Pea protein is not without fault and suffers from some issues related to water solubility, acid solubility, bitter taste, beany aroma, and poor functionality relative to traditional proteins (Klemmer, Waldner, Stone, Low, & Nickerson, 2012). Many strategies have been investigated to enhance the properties of plant proteins. Much research has been done on methods of modification such as physical, chemical, and biological (Arzeni, Martinez, Zema, Arias, Perez, & Piloof, 2012; Boye, Aksay, Roufik, Ribereau, Mondor, Farnworth, & Rajamohamed, 2010; Klassen & Nickerson, 2012). Pea protein is primarily composed of storage proteins which contain compact tertiary and quaternary structures which are stabilized by disulfide bonds,

hydrogen bonds, Van der Waals interactions, and hydrophobic conformations (Lam, Karaca, Tyler, & Nickerson, 2018). The compact nature of pea proteins provides resistance to structural and chemical changes. Effective modification methods must be capable of disrupting these compact structures without destroying the protein.

Ultrasound treatment is a non-thermal physical process that has been shown to be effective in multiple food operations. Ultrasound has shown promising results in a variety of applications, including improving food preservation, thermal treatments, and the modification of textures and viscosity (Awad, Moharram, Shaltout, Asker, & Youssef, 2012; Kentish & Feng, 2014; Vilkhu, Mawson, & Simons, 2008). How ultrasound treatment impacts pea protein functionality has just begun to be studied. Most research focuses on emulsion and foaming property enhancement but with little attention to food applications.

This study attempts to test the thermal and interfacial properties of pea protein after physical modification by high power ultrasound and under various food conditions. The end purpose of these experiments is to enhance the understanding and application of pea protein in beverage and bakery applications. To function as a successful substitute for, or alternative to, main stream proteins such as soy protein, pea protein must be capable of having good emulsifying activity and foaming capacity, as well as reasonable stability while in complex solutions with salt and sugars. For incorporation in beverage products, pea protein must have the ability to bind water and improve textural properties of food. In this project, the changes to pea protein functionality were tested in angel food cakes and model emulsion systems.

It is hypothesized that pea protein modification by high intensity ultrasound treatment could change the physicochemical properties of pea protein. The cavitations mechanism could reduce protein aggregate size, disrupt quaternary and tertiary structures, rearrange conformation and lead to improved protein functionality. To test these hypotheses, the following objectives were proposed for this thesis study:

1) To evaluate the changes in thermal properties, particle size, and aggregation patterns under different food ingredient conditions;
2) To analyze the influence of ultrasound processing on protein structure and thermal properties via solubility measurement, particle size, surface tension, surface sulfhydryl groups, surface hydrophobicity;
3) To test the foaming ability of ultrasound treated pea protein and the ability to replace egg in angel food cake, focusing on product texture and color;
4) To investigate the impact of ultrasound treatment on ability of pea protein to function as an emulsifier in sunflower oil water emulsions.

CHAPTER 2.

Literature Review

2.1. Introduction to Peas

Yellow and green peas (*Pisum sativum L.)* are legume seeds and are part of a group of plants known as pulses. Lentils, beans, and peas are high in protein content and have often been used in supplemental diets for children, animal feed, and extruded products (Aguilera & Kosikowski, 1976; Akinyele, Love, & Ringe, 1988). Peas are grown throughout the world and are being investigated for a variety of applications, such as animal feed, gluten-free starch, and traditional protein replacement. Concentrate and isolate pea proteins are used for their functionality in food systems. The demand for protein is projected to be doubled by 2050, triggering concerns over sustainability, availability, and food security (Henchion, Hayes, Mullen, Fenelon, & Tiwari, 2017). Plant-based proteins have the potential to meet this growing demand and researching their functionality and modification can increase their usefulness.

2.2. Allergen Status

It is estimated that half of all protein sources will be hypoallergenic by the year 2054 (Tarver, 2016). Milk is the most common allergy in population before age 16 (Branum & Lukacs, 2008). Milk is a household staple for a majority of the population, but total dairy sales are decreasing. Milk is being replaced by non-dairy alternatives with 56% of consumers switching to plant-based milk products (Mintel, 2018). Eggs are the second most common food that triggers allergic reactions in adults, and the CDC displayed that children are more susceptible to egg allergies (Branum & Lukacs, 2008).

Egg allergies are often believed to disappear after puberty, but recent studies have shown that a significant amount of children keep an egg allergy throughout adulthood (Pablos-Tanarro, Lozano-Ojalvo, Molina, & López-Fandiño, 2018). There is little available allergen data on the prevalence of allergy to peas. Food allergies in the United States impact 2% of adults and 4-8% of children, of these allergies, pea allergies are estimated to be less than 1% (Branum & Lukacs, 2009; Goldstein & Goldstein, 2009). Pea proteins are not classified as major food allergens in both the United States and European Union (Lefranc-Millot, 2018; Sell, Steinhart, & Paschke, 2005). Peas contain no gluten or lactose, and are therefore appealing to those with celiac's disease, gluten sensitivities, or lactose intolerance. As demand for protein increases, so will demand for low allergen protein sources. Investigation into the replacement of egg and milk proteins by pea protein could benefit at-risk populations.

2.3. Carbohydrates, Lipids, Trace Compounds

Peas are composed of carbohydrates (35-40% amylopectin; 24.0-49.0% amylase) and dietary fiber (10-15% insoluble and 2-9% soluble) in the range from 60 to 65%, which also includes non-starch polysaccharides such as sucrose, oligosaccharide, and cellulose (Dahl, Foster, & Tyler, 2012; Simsek, Tulbek, Yao, & Schatz, 2009). Pea carbohydrates, their function, and the roles they play in a variety of systems have been studied in depth (Hood-Niefer & Tyler, 2010; Lu, Donner, & Liu, 2018; Nielsen, Sumner, & Whalley, 1980; Penetrometer, 1983; Periago, Vidal, Ros, Rincón, Martínez, López, et al., 1998; Wang, Bhirud, Sosulski, & Tyler, 1999). Depending on growing conditions, time of harvest, and species, peas other constituents are 1.5-2% lipids, and less than 1% of anti-nutrients, vitamins, and minerals. Anti-nutrients, such as saponins,

phytate, and lectins are often found within the pea seed. These anti-nutrients can be reduced with heating, chemical and physical treatments (Josephine & Janardhanan, 1992).

2.4. Pea Proteins

Pea proteins can be found in a variety of forms (flour, concentrate, and isolate). Concentrates contain 50% protein content, while isolates will have 70-90% protein depending on protein extraction technique (Stone, Karalash, Tyler, Warkentin, & Nickerson, 2015). Protein concentrates can be generated from de-hulled peas and air classification (Schutyser, Pelgrom, Van der Goot, & Boom, 2015). Isolates can be generated through systematic spray drying or iso-electric precipitation (Aluko, Mofolasayo, & Watts 2009).

The amino acid composition of pea proteins varies based on preparation, pea protein concentrate has a protein digestibility-corrected amino acid score (PDCAAS) of 54.07 and pea protein isolate has a score of 52.56. As a comparison protein isolates from soy, lentil, fava bean have scores of 100, 68.14, and 43.29 respectively (Nosworthy, Tulbek, & House, 2017). Pea albumins contain more essential amino acids (tryptophan, lysine) compared to the globulin fraction which contain higher amounts of phenylalanine, and isoleucine (Swanson, 1990). Pea protein's limiting amino acids are methionine and cysteine (Aluko, Mofolasayo, & Watts 2009).

The plant globulins from soy, wheat, rice, and pea share similar secondary structure, high amounts of β-sheet and low in α-helix (Tang, 2017). Plant globulins are classified as a β-type protein (Lin, Tay, Yang, & Li, 2017).

The protein content of peas varies from strain to strain but on average is 23.1-30% in the seed. Pea proteins can be classified by their solvent solubility. Albumins are the major water-soluble protein and comprise 10-20% of the total protein content. Globulins are salt-soluble storage proteins composing 70-85% of the total protein content. Globulins are further classified into legumin and vicilin proteins (Fig 2.1). Legumin and vicilin are similar in structure and primary structure as glycinin and β-conglycinin found in soy (Duranti & Gius, 1997). Prolamins and glutelins are other minor storage proteins found in peas (Saharan & Khetarpaul, 1994).

Legumin is a hexamer protein (300-400 kDa) and a sedimentation coefficient of 11S. Within the hexamer, each of the six subunits is composed of an acid-basic subunit linked by a disulfide bond (Mertens, Dehon, Bourgeois, Verhaeghe-Cartrysse, & Blecker, 2012). The acidic subunit is composed of glutamic acid and contains an N-terminal group of leucine, while the basic chain contains higher amounts of alanine, and leucine and has glycine at the N-Terminal (Shand, Ya, Pietrasik, & Wanasundara, 2008). The hydrophobic amino acids are located in the molecule's interior, while acidic amino acids generally locate acidic amino acids on the exterior of the molecule. As a storage protein, the quaternary structure is compact and heat-stable (Lam et al., 2018). Thermal transition starts around 90 °C depending on conditions. Pea legumin keeps the hexamer quaternary structure at neutral pH and strong ionic strength (0.1 M NaCl), but will disperse at extreme pH into monomers, dimmers, and trimers. Complete dissociation can be achieved at and below pH 2.5 and above 12.0 (Gueguen et al., 1988). The legumin amino acid profile is notable for its cysteine residues which allow for disulphide bonds (Shewry, Napier, & Tatham, 1995)

Vicilin proteins are trimers (150-170 kDa) and with a 7S sedimentation coefficient. Each monomer of the trimer is composed of a 50 kDa subunit with three parts held together by hydrophobic interaction (Shand, Ya, Pietrasik, & Wanasundara, 2008). N-terminal amino groups are represented by serine, glutamic acid, and aspartic acid (Sikorski, 2001). Pea vicilin lacks cystiene and cannot form disulphide bonds (Shewry, Napier, & Tatham, 1995). The thermal transition varies on ionic strength, around 70°C at low salt concentrations and 80°C under high concentrations (Kimura et al., 2008).

The functional properties of a protein vary on intrinsic and extrinsic factors, the most important is solubility, which directly impacts the proteins ability to function as a foaming, emulsification and gelation agent.

2.5. Protein Functionality

The functional properties of a protein vary on intrinsic and extrinsic factors, the most important is solubility, which directly impacts the proteins ability to function as a foaming, emulsification and gelation agent.

2.5.1. Solubility

Protein solubility is the most important functional property for a potential food protein. Solubility is the equilibrium between hydrophobic and hydrophilic regions' interaction with the solvent. In water, hydrophilic amino acids are attracted toward the solvent while the hydrophilic are oriented away from the solvent to reduce free energy. Hydrophobic areas unable to be buried in the protein's interior reduce solubility (Damodaran, 2008). Protein needs to be soluble to be functional in food systems; other properties such as foaming, gelation, and emulsification are impacted by the solubility of

proteins (Bera & Mukherjee, 1989). In aqueous solution, pea proteins exist in a folded storage conformation with most of the hydrophobic amino acids within the protein structure. This association is often followed by a decrease in Gibb's free energy. Because of steric hindrance and protein-protein repulsion, a smaller percentage of hydrophobic amino acids are located in patches on the surface of the protein.

Solubility is influenced by extrinsic factors, especially pH, temperature, ionic strength, and total concentration. The lowest solubility is found at the isoelectric point (pI), the point at which the protein carries a zero net charge, resulting in limited electrostatic repulsive forces between proteins (Pelegrine & Gasparetto, 2005). Hydrophobic interaction at the pI can cause aggregation and eventual precipitation (Mahadevan & Hall, 1990). Solubility increases at pH above and below the pI because of increased electrostatic repulsion.

Salt concentration is a major factor influencing protein solubility, hence, functionality. Salt denaturation is attributed to the binding or interaction of salts with charged residues. The binding of salts increases the net charge of the protein, increased repulsive forces, decreased conformation stability, and an increase in Gibb's free energy (Ragab, Babiker, & Elitnay, 2004). The presence of salts can act as a double layer around the protein, reducing the electrostatic repulsion forces, but at too high concentrations will result in aggregation. The type and concentration of salt dictates how it will impact protein solubility. Thiocyanate, barium and calcium salts have been shown to assist in protein-water solubility by forming hydration layers (Mahadevan & Hall, 1990). Ammonium and potassium salts disrupt the hydration layer and result in a loss of solubility. Chloride salts have been shown to induce denaturation at lower concentrations

than citrate, sulfate, and phosphate salts (Shih, Prausnitz, & Blanch, 1992). Magnesium and calcium salts have been shown to depress total protein solubility as concentration increases. The interaction between most salts and protein occurs at hydrophobic patches or charged amino acids on the surface of proteins (Inyang & Iduhm, 1996).

Pea protein displays a typical u-shaped pH-solubility, with moderate solubility below the pI and higher solubility above. The pI of legumin was found to be at pH 4.8 (α-chain: pH 4.5–4.9; β-chain: pH 8.4–8.8), and at pH 5.5 for vicilin (Aluko, Mofolasayo, & Watts, 2009). Other studies have shown that pea protein solubility can be improved by pH shifting, ultrasonication, combination with carbohydrates, and chemical modification (Adebiyi & Aluko, 2011; Farnworth et al., 2010; Liu, Elmer, Low, & Nickerson, 2010).

2.5.2. Emulsification

Emulsions are a mixture of two immiscible liquids stabilized by an emulsifier and are present in communicated meat products, bakery batters, mayonnaises, and dressings (McClements, 2015). Emulsifiers are molecules that interface between the two liquids and prevent the separation of the liquids from occurring. Successful emulsifiers are often amphiphilic and surface active. For proteins to function as an effective emulsifier, it requires the proper balance between polar and non-polar residues, solubility, surface hydrophobicity, and stability in solution (Belitz, Grosch, & Schieberle, 2004). Smaller particle size, high surface activity, surface charge, solubility, and flexibility are correlated with improved emulsifying characteristics (Sharif et al., 2018). Globular proteins are more rigid and require more time to associate at the water-oil interface. Emulsions at the pI and high ionic strength are weakened because of the suppression of electrostatic repulsion (McClements, 2015). Oil selection and protein processing can influence

stability, unfolded proteins have more hydrophobic groups exposed, and more polar oils allow for more favorable associated.

Emulsions can be measured by the emulsifying activity index (EAI), which measures the area that can be stabilized per weight unit of protein. The ability for the emulsion to resist collapse and separation is known as the emulsion stability index (ESI). Emulsion capacity (EC) is the measurement of the maximum amount of oil that can be trapped by the weight unit of the protein (McClements, 2015). Measurement methods vary between authors and values often reported with different units, making comparison less direct.

Pea protein emulsion characteristics have been investigated by several researchers (Gharsallaoui, Saurel, Chambin, Cases, Voilley, & Cayot, 2010; Humiski & Aluko, 2007; Johnson & Brekke, 1983; Liang & Tang, 2014). In unprocessed pea protein, vicilin (7S) displays better emulsifying properties than legumin (11S). The flexible nature of vicilin allows for favorable rearrangement of the adsorbed-proteins at the water-oil interface (Tang, 2017). pH has a major impact on the emulsification functionality. The lowest quality emulsions are at the pI, above and below the pI increase as protein's ability to dissociate and become more amphiphilic. Surface hydrophobicity has been shown to be linked to higher emulsifying properties in a variety of legume proteins. Commercial pea protein was reported to have a higher ESI value at neutral and alkaline pH compared to acidic pH, and this was attributed to cohesiveness of interfacial protein layer (Aluko, Mofolasayo, & Watts, 2009). Under acidic conditions, the drop in ESI was due to decreased solubility and a more folded protein structure.

Pea protein is often compared to soy protein. Results are varied, early studies showed that pea protein was less effective as an emulsifier than soy but was still capable of use in mayonnaise emulsions (McWatters & Holmes, 1979). A comparison of soy and pea showed similar EAI and ESI across a variety of pH conditions (Barac, Pesic, Stanojevic, Kostic, & Bivolarevic, 2015). Most investigations had a high variance because of genotype differences, processing, and extraction conditions. Freeze-dried samples were shown with lower EAI and ESI compared to spray dried pea protein (Hoang, 2012). The authors attribute this to partial unfolding during processing. It has been reported that NaCl addition will increase emulsion ability but lower stability with increasing concentrations (Tian, 1998).

2.5.3. Gelation

Protein gelation is one of the most important functional properties used to change the structure and texture of foods. Examples of gelation can be seen in confectionary, meat products, bakery, and egg products. The texture of foods and consumer acceptance is closely linked (Szczesniak, 2002). Matrix formation in a protein gel system is essential to moisture retention, stabilization of phases, and flavors. Protein matrixes are classified into two categories: random aggregate opaque gels and ordered aggregates with high degrees of transparency (Hermansson & Langton, 1988).

Globular proteins such as egg proteins and pea proteins are capable of gelation upon heating (McWatters & Holmes, 1979). Gel formation depends on hydrophobic groups on the interior exposure and ability to interact and develop a 3-D network. Gel formation depends on concentration, water amount and availability, ionic strength, time, temperature, pH, and co-solutes (Raikos, Campbell, & Euston, 2007). The general

process is that native protein is heated until denaturation; during denaturation, S-S bonds are formed and the hydrophobic interior is exposed. Proteins then aggregate and develop into a matrix that depends on protein concentration, processing temperature, and time. Evidence suggests that proteins unfold without the breaking of covalent bonds and then interact via hydrogen, covalent, ionic, electrostatic, and hydrophilic bonds (Clark, Kavanagh, & Ross-Murphy, 2001). Pea protein has been reported to have inferior gelling properties compared to soy proteins. Pea proteins were found to form unstructured gels; more of a paste instead of a rigid gel with lower elasticity has been reported (Adebiyi & Aluko, 2011; Sun & Arntfield, 2010). It has been reported that pea variety can play a large role in gelation; Solara peas were found to be able to form turbid gels at a minimum protein concentration of 10% (w/v) while Supra peas minimum gel concentration was 14% (w/v) and produced transparent gels. As noted above, pH and salt concentration change the gelation characteristics of pea protein. The firmest gels were found to form at pH 4.0 in 0.3 M NaCl (Sun & Arntfield, 2010).

2.5.4. Foaming

Foam is generated by the entrapment and dispersion of a gas in a continuous or semi-solid phase. The two properties used most often to describe foams are foaming ability and foam stability. The foaming ability is a measurement of how much gas can be incorporated in a fixed volume of solution. The foam stability of a solution is defined by the ratio of bubbles development to the disappearance of bubbles via coalescence or collapse. Bubbles are generated in different ways; mechanical whipping, super saturation of a liquid with gas (soda), fermentation (Hailing & Walstra, 1981). In this thesis, mechanical whipping was chosen as it is most similar to potential industrial application.

Once foam is formed, bubbles will combine and aggregate because of gravity and density changes. Disproportionation is the shrinking of small bubbles into larger bubbles because of differences in pressure. Gas will diffuse from small bubbles into larger bubbles. As bubbles combine, liquid drains through the channels between the bubbles. This process can be slowed down by increasing the viscosity of the solution. The stability of foam is defined by the matrix that originates between the coalescence of bubbles. Strong matrixes have been linked to a balance between electrostatic repulsion and attractive forces (hydrogen bonds, Van der Waals) (Parnell, Feeding, Luck, and Davis 2002).

Protein foams are dependent on several principles and structural changes of protein subunits. The adsorption of protein at the air-water interface, the orientation of adsorbed proteins at the air and water interface, and the development of a cohesive matrix with other proteins stabilized by hydrogen bonding, electrostatic, and hydrophobic attraction (Li, Le Brun, Agyei, Shen, Middelberg, & He, 2016). How well proteins interact at the air-water interface is predicated by the properties and the conditions of the solution, which dictate the foaming properties. During foam generation, proteins are subject to structural changes which increase viscosity, elasticity, and strength due to protein aggregation and coagulation, excessive structural changes will lead to destabilization of the foam (Kinsella, 1981). The optimal proteins to form and maintain foams have a low molecular weight, high surface hydrophobicity, acceptable solubility, and ability to be modified (Damodaran, 2008). Pea proteins are primarily globular proteins and have the hydrophobic amino acids within the core of the protein. Exposure of hydrophobic regions can increase the surface activity of proteins (Murray, Durga,

Yusoff, & Stoyanov, 2011). The balance of electrostatic forces between proteins is critical to foaming capacity and foam stability. It was found that the most stable foams were generated at the pI, as protein-protein interaction is at its highest. Stable proteins require rapid adsorption to the interface and must be elastic to allow for some deformation (Suo, Jin, Jiang, Dayton, & Jing, 2017).

Native pea protein has been shown to have the best foaming properties at pH 5 and 7 (Fuhrmeister & Meuser, 2003). The foam stability was shown to be greater than soy protein at pH 5.0. Pea protein was found to be more flexible than soy protein at pH 3.0 and 7.0 (Aluko, Mofolasayo, & Watts, 2009). Processing conditions can modify the protein conformation, protein size, and solubility, thereby impacting foaming properties. Ultra-filtration has been shown to yield a foaming capacity (FC) of 95-105% (Boye et al., 2010). Our preliminary study showed that ultrasound treatment of pea protein could improve the foaming activity from 58% to 73.3% with increased amplitude. Soy protein shows less stability in a wide range of pH (3-8) than pea protein (Barac et al., 2015). Pea protein that was freeze-dried has been showed to have lower FC and foam stability (FS) values than spray-dried, which is attributed to changes in protein solubility (Hoang, 2012). The same authors showed that treatment with transglutaminase can improve FC and FS regardless of extraction method.

The acceptability and physicochemical of bakery products such as cakes, muffins, and meringues rely on the foaming properties of their raw ingredients. Eggs are a key ingredient in bread and cake baking. The texture of cakes is defined by the ability of the protein to generate large foam and coagulate into an ordered matrix (Abu-Ghoush, Herald, & Aramouni, 2010).

2.6. Ultrasound Processing

Ultrasound is a novel processing aid that has been used in a variety of industrial sectors for many years. The low energy high frequency is often used in medical imaging and as an analytical technique in the food industry to measure the structural, textural, and composition of food (Vilkhu et al., 2008). Low frequency high energy ultrasound is used in the modification of properties of food ingredients. The focus of this review will be on low frequency high energy, as is what is most commonly used in ingredient modification.

The application of ultrasound within the food industry is a developing field. Most applications are liquid-liquid and solid-liquid applications because of the ease in which ultrasonic waves can transfer in liquid mediums. Applications vary from brining, osmotic dehydration, heat transfer, extraction, emulsification, and fermentation (Ojha, Mason, O'Donnell, Kerry, & Tiwari, 2017; Paniwnyk, Alarcon-Rojo, Rodriguez-Figueroa, & Toma, 2017). Additional applications are covered in other, extensive reviews (Chemat, Rombaut, Sicaire, Meullemiestre, Fabiano-Tixier, & Abert-Vian, 2017; Musielak, Mierzwa, & Kroehnke, 2016; O'sullivan, Park, Beevers, Greenwood, & Norton, 2017).

Sound waves of frequency X > 18-20 kHz are classified as ultrasound waves. A transducer is used to convert electrical energy into mechanical energy. In ultrasound, the transducer is referred to as the tip, the point at which acoustic waves are generated. The tip vibrates while submerged and energy is delivered to the medium by acoustic waves (Maruyama, Wagh, Gioielli, da Silva, & Martini, 2016).

When applied to a liquid, acoustic waves are generated, which are thought to be sinusoidal and dependent on frequency and time. The acoustic waves result in the

expansion and contraction of bubbles during the ultrasound cycle (Zhang, Zhu, & Sun, 2018). The implosion of the bubbles results in the generation of turbulence and high particle collisions. Cavitation threshold pressure is the resistance of a material to the generation and propagation of acoustic waves. Viscous material such as honey or a higher concentration of protein have a higher cavitation threshold and thus resists ultrasonic treatment (Atchley, Frizzell, Apfel, Holland, Madanshetty, & Roy, 1988). Acoustic waves are scattered by bubbles as they generate. These bubbles behave like mirrors bouncing acoustic waves causing effective absorption of acoustic waves (O'sullivan et al., 2017). Generation and cavitations is greatest at and near the tip, with exponential decays with distance from the tip. The importance of proper positioning and container size is important for adequate processing (Jawale & Gogate, 2018; Sancheti & Gogate, 2017)

An established application of high power ultrasound is the reduction of particle size of a variety of protein aggregates (soy, black bean, mung bean, pea, wheat) and improvements to solubility (Cheng, Zhang, Xu, Adhikari, & Sun, 2015; Dangvilailux & Charoensuk, 2017; McCarthy, Murphy et al., 2016; Suo, Jin, Jiang, Dayton, & Jing, 2017). Size reduction of aggregates is associated with structural changes and disruptive of non-covalent interactions. Ultrasound treatment does not seem to cause lysis of the primary structure for a large number of proteins. The distance between adjacent protein aggregates is increased upon size reduction, decreasing the bulk viscosity. Besides the physical effect, radicals H• and •OH can be generated (Ince, Tezcanli, Belen, & Apikyan, 2001; Hu et al., 2013).

Ultrasound treatments of proteins can cause changes in quaternary and tertiary structures, resulting in a modification of the functional parameters of the proteins (Fig. 2.2). Potential changes include reduction of viscosity, increased surface hydrophobicity, improvements to emulsion stability and ability, foaming capacity and stability, and gelation. The disruption of non-covalent forces resulting in the dispersal of aggregates, energy is often not enough to lysis peptides. Most experiments and understandings on ultrasound treatment are performed at lab scale; further work is required to understand changes needed when scaling up to the food industry.

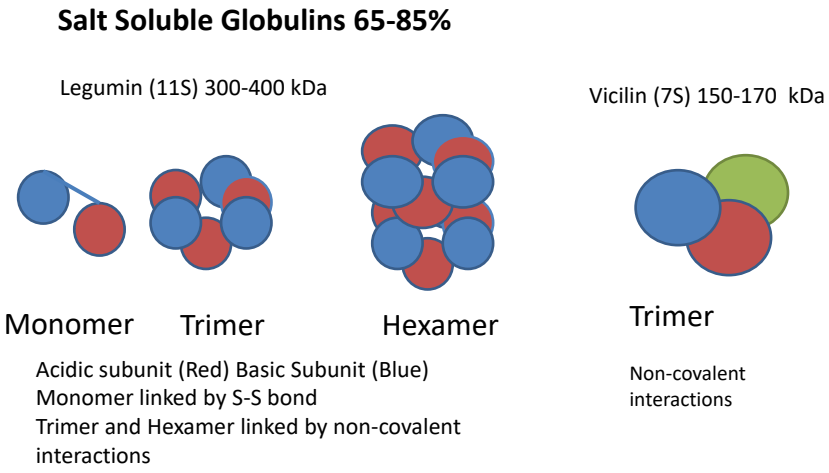

Figure 2.1. Major globulins in pea protein.

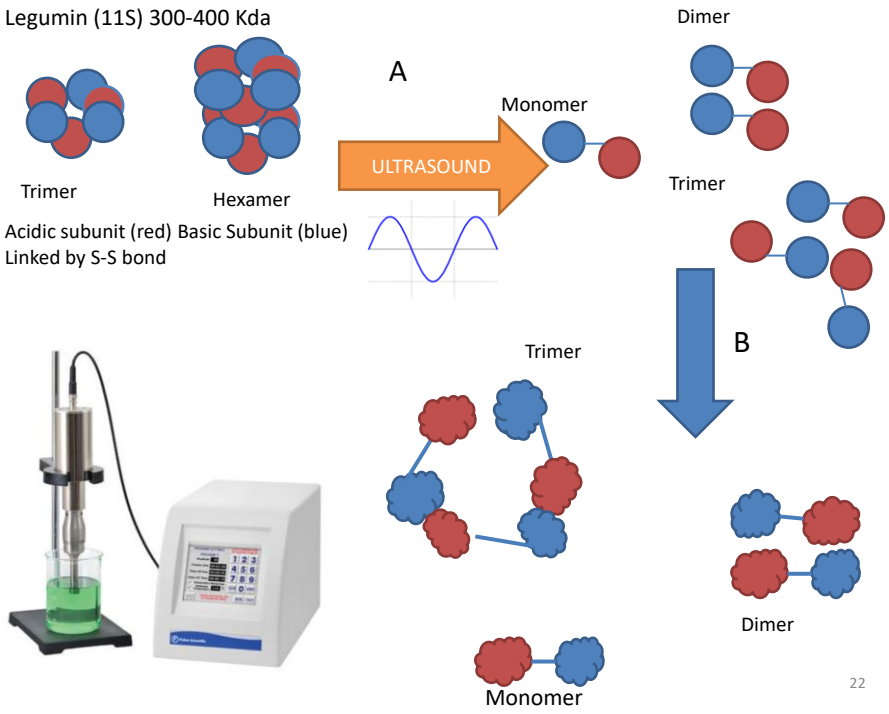

Figure 2.2. Possible ultrasound effect on pea storage globular proteins.

CHAPTER 3.

Effects of pH and Chloride Salts on The Thermal Stability and Aggregation of Pea Protein

Summary

The thermal stability and aggregation properties of pea protein isolate (PPI) were evaluated. Pastes of PPI were adjusted to pH 4–8, and the PPI at pH 6.0 was treated with 0, 0.1, and 0.6 M NaCl or 0, 5, 10, 50, 100, and 200 mM $CaCl_2$. Differential scanning calorimetry (DSC) was applied to measure the thermal stability of 14% protein samples with a 10°C/min heating rate. Heat-induced aggregation was analyzed on 0.5 mg/mL protein solutions using dynamic turbidity testing (600 nm), and the particle size of the aggregates was measured with a Zetasizer. The DSC analysis showed a trend of decreasing onset (T_0) and maximum (T_{max}) melting temperatures and the enthalpy (ΔH) of denaturation with increasing the pH from 4.0 to 8.0 ($P < 0.05$), suggesting conformation destabilization. Increases in concentrations of NaCl increased the T_0 and T_{max} and lowered the ΔH ($P < 0.05$). $CaCl_2$ addition decreased the ΔH; however the effect was concentration dependant. Increasing the NaCl concentration or $CaCl_2$ concentration rendered the protein vulnerable to aggregation upon heating. Zetasizer results agreed with the turbidity measurements for the divalent salt treatment where the particle size increased from 255 nm (0 mM $CaCl_2$) to above 2000 nm at 200 mM $CaCl_2$ ($P < 0.05$). The results show that heat-induced structural unfolding and aggregation of pea protein are sensitive to pH and vary with the type and amount of salts.

3.1. Introduction

Soy protein dominates the plant protein market, but there is a growing desire for alternative protein sources with similar functional and nutritional characteristics. Globular proteins play a functional role in many foods due to their textural and nutritional value (Sun & Arntfield, 2011). Pea (*Pisum sativum L.*) is a potential alternative protein source with major globulin proteins comparable to soy proteins (Stone, Karalash, Tyler, Warkentin, & Nickerson, 2015). Pea protein is primarily composed of the globular proteins vicilin (7S), legumin (11S), and minor amounts of albumin (2S) (Fig. 2.1). Despite peas inexpensive cost, protein quality, and functionality, peas are underutilized.

Alterations of the protein structure may change the thermal profile, aggregation properties, and particle size. In food thermal processing, pea proteins undergo structural unfolding to expose reactive groups and reaction of unfolded subunits into functional aggregates (Shand, Ya, Pietrasik, & Wanasundara, 2008). The pH, presence of ionic species and their strength, heating temperature, and heating time are main factors that affect aggregation pattern of globular proteins (Matsumura, Chanyongvorakul, Mori, & Motoki, 1995). The structural characteristics of plant proteins have received many studies, but specific research into the thermal properties of pea protein is limited. Knowledge about thermal properties may be useful for appropriate heat processing and product development.

Interactions between pea proteins and other co-solutes may impact their thermal properties. Salts are added to food for a variety of reasons, such as textural modification, functional modification, nutritional value, and sensory characteristics. Calcium is a necessary nutrient and its inclusion has been shown to change the structure and

functionality of plant proteins (Lawal, 2009). The thermal behavior of pea protein has been studied by several investigators, evaluating different extraction methods, milling techniques, heating times, and salt concentrations (Shand, Ya, Pietrasik, & Wanasundara, 2008; Sun & Arntfield, 2011; Sun & Arntfield, 2010). However, few studies have been performed on the impact of divalent salts and pH.

The objective of this experiment was to investigate the effect of different pH, $CaCl_2$, and NaCl concentrations on the thermal and aggregation properties of pea protein. The thermal profiles under different pH (2-8), NaCl (0-0.6 M), and $CaCl_2$ (0-200 mM) concentrations were analyzed. To examine the aggregation behavior, turbidity was measured optically at 600 nm after heat treatment at pH 6.0 under different salt conditions. To confirm the observed aggregation, the particle size was measured to determine size of protein aggregates.

3.2. Materials and Methods

Pea protein isolate (PPI, NUTRALYS® S85F, 80% pea protein based on dry basis) in powder form was provided by Roquette America Inc. (Geneva, IL, USA) or produced using a wet extraction process from dry yellow peas also donated by Roquette (Fig. 3.1). The protein isolate was not further purified but used as is. The PPI was stored in a refrigerator at 4 °C before use. All other reagents and chemicals, including NaCl and $CaCl_2$, were purchased from VWR (Radnor, PA, USA), Sigma-Aldrich (St. Louis, MO, USA), or Fisher Scientific (Pittsburgh, PA, USA) and were of analytical or higher grade.

3.2.1. Mineral analysis

Prior to experimentation the background mineral levels of the laboratory deionized (DI) water and the pea protein samples were tested via inductively coupled plasma mass spectrometry to determine potential interfering elements, specifically divalent salts of magnesium and calcium, and sodium (iCAP 7600 ICP-OES, ThermoFisher Scientific, Waltham, MA, USA). Levels were found to be for all three elements in the PPI and DI water. DI water had X < 0.1 ppm for $Ca^{2+,}$ Mg^{2+}, and Na^{+}. The mineral contents in PPI were 350, 470, and 580 ppm for Ca^{2+}, Mg^{2+}, and Na^{+}, respectively.

3.2.2. Conformational stability (DSC)

Thermal analysis was conducted using a 2920 modulated differential scanning calorimeter (DSC) of TA Instruments (New Castle, DE, USA). Aqueous pastes of PPI (14% protein) were adjusted to pH 4–8 with 1 M HCl or 1 M NaOH, and the PPI at pH 6.0 was treated with 0, 0.1, and 0.6 M NaCl or 0, 5, 10, 50, 100, and 200 mM $CaCl_2$. The PPI paste samples were weighed (approx 17-20 mg) into hermetic anodized aluminum sample pans and heated with a 10°C/min heating rate. An empty pan was used as the reference. Three replications were performed with each sample. The enthalpy of denaturation ΔH (J/g of protein) and the onset temperature T_0 (°C) as well as temperature at maximum transition T_{max} (°C) were calculated with the data analysis software supplied by TA Instruments.

3.2.3. Particle size

The volume-weighted mean diameters ($D_{4,\ 3}$) of soluble protein aggregates were measured by dynamic light scattering (DLS) using a ZetaSizer Nano-S90 (Malvern Instruments Limited, Worcestershire, UK) with the following settings: measurement

angle, 90°; equilibration time, 120 s; number of runs, 3; run duration, 10 s; number of measurements, 3; and delay between measurements, 10 s. Samples were diluted 500-fold with DI water before measurement. The measurement was conducted at 23 °C, and the liquid viscosity and index of refraction was set according to water, which were 0.933 and 1.333, respectively.

3.2.4. Turbidity

PPI solutions (0.5 mg/mL) were treated with 0, 0.1, and 0.6 M NaCl or 0, 5, 10, 50, 100, and 200 mM $CaCl_2$ and the pH was adjusted to 6.0. Solutions were then heated in test tubes (10 x 75 mm) from 30 to 100 °C, removing tubes every 10 °C. Aliquots were cooled to approximately 4 °C in an iced water bath immediately after removal. Cooled protein suspensions were well mixed by inverting the test tube and the absorbance was immediately measured at 600 nm against blank water at room temperature.

3.2.5. Statistical analysis

Data with at least three independent trials (n = 3) each with freshly prepared protein solution was analyzed using the general linear model procedures of the Statistix 10 software package (Analytical Software Inc., St. Paul, MN, USA). Analysis of variance (ANOVA) was performed to determine treatment effect. When significant treatment effects ($P < 0.05$) were found, their means were separated by Tukey's honest significance test.

3.3. Results and Discussion

3.3.1. Conformational stability (DSC)

The two main globular proteins in pea protein are vicilin (7S) and legumin (11S). A single endothermic peak with a T_{max} of 83-88 °C was observed in the thermographs of all experiments (Fig. 3.2). The lack of other endothermic peaks indicates that denaturation might have occurred when manufacturing this PPI, specifically the legumin subunit. As reported by other researchers, the thermal transition of vicilin depends on ionic strength, around 70 °C at low salt concentrations (x < 0.05 M NaCl) and 80°C under high concentrations (x > 0.1 M NaCl) and neutral pH (Kimura et al., 2008). Thermal transition of legumin begins at 90 °C depending on conditions. Pea legumin retains a hexamer quaternary structure at neutral pH and strong ionic strength (0.1 M NaCl) but will disperse at extreme pH into monomers, dimmers, and trimers. Complete dissociation can be achieved at and below pH 2.5 and above 12.0 (Gueguen, Chevalier, & Schaeffer, 1988). Dissociation of subunits into monomers, dimers, and trimers will reduce the enthalpy of denaturation.

The specific thermal profiles of pea protein under various pH are presented in Table 3.1. Adjustments to the pH showed that decreasing the pH resulted in a significant increase of the T_0 temperature, from 73.8 °C to 77.4 °C for pH 8 and 4, respectively. The ΔH increased as the pH became more acidic. The protein-protein repulsion is minimized at the iso-electric point (pI), allowing for more aggregation to occur. The aggregates require more energy (ΔH) to denature due to increased intermolecular forces (hydrophobic interaction and van der Waals forces) resulting in more stability as shown in the increased T_{max} and T_0. These results are similar to what is observed in whey and soy proteins, denaturing at higher temperatures the more acidic the pH (Bernal & Jelen 1985).

The effect of sodium chloride addition is reported in Table 3.2 and Fig. 3.3. As NaCl concentration increased, the T_0 and T_{max} increased while ΔH decreased from that of control (0 M). The increase in T_0 could be attributed to stabilization of the Na^+ and Cl^- ions in the form of a bi-layer, rendering the protein resistant to increasing thermal temperatures. NaCl is theorized to provide charge-shielding reducing protein-protein interactions and supporting hydrophobic arrangements. The increase in the thermal stability with NaCl allows for resistance to thermal denaturation. However, once the salt barrier is overcome, the protein rapidly denatures due to the increased energy at the higher temperature. These observations agree with previous studies showing pea legumin, fava bean, and soybean proteins thermal transition temperatures can be increased at high NaCl concentrations (0.3-0.6 M) (Adebiyi & Aluko, 2011; Artfield et al., 1986; Kimura et al., 2008; Message, Sok, Assifaoui, & Saurel, 2013; Zheng, Matsumura, and Mori, 1993).

Addition of calcium chloride to PPI at pH 6.0 resulted in no significant changes in T_0 and T_{max}. Results can be seen in Table 3.3 and Fig. 3.4. The addition of calcium chloride decreased the ΔH required to denature the protein. The relationship is not linear in nature, but follows the general trend that increasing concentration decreases the energy required. Divalent salts have been shown to destabilize the charges of some plant proteins, promoting denaturation and aggregation by interaction with hydrophobic patches (Shih, Prausnitz, & Blanch, 1992). In soy protein, β-conglycinin has been shown to be destabilized by $CaCl_2$ between 5-20 mM (Speroni, Anon, & de Lamballerie, 2010). The same authors reported a stabilizing effect on glycinin at 0-25 mM concentrations. The concentration effect seems to be specific to each protein's electrostatic interaction.

At lower concentrations Ca^{2+} ions could potentially interact with reactive groups forming cross-bridges within and between protein subunits providing stabilization. At certain concentrations the intermolecular hydrophobic association with ions becomes overwhelming, resulting in destabilization, denaturation, and aggregation.

3.3.2. Particle size

Dynamic light scattering (DLS) measured the size of pea protein aggregates as hydrodynamic diameter. The size is the diameter of a sphere having a comparable translational diffusion coefficient as the observed particle. The particle size of pea protein at different pH is reported in Table 3.1. The largest particles were observed at pH 5. The pI of legumin has been shown to be pH 4.8 (α-chain: pH 4.5–4.9; β-chain: pH 8.4–8.8) and pH 5.5 for vicilin (Aluko, Mofolasayo, & Watts, 2009). The results were as expected because protein-protein repulsion is minimized at the pI allowing for more aggregation to occur.

The particle size increased with salt concentration regardless of salt type (Tables 3.2, 3.3). The increase in particle size with NaCl is attributed to the bi-layer disruption reducing differences between surface charges allowing for increased protein-protein aggregation (Shand et al., 2008). The Ca^{2+}-induced protein aggregation is attributed to electrostatic shielding, hydrophobic interaction, and cross-linking (Li Tay, Yao Tan, & Perera, 2006). The primary mechanism is believed to be that Ca^{2+} interacts with the surface hydrophobic groups, promoting unfolding and aggregation, resulting in large protein aggregates.

3.3.3. Turbidity

Sample turbidity was quantified as absorbance measured at 600 nm and is reported in Figs. 3.5, and 3.6. Lower turbidity has been shown to correlate with smaller particles due to the reduction in light scattering. Sodium chloride concentration did display significant effect on turbidity, which are similar to previously reported values in soy and pea protein (Kimura et al., 2008; Molina & Wagner, 1999). As NaCl concentration increased, turbidity increased (Fig. 3.5). The modification of the bi-layer around charged groups by salts may suppress electrostatic repulsion, resulting in aggregation at higher ionic strength (Damodaran & Kinsella, 1982).

Calcium chloride addition was observed to increase turbidity with increasing concentration. Calcium's electrostatic interactions with protein can cause destabilization (Xiong, 1992). The increased calcium levels resulted in lower ΔH with concentration (Fig. 3.4). Increasing Ca^{2+} concentration resulted in larger particles as shown by particle size measurement (Table 3.3). These larger unfolded protein aggregates correlated with the increase in turbidity (Fig. 3.6). $CaCl_2$ is likely interacting with the hydrophobic patches on the surface of PPI, resulting in partial unfolding and aggregation. These results are similar to turbidity changes found by other researchers (Li Tay, Yao Tan, & Perera 2006; Molina & Wagner, 1999; Sorgentini, Wagner, & Anon, 1995).

3.4. Conclusion

Thermal aggregation characteristics of pea protein were influenced by the ionic strength (salt concentration), ionic species, and pH of the protein pastes. As pH increases (becomes more basic) the thermal stability deceases, requiring less energy to denature. NaCl increases the thermal stability with increasing concentration while the divalent salt,

$CaCl_2$ had the opposite effect on thermal stability. Both sodium and calcium salts resulted in increased particle size and turbidity with increasing concentration. Understanding how pH, salt type, and concentration can impact the aggregation and thermal profiles can allow for optimal application in a variety of systems.

NUTRITIONAL DATA*

(Average values for 100g of commercial product)

	Range	*Unit*	Methods
Dry substance	93	g	Dessiccation at 130°C
Protein	80	g	Dumas
Carbohydrates	**3**	**g**	
- Digestible carbohydrates	2	g	HPLC
- Sugars (mono & disaccharides) of which sucrose	< 0.1	g	HPLC
- Other carbohydrates	-	g	HPLC
- Indigestible carbohydrates		g	
- Dietary fiber	1	g	HPLC
Extractible fat	1	g	Ether extraction
Total Fat	**6**	**g**	Hexane extraction
- Saturated fat	1	g	Gas chromatography
- Mono-unsaturated fat	1	g	Gas chromatography
- Poly-unsaturated fat	4	g	Gas chromatography
- Cholesterol	0	g	Gas chromatography
- Trans fatty acids	0	g	Gas chromatography
Ash	**4**	**g**	Inductively coupled plasma
- Sodium	1.5	g	Inductively coupled plasma
- Phosphore	1.1	g	Inductively coupled plasma
- Potassium	0.2	g	Inductively coupled plasma
- Calcium	0.05	g	Inductively coupled plasma
Calories	395	kcal	
	1653	kJ	

(*): typical values are given for information, and should not be considered as contractual.

FOOD GRADE.

ROQUETTE

Date of issue: 31/08/2010 - 21.2004 - V 7 by Quality Customer Department

ROQUETTE FRERES, 62136 LESTREM FRANCE, TEL 03.21.63.36.00, TELEX 810658 F, TELECOPIE 03.21.63.39.50
SOCIETE ANONYME AU CAPITAL DE 8.812.908 RCS ARRAS 357.200.054 SIEGE SOCIAL 62136 LESTREM FRANCE TVA FR 46357200054

Figure 3.1. Proximate analysis of Roquette pea protein isolate. Data were provided by Roquette America Inc. (Geneva, IL, USA).

Table 3.1. Thermal profile data and particle size for pea protein at different pH*

pH actual & (target)	Particle size (nm)	T_0 (°C)	T_{max} (°C)	ΔH (75-100 °C) (J/g protein)
4.2 (4)	379 ± 45^{A}	77.4 ± 0.2^{A}	87.7 ± 0.2^{A}	10.00 ± 0.82^{A}
5.1 (5)	360 ± 31^{B}	77.7 ± 0.4^{A}	87.8 ± 0.3^{A}	8.70 ± 0.05^{AB}
6.04 (6)	280 ± 21^{C}	77.5 ± 0.3^{A}	87.5 ± 0.3^{AB}	8.16 ± 1.20^{AB}
7.01 (7)	305 ± 11^{C}	76.2 ± 0.1^{B}	86.5 ± 0.4^{B}	7.69 ± 0.40^{B}
7.53 (8)	310 ± 31^{C}	73.8 ± 0.2^{C}	83.9 ± 0.3^{C}	7.45 ± 0.26^{B}

*Means ± SD having a common superscript letter in the same column are not significantly different ($P < 0.05$).

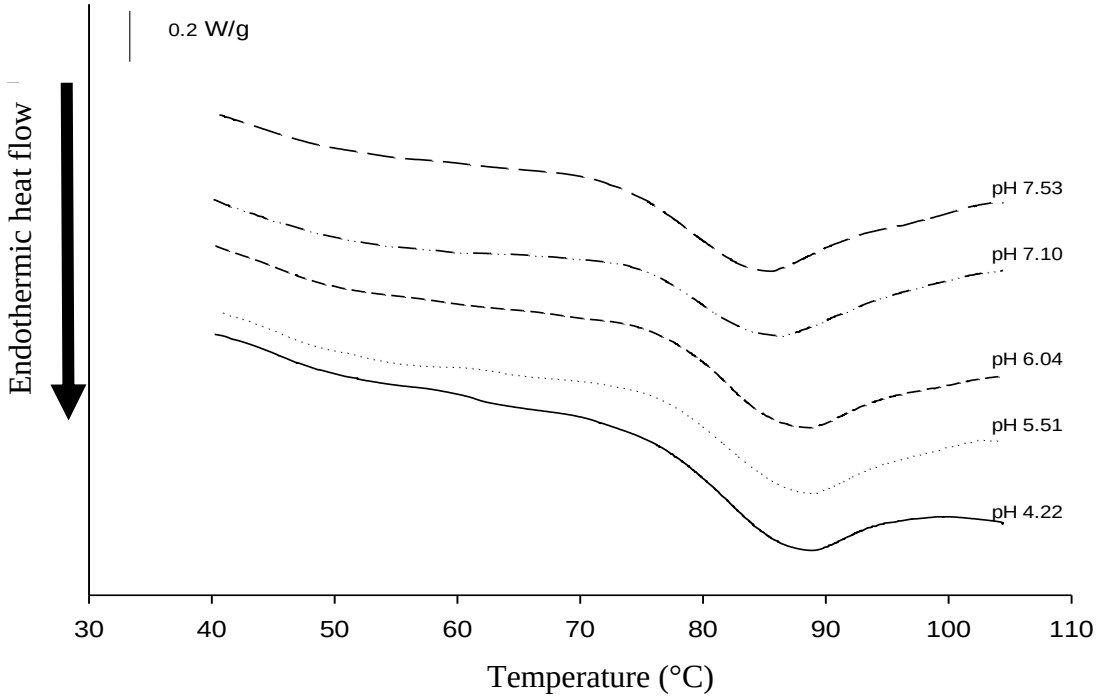

Figure 3.2 DSC thermograms of pea protein pastes (14% w/v) at various pH heated at 10 °C/min.

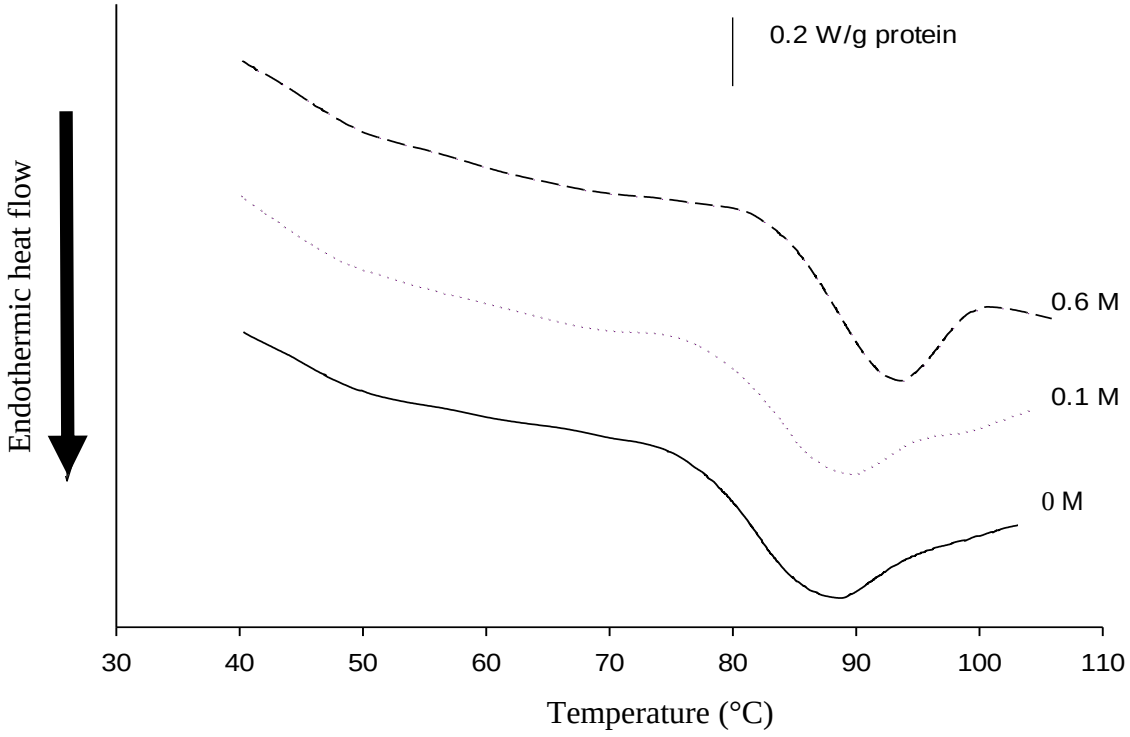

Figure 3.3. DSC thermograms of pea protein pastes (14% w/v, pH 6.0) heated at 10 ^{o}C/min at various NaCl concentrations.

Table 3.2. DSC thermal data for 14% pea protein pastes at different NaCl concentrations*

NaCl (M)	Particle size (nm)	T_0 (°C)	T_{max} (°C)	ΔH (75-100 °C) (J/g protein)
0	255 ± 41^{C}	77.5 ± 0.3^{C}	87.5 ± 0.3^{C}	9.08 ± 0.06^{A}
0.1	313 ± 45^{B}	80.7 ± 0.4^{B}	88.4 ± 0.4^{B}	6.68 ± 0.05^{B}
0.6	414 ± 36^{A}	93.5 ± 0.7^{A}	91.9 ± 0.7^{A}	$7.35 \pm .06^{B}$

*Means ± SD having a common superscript letter in the same column are not significantly different ($P < 0.05$).

Table 3.3. DSC thermal data for 14% pea protein pastes at different $CaCl_2$ concentrations*

$CaCl_2$ (mM)	Particle size (nm)	T_0 (°C)	T_{max} (°C)	ΔH (75-100 °C) (J/g protein)
0	255 ± 41^{F}	77.7 ± 0.5^{AB}	87.7 ± 0.4^{B}	10.65 ± 0.10^{A}
5	313 ± 51^{E}	77.6 ± 0.2^{B}	87.7 ± 0.2^{B}	7.49 ± 0.02^{CD}
10	614 ± 51^{D}	78.2 ± 0.1^{A}	88.8 ± 0.1^{B}	9.29 ± 0.18^{B}
50	850 ± 61^{C}	77.9 ± 0.6^{AB}	87.7 ± 0.2^{B}	8.62 ± 0.81^{BC}
100	1078 ± 85^{B}	77.8 ± 0.2^{B}	87.9 ± 0.1^{B}	8.79 ± 0.19^{B}
200	2078 ± 120^{A}	77.8 ± 0.2^{B}	87.9 ± 0.1^{B}	8.79 ± 0.19^{B}

*Means ± SD having a common superscript letter in the same column are not significantly different ($P < 0.05$).

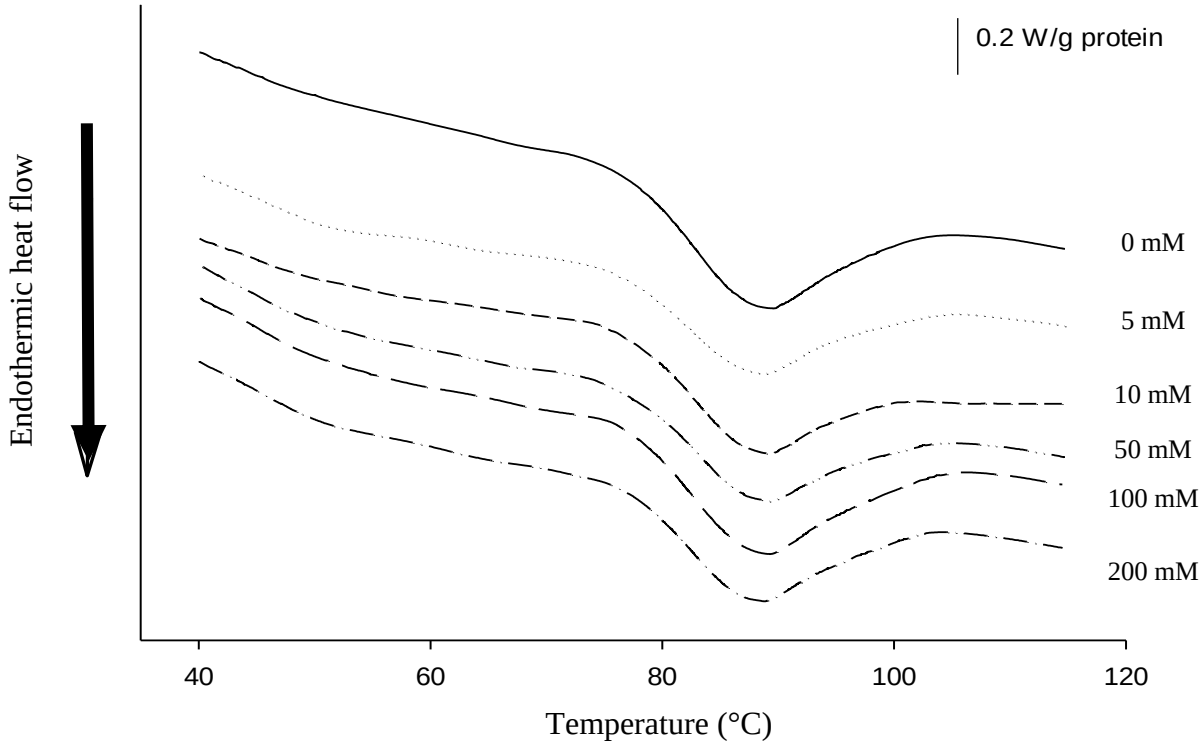

Figure 3.4. DSC thermograms of pea protein pastes (14% w/v, pH 6.0) heated at 10 °C/min at various $CaCl_2$ concentrations.

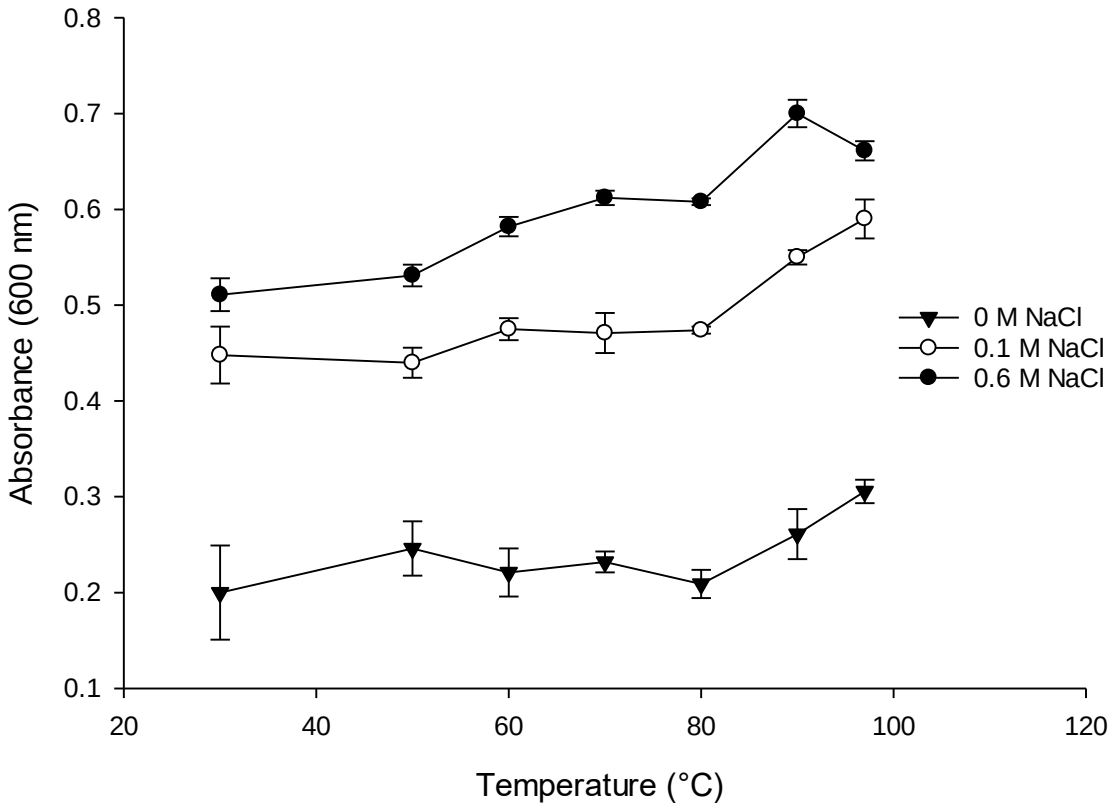

Figure 3.5. Turbidity of pea protein suspensions (0.5 mg/mL protein, pH 6.0) treated with different concentrations of NaCl.

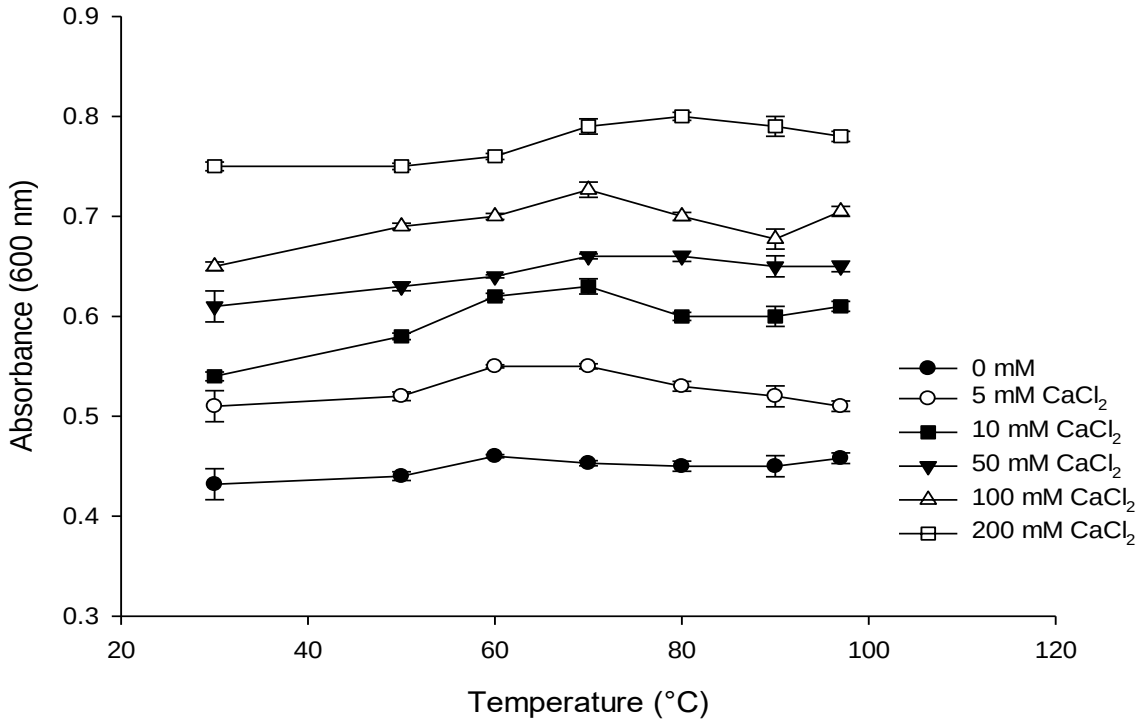

Figure 3.6. Turbidity of pea protein suspensions (0.5 mg/mL protein, pH 6.0) treated with different concentrations of $CaCl_2$.

CHAPTER 4.

Modification of Physicochemical Properties of Pea Protein by High Intensity Ultrasound Treatment

Summary

The altercation of the physiochemical properties of pea protein isolate (PPI) induced by ultrasound were studied under various processing conditions. Particle size and solubility were measured and used to determine optimum processing parameters. The turbidity of PPI, which indicates protein aggregation, with various concentrations of NaCl, $MgCl_2$, or $CaCl_2$ was measured as absorbance. The structural changes were studied by measuring the surface hydrophobicity, disulfide bonds, surface sulfhydryl groups, ζ-potential, and tryptophan fluorescence. The optimum parameters were determined to be 50% amplitude (60 W cm^{-2}, 20 kHz) for 5 second pulsed cycles for a total of 3 min due to the significant improvements to solubility and particle size reduction. Ultrasound treatment increased solubility across a range of pH (2-10), and salt concentrations. At pH 7.0 and 0.6 M NaCl, solubility increased from 48% to 73%. Ultrasound treated PPI had a higher turbidity with divalent salts, likely due to the increased solubility combined with divalent cation induced aggregation. The tryptophan intensity was higher in ultrasound treated PPI indicating a change in conformation. The surface characteristics all significantly changed after ultrasound treatment, surface hydrophobicity (increased 93 to 206), ζ-potential (-24.2 to -31.4), and surface sulfhydryls (23.8 to 43.9 μM/g soluble protein) ($P < 0.05$). Evaluation of how the structural changes impact functionality is the next logical step.

4.1. Introduction

The complex chemical structure of proteins allows for functionality as surface active agents in foam, encapsulation, viscosity modification, and gelation applications. Techniques that change the functionality of proteins without chemical addition are being investigated (e.g., ultrasound, electric field, and irradiation). The food industry has been driven to find alternatives to traditional production methods and ingredients while retaining functionality (Asioli, Aschemann-Witzel, Caputo, Vecchino, Neas, & Varela, 2017). Interest in using plant sourced protein has increased due to the reduced impact on the environment, ethical concerns, and lower relative cost (Yildiz, Ding, Andrade, Engeseth, & Feng, 2018). Pea protein has emerged as a potential replacement for traditional protein sources. Pea proteins main advantages are having low allergenicity, high antioxidant potential, and similar nutritional profiles to other legume proteins (Sanchez-Monge, Lopez-Torrejón, Pascual, Varela, Martin-Esteban, & Salcedo, 2004). It is possible to extend pea protein's utilization in food applications through chemical, physical, and enzymatic modification.

Pea protein is a major plant protein being investigated as an alternative because of its similar nutritional and functional properties to soy (Jiang et al., 2017). The protein content of peas varies (23.1-30%) in unprocessed seed. Pea proteins can be classified by their solubility. Albumins are the major water-soluble protein and comprise 10-20% of the total protein content. Globulins are salt-soluble storage proteins composing 60-85% of the total protein content. Globulins are further classified into legumin and vicilin proteins. Prolamins and glutelins are other proteins found in small amounts in peas (Saharan & Khetarpaul, 1994).

The two most influential proteins in pea protein are legumin and vicilin. Legumin is a hexamer protein (300-400 kDa) with a sedimentation coefficient of 11S. Within the hexamer, each of the six subunits is composed of an acid-basic subunit covalently linked by a disulfide bond (Gueguen, Chevalier, & Schaeffer, 1988; Mertens, Dehon, Bourgeois, Verhaeghe-Cartrysse, & Blecker, 2012). Vicilin proteins are trimers (150-170 kDa) and 7S sedimentation coefficient. Each monomer of the trimer is composed of a 50 kDa subunit held together by hydrophobic interactions (Shand, Ya, Pietrasik, & Wanasundara, 2008). Pea vicilin lacks cystiene and cannot form disulphide bonds (Shewry, Napier, & Tatham, 1995).

Ultrasound technology is the application of sound waves at a frequency above the threshold of human hearing (X > 16 kHz). High-intensity ultrasound is being investigated for its ability to alter the properties of food while being a chemical and thermal-free process. The principal mechanism is the formation and collapse of cavitation bubbles. These bubbles form and collapse, creating micro-events of extreme temperature and pressure (Chemat & Khan, 2011). These micro-events can result in shearing and turbulence in the solution. The combined effect of temperature, pressure, and shearing leads to changes in food products (McClements, 1995). The use of ultrasound on food proteins has been a growing area of research. Applications are diverse and include brining, osmotic dehydration, heat transfer, extraction, emulsification, and fermentation (Ojha, Mason, O'Donnell, Kerry, & Tiwari, 2017; Paniwnyk, Alarcon-Rojo, Rodriguez-Figueroa, & Toma, 2017). Additional applications are covered in extensive reviews (Ojha et al., 2017; O'Sullivan, Park, Beevers, Greenwood, & Norton, 2017). The ultrasonic process has been shown to induce partial unfolding of proteins thus exposing more

hydrophobic regions towards the surface of the protein which correlates with increased solubility. Ultrasound has been shown to disrupt the protein quaternary and tertiary structures and reduce the molecular weight in certain proteins (Jiang, Ding, Andrade, Rababah, Almajwal, & Abulmeaty, 2017).

Understanding the physiochemical changes brought on by ultrasonic processing of PPI may lead to improved application in the food industry. The purpose of this experiment was to evaluate the effect of ultrasound treatment on physicochemical properties of PPI. Examining potential changes in solubility, turbidity, and various structural properties was a primary objective of the present investigation.

4.2. Materials and Methods

4.2.1. Materials

Pea protein isolates (PPI, NUTRALYS® S85F, 80% pea protein based on dry basis) in powder form was provided by Roquette America Inc. (Geneva, IL, USA), and was produced using a wet extraction process from dry yellow peas. The protein isolate was not further purified but used as is. The PPI was stored in a refrigerator at 4 °C before use. All other reagents and chemicals were purchased from VWR (Radnor, PA, USA), Sigma-Aldrich (St. Louis, MO, USA), or Fisher Scientific (Pittsburgh, PA, USA) and were of analytical or higher grade.

4.3.2. Ultrasound treatment

Pea protein suspensions (5% w/v) were obtained by dissolving PPI in deionized water under stirring at 25°C for 1 hour. Ultrasound treatment was applied to 25 mL of suspension in 30 mL beakers using a Q700 Sonicator (Qsonica Sonicators, Inc.,

Newtown, CT, USA) at 10%, 50%, and 100% amplitude, 5 second pulsed cycle for a total of 1, 3, and 5 minutes of sonication. An ultrasonic probe of 1/2" (12 mm) diameter was used to deliver acoustic energy into the sample. The probe was inserted into the solutions at a depth of 1-inch. Heat produced by ultrasonication may cause protein denaturation (Kent & Doherty, 2014). In order to avoid overheating, an iced water bath was used to cool the samples. An integrated temperature probe was also used to monitor the samples with a programmed shutdown of 50 °C.

4.2.3. Solubility

The solubility of ultrasound treated samples in comparison with the respective controls was investigated with three salts at different ionic strengths (0, 0.1, 0.6 M NaCl, 5, 10, 50, 100, and 200 mM $CaCl_2$ or $MgCl_2$ at pH 7.0) and nine pH levels (2.0-10.0). Specifically, sample proteins were dissolved (2% w/v) in deionized water. pH was adjusted with 1.0 M HCl or 1.0 M NaOH after ultrasound treatment (60 W/cm^{-2}, 3 min). Aliquots of proteins suspensions were then diluted to final concentrations of 5 mg/mL with deionized water. The solutions were centrifuged at 10,000g for 10 min at 21°C. Protein concentration of the supernatants and entire suspension was determined according to the Biuret method (Gornall, Bardwill, & David, 1949). Solubility was calculated as the percent distribution of protein in the supernatant over the total protein content in the dispersion.

4.2.4. Turbidity

A turbidity experiment was carried out to determine the susceptibility of ultrasound treated proteins to thermal insolubilization and aggregation. Aliquots of 5 mL each of dilute protein solutions (2 mg/mL in 25 mM sodium phosphate buffer, pH 7.0) at

different ionic strengths (0, 0.1, 0.6 M NaCl, 5, 10, 50, 100, and 200 mM $CaCl_2$ or $MgCl_2$) were placed in test tubes. The tubes were closed with screw caps to prevent evaporation of water during heating. Samples were heated at 1 °C /min in a programmable water bath (Haake L D3 heating circulator, Fisher Scientific, Waltham, MA). When a target temperature was reached (30, 50, 60, 70, 80, 90, 97 °C), three tubes (triplicate) were removed and immediately chilled in an ice slurry. Cooled protein suspensions were well mixed by inverting the test tube and the absorbance was immediately measured at 600 nm against blank water at room temperature.

4.2.5. Particle size

The volume-weighted mean diameters ($D_{4,3}$) of soluble protein aggregates were measured by dynamic light scattering (DLS) using a ZetaSizer Nano-S90 (Malvern Instruments Limited, Worcestershire, UK) with the following settings: measurement angle, 90°; equilibration time, 120 s; number of runs, 3; run duration, 10 s; number of measurements, 3; and delay between measurements, 10 s. DLS is a technique used to analyze particle size by measuring Brownian motion. Samples were diluted 500-fold with Deionized water before measurement. The measurement was conducted at 23-25 °C, and the liquid viscosity and index of refraction was set according to water, which were 0.933 and 1.333, respectively.

4.2.6. Tryptophan fluorescence

The protein concentration of control and ultrasound treated suspensions was diluted to 1 mg/mL in a 50 mM phosphate buffer at pH 7.0. Tryptophan fluorescence was measured with a FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon Inc., Edison, NJ, USA) at a 295 nm excitation wavelength (slit width 5 nm) and a 300-500 nm emission

wavelength (slit width = 5 nm) at a 10 nm/s scanning speed. The phosphate buffer used to dissolve PPI was used as blank solution for all samples

4.2.7. Surface hydrophobicity (H_o)

Surface hydrophobicity was measured using the 1-anilino-8-naphthalenesulfonate magnesium salt (ANS) (Sigma Chemical Co.) fluorescence probe. Because fluorescence intensity (FI) is directly proportional to pea protein concentration in the range from 0.005 to 0.5 mg/ mL, control and ultrasound treated samples were diluted with 0.01 M phosphate buffer (pH 7.0) to yield final concentrations of 0.02, 0.04, 0.06, 0.08, and 0.1 mg/mL before reaction with 20 μL of ANS (8 mM in 0.1 M phosphate buffer, pH 7.0). FI was measured with an emission wavelength of 484 nm and an excitation wavelength of 365 nm (both with a slit width 5 nm) on a FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon Inc., Edison, NJ, USA). The initial slope of the FI versus protein concentration plot (calculated by linear regression analysis) was used as an index of protein hydrophobicity.

4.2.8. Surface sulfhydryl and disulfide bonds

Determination of surface SH groups were carried out using 5,5′-dithiobis-(2-nitrobenzoic acid) DTNB (Thannhauser, Konishi, & Scheraga, 1984). PPI samples were diluted to 2.0 % (w/v) then stirred for 1 h. Absorbance at 412 nm was measured after incubating the solution with DTNB for 15 min in the dark at 25 °C. Reagent blank and sample blank were prepared to correct for color from reagents and protein solution. Total surface sulfhydryl (SH) content was then calculated by the molar absorption coefficient of 13.6 mM^{-1} cm^{-1}. The results were expressed as μmol SH g^{-1} protein. Disulfide bonds in proteins were determined by reacting with disodium 2-nitro-5-thiosulfobenzoate (NTSB) as described by Damodaran (1985). Protein samples were diluted to 5 mg/mL

protein with 25 mM phosphate buffer (pH 6.0) containing 0.6 M NaCl. A 100-µL aliquot of diluted protein solution in triplicate was mixed with 1.5 mL NTSB assay solution (freshly made) and incubated in the dark at room temperature for 25 min. Absorbance at 412 nm was then measured. A molar absorption coefficient of 13600 M^{-1} cm^{-1} was used for calculation. Because NTSB reagent forms chromophoric derivatives with both surface sulfhydryl groups and disulfide bonds, the numbers calculated from the absorbance readings represented the total concentrations of sulfhydryl and disulfide groups in the samples. Disulfide content was estimated by subtracting the surface sulfhydryls (obtained from sulfhydryl assay) from the total content.

4.2.9. Zeta potential

Protein suspensions were diluted to (0.05 wt.%) and adjusted to pH 7.0. The solutions were placed in a standard four-sided, 1 cm polystyrene cuvette and a parallel plate electrode (0.45 cm^2square platinum plates with a 0.4 cm gap) was inserted. The cuvette was placed in a temperature-controlled holder (25 °C). The electrophoretic mobility was measured by PALS (ZetaPALS, Brookhaven Instruments, Holtsville, NY, USA). Each measurement was the average of 50 (five sets of 10) measurements and the entire experiment was conducted in triplicate. The ζ-potential was calculated from the electrophoretic mobility using the Smoulokowski model (assuming the double layer thickness is much less than the particle size) (Hunter, 2001).

4.2.10. Statistical analysis

All experiments were repeated with at least three independent trials (n = 3) each with freshly prepared protein solution. Data was analyzed using the general linear model procedures of the Statistix 10 software package (Analytical Software Inc., St. Paul, MN,

USA). Analysis of variance (ANOVA) was performed to determine treatment effect. When significant treatment effects ($P < 0.05$) were found, their means were separated by Tukey's honest significance test.

4.3. Results and Discussion

4.3.1. Effect of ultrasound conditions on protein solubility and particle size

Solution viscosity, processing time, and amplitude (power) are the three main factors which impact the ability for reproducible results during ultrasonic processing. Ultrasound treated PPI had significant changes with all treatments (Fig. 4.1). The largest increase in solubility occurred at 5 min 100% amplitude (120 W cm^{-2}). However, to avoid damaging the probe the instrument required significant cooling time between samples. The 3 min 50% amplitude (60 W cm^{-2}) yielded similar results (75.3% solubility compared to 80.1%) and was therefore selected to use throughout the study in the interest of efficiently processing as many samples as possible during the course of a day. The application of ultrasound resulted in significant particle size reduction with the greatest reduction occurring at 5 min 100% amplitude (Fig. 4.2). The turbulence, pressure, and temperature generated by bubble formation and collapse are likely causes for the particle size reduction and subsequent increase in solubility (Fig. 2.2).

4.3.2. Influence of pH on protein solubility

pH is of critical importance for protein solubility and application in the food industry. Ultrasound improved the solubility of pea protein across pH 2-10 (Fig. 4.3). This could be due to the disruption of quaternary and tertiary structures, their partial unfolding, and reduction of aggregate size. Mechanical dissolution of protein into solution by the rapid

formation and collapse of bubbles could also partially explain the increase in solubility. The pH-solubility profile of control and ultrasound treated PPI exhibited a typical U-shaped curve found in most globular proteins. The results are similar to other studies of legume proteins (fava, soy, and pea) (Jiang et al., 2017; Martínez-Velasco, Lobato-Calleros, Hernández-Rodríguez, Román- Guerrero, Alvarez-Ramirez, & Vernon-Carter, 2018; O'Sullivan et al., 2017).

4.3.3. Influence of chloride salts on protein solubility and particle size

Ionic strength was investigated for impacts on solubility and particle size. The NaCl concentration did not have a significant effect on the solubility of ultrasound treated or control PPI (Fig. 4.4). Particle sizes were larger with increasing NaCl concentration (Fig. 4.5), and this is attributed to the disruption of electrostatic repulsion thus allowing for increased protein aggregation. Ultrasound treated PPI had smaller particles under all salt concentrations due to protein aggregate dispersal, and partial unfolding caused by turbulence and shearing forces.

Divalent chloride salts ($MgCl_2$ and $CaCl_2$) were added into the solutions of control and ultrasound treated PPI (Fig. 4.6). Rapid precipitation occurred in control PPI samples while ultrasound treated samples displayed no visible separation until after centrifugation. Divalent salts decreased protein solubility with increasing salt concentration regardless of treatment. Divalent salts have been shown to destabilize the charges of some plant proteins, promoting denaturation and aggregation by interaction with hydrophobic patches (Shih, Prausnitz, & Blanch, 1992). The destabilization and increase in free energy caused by the divalent salts can explain the overall decrease of

solubility. In Fig. 3.4, increasing $CaCl_2$ concentration correlated with a decrease in ΔH indicating some degree of denaturation, which corresponds with decreased solubility.

$CaCl_2$ addition resulted in the largest particles regardless of treatment (Fig. 4.7). $MgCl_2$ increased particle size with concentration but resulted in smaller particles sizes when compared to $CaCl_2$ at the same concentration. At 100 mM, particle size was approx 500 nm for $MgCl_2$ and above 2000 for $CaCl_2$. It was observed that storage proteins have an affinity for divalent salts above their pI which agrees with what was observed in this experiment (Sakakibara & Noguchi 1977).

4.3.4. Thermal aggregation of protein as influenced by chloride salts

Heat-induced aggregation was quantified as absorbance measured at 600 nm. A lower turbidity has been associated with smaller particles due to the reduction in light scattering.

NaCl at concentrations of 0.0, 0.1, 0.6 M was added to PPI suspensions. Control PPI turbidity an increased with increasing NaCl concentrations (Fig. 4.8). Ultrasound treatment significantly decreased turbidity when no salts were present. This can be explained by the disruption of larger protein aggregates by ultrasonic cavitations (Fig. 4.2). Na^+ and Cl^- ions interact with protein, i.e., the weakening of charge repulsions of exposed ionic groups, allowing for closer association and aggregation. As temperature increases, the electric double layer around the protein surface would be removed (Jiang et al., 2014). This aggregation effect was not observed in ultrasound treated PPI. The reduction in particle size and increased solubility likely made ultrasound treated PPI less susceptible to the NaCl ionic disruption of surface charge and the aggregation it can induce.

Upon treatment with divalent cation salts, ultrasound treated PPI exhibited a significant increase in turbidity when compared to control PPI (Fig. 4.9; Fig. 4.10). Divalent salts addition resulted in conformational instability, aggregation, and subsequently increased turbidity. Binding of Ca^{2+} and Mg^{2+} to PPI is primarily attributed to electrostatic interaction with hydrophobic groups and negatively charged amino acids, e.g., aspartic and glutamic acids (Agboola & Dalgleish, 1995). Ultrasound treatment decreased the particle size of PPI (568 nm to 220 nm) and exposed additional charged amino acids and hydrophobic groups. The increase in turbidity could be attributed to the combined effect of higher solubility (Fig. 4.1) of ultrasound treated PPI and promotion of protein aggregation due to increased divalent electrostatic interaction with the newly exposed groups.

4.3.5. Tryptophan fluorescence

The intensity of intrinsic tryptophan fluorescence was assessed as a measurement of protein conformational changes. The emission spectra of ultrasound treated and control PPI are reported in Fig. 4.11. Ultrasound treated PPI experienced a fluoresce shift, revealing that tryptophan residues were less exposed than in the control samples. This was unexpected; most physical processes result in a decrease in fluorescence, showing the exposure of the buried inner hydrophobic groups and tryptophan. Two possible explanations are hypothesized. Firstly, the disruption of the quaternary structures of vicilin and legumin proteins allowed for a greater amount of tryptophan and hydrophobic groups to be exposed but tryptophan residues are more buried than the native conformations due to new aggregation arrangements. Secondly, the control PPI has larger particles (Fig. 4.2), which resulted in the excitation to be blocked. The ultrasound

treated samples had smaller particles and increased solubility, resulting in less excitation being blocked.

4.3.6. Surface hydrophobicity (H_o*)*

Surface hydrophobicity (H_o) is related to the shape, size, amino acids and sequence, and intermolecular interactions (Feng, Li, Li, Zhai, Song, & Jiang, 2002). Tertiary structures of proteins highly depend on the hydrophobic and hydrophilic side chains. Hydrophobic groups attempt to reduce free energy by orienting themselves towards the core, but some regions remain on the exterior (Kinsella, 1981). Fluorescent probes are used to measure H_o, 8-anilino-1-naphthalenesulfonic acid (ANS) for aromatic residues and cis-parinaric acid (CPA) for aliphatic residues (Alizadeh-Pasdar & Li-Chan, 2000). Changes in H_o can indicate protein unfolding and changes to hydrophobic regions.

H_o of control and ultrasound treated PPI samples is summarized in Table 4.1. The H_o of control PPI was 93.1 ± 7.1. After ultrasound treatment the H_o of pea protein increased substantially ($P < 0.05$) to the range of 206 ± 13. It is possible that hydrophobic residues in PPI were exposed due to quaternary aggregate dispersal. This disruption of the quaternary structure is linked to tertiary structural rearrangement and partial unfolding. This rearrangement could explain the smaller particle size and increase of hydrophobic regions observed.

4.3.7. Surface sulfhydryls and disulfide bonds

After ultrasound treatment, an increase in surface sulfhydryl groups (SH) was found (Table 4.1). The content of exposed SH in control PPI samples was 24 µM/g protein; ultrasound treatment increased SH to 44 µM/g protein. This is attributed to either an increase of SH exposed towards the solvent environment or the cleavage of disulfide

bonds. Mechanically, the increase observed after treatment is believed to be caused by the generation and collapse of gas bubbles, turbulence, and shear forces by ultrasound (Chandrapla, Zisu, Kentish, & Ashokkumar, 2012). Previous studies have shown that SH content of pea protein is 3-70 μM/g protein and can be increased by ultrasonic processing (O'Sullivan et al., 2017; Jiang et al., 2017). Disulfide bond content was unchanged after ultrasound treatment, suggesting an increase in the amount of SH exposed to the environment rather than disulfide bond cleavage (Table 4.1). Multiple studies on pea, soy, and rice proteins report increases in SH exposure after ultrasound treatments and that cleavage of covalent bonds is uncommon (Hu et al., 2013; Zhang-cun, Wei-huan, Xue-wei, Jian-qiang, Chang-wen, & Sheng-wen, 2012). The decrease in particle size, and increased H_o support the hypothesis that ultrasound partially unfolds and dissociates PPI aggregates resulting in more SH groups exposed.

4.3.8. Zeta potential

The surface charge of protein molecules is attributed to the ionization of specific amino acid residues. The charge of a protein is impacted by ionic strength, pH, and co-solutes (Malhotra & Coupland, 2004). ζ-potential of control and ultrasound treated PPI was found to be -24.2 ± 2.4 mV and -31.4 ± 2.5 mV, respectively (Table 4.1). The change in zeta potential of ultrasound treated PPI is ascribed to structural dispersal and rearrangement that resulted from ultrasonic shear forces and turbulence. The dispersal of protein aggregates will expose previously buried charged groups, resulting in an increase in ζ-potential. A large absolute value ζ-potential correlates with increased electrostatic repulsion and distance between particles, leading to greater stability in solution (Tamnak, Mirhosseini, Tan, Ghazali, & Muhammad, 2016). This increase in surface charge in

conjunction with particle size reduction, increased H_o, and exposed SH groups could explain the increase in solubility.

4.4. Conclusions

The evaluation of ultrasound treatment on PPI and its effects on molecular structure and functionality was performed. A treatment of 3 min 50% amplitude (60 W cm^{-2}, 20 kHz) was chosen for significant differences in solubility, particle size reduction, and feasibility. With increased time and amplitude, water solubility increased across a wide range of pH (2-10) while particle size decreased. NaCl did not have an effect on the solubility of control or treated PPI at any concentration. Divalent salts had a negative effect on the solubility of PPI, but ultrasound treated PPI was less susceptible to the destabilization effects possibly due to the decreased particle size and changes to structural characteristics. The turbidity of ultrasound treated PPI was not impacted by NaCl concentration while turbidity increased significantly in control PPI. Ultrasound treated PPI presented a higher turbidity in solutions containing divalent salts ($MgCl_2$ and $CaCl_2$), likely due to increased solubility combined with aggregation induced by the salts. Tryptophan fluorescence had unexpected results showing that the tryptophan residues were less exposed after ultrasound treatment. It is theorized that this is due to the dissociated subunits aggregating in new patterns which hide more tryptophan or the larger particles in control PPI result in blocking of emission. Surface hydrophobicity, sulfhydryl groups, and ζ-potential increased while disulfide bonds remained constant. These structural changes along with particle size reduction support that ultrasound induced the dissociation of quaternary and tertiary structures and their partial unfolding.

The solubility and structural changes may allow pea protein to be used as a functional ingredient in beverages or bakery products.

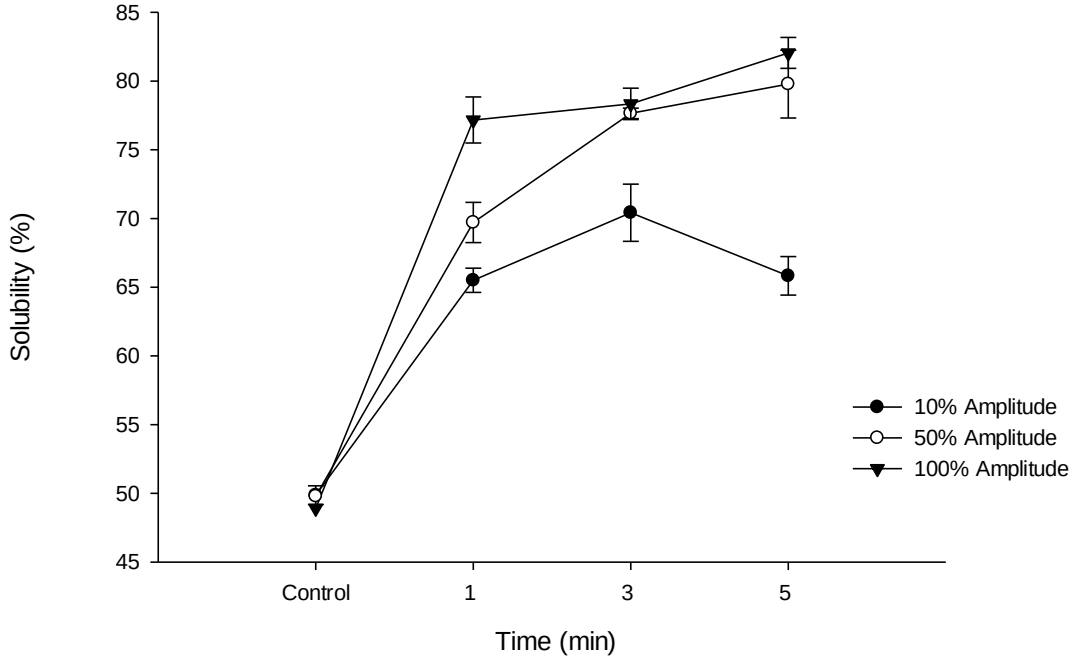

Figure 4.1. Solubility of pea protein isolate suspensions (5% w/v, pH 7.0) at various ultrasonic processing times and amplitudes.

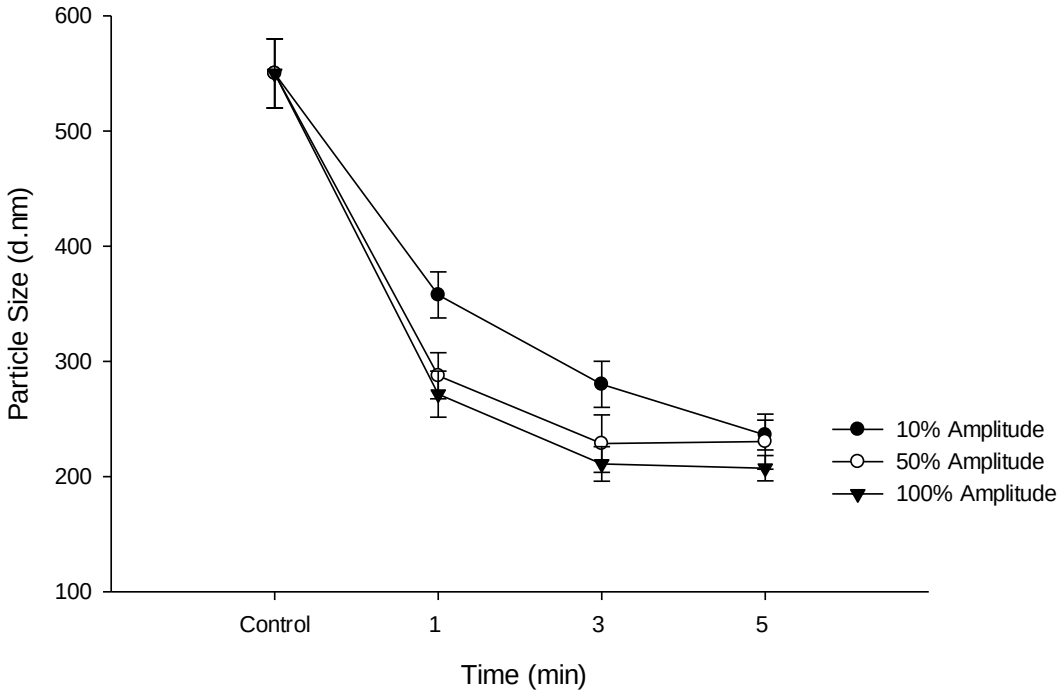

Figure. 4.2. Particle size of pea protein particle size of pea protein isolates (5% w/v, pH 7.0) at various ultrasound times and amplitudes.

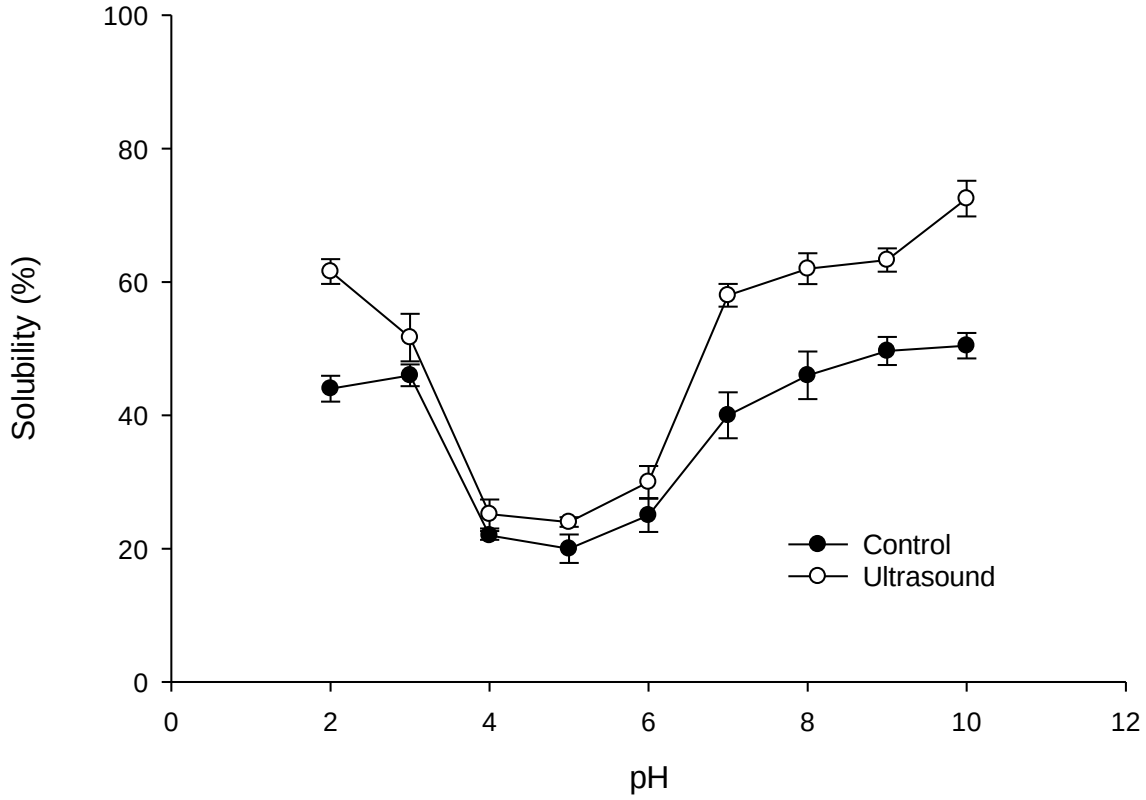

Figure 4.3. Solubility of pea protein suspensions (5.0 mg/mL) at various pHs (2-10).

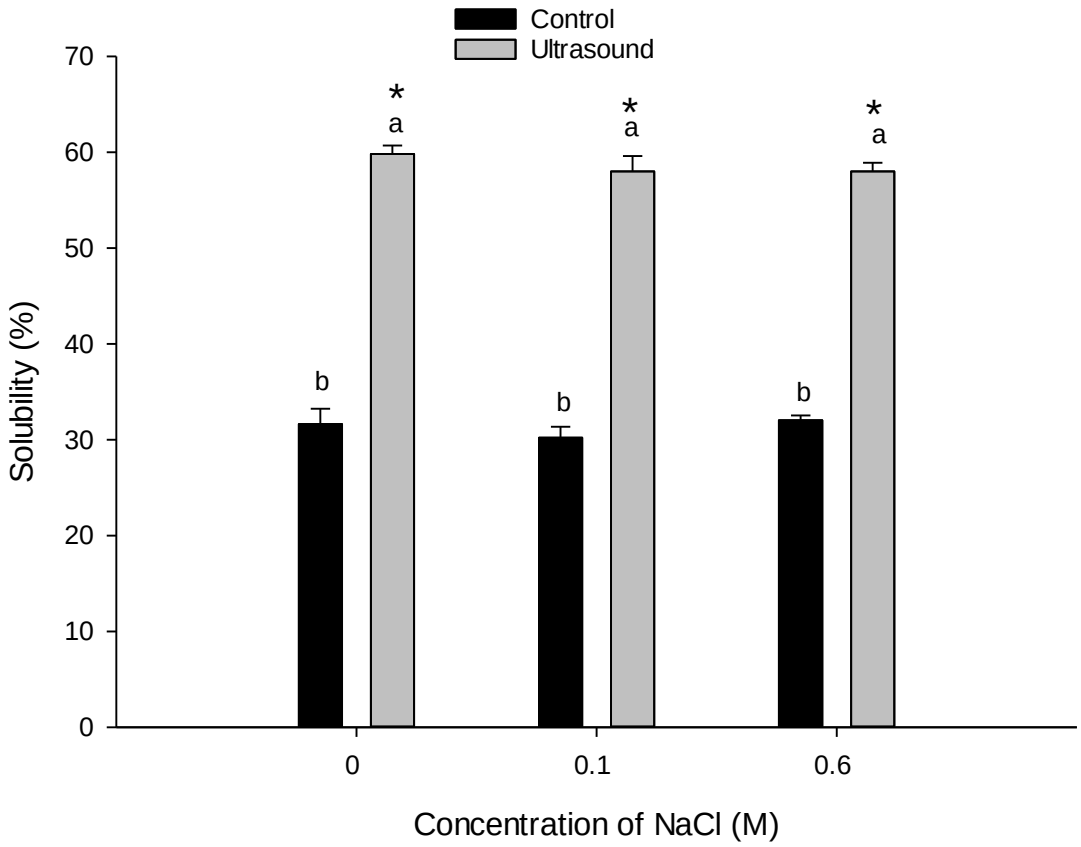

Figure 4.4. Solubility of pea protein suspensions (5% w/v) at pH 7.0 with various NaCl concentrations. * Denotes significant difference ($P < 0.05$) between treatments; a-c denote significant differences ($P < 0.05$) within the same treatment.

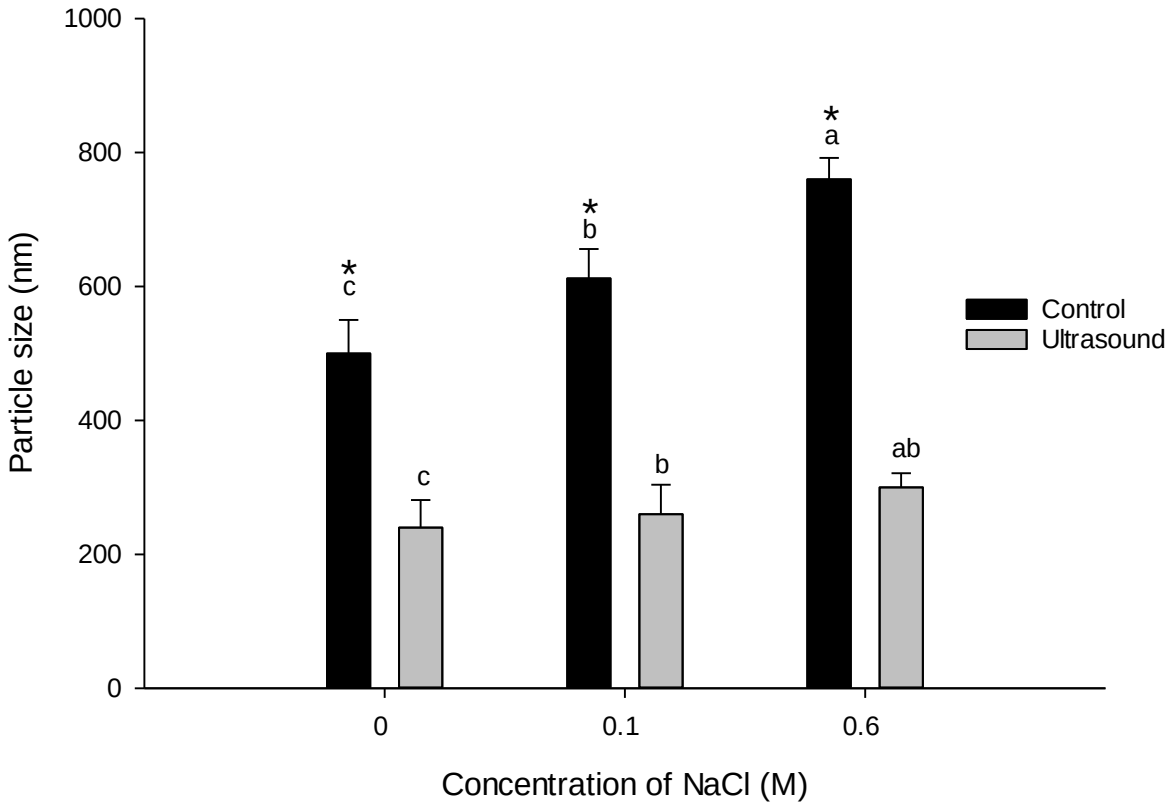

Figure 4.5. Particle size of pea protein suspensions (5% w/v) at pH 7.0 with various NaCl concentrations. Denotes significant difference ($P < 0.05$) between treatments; a-c denote significant differences ($P < 0.05$) within the same treatment.

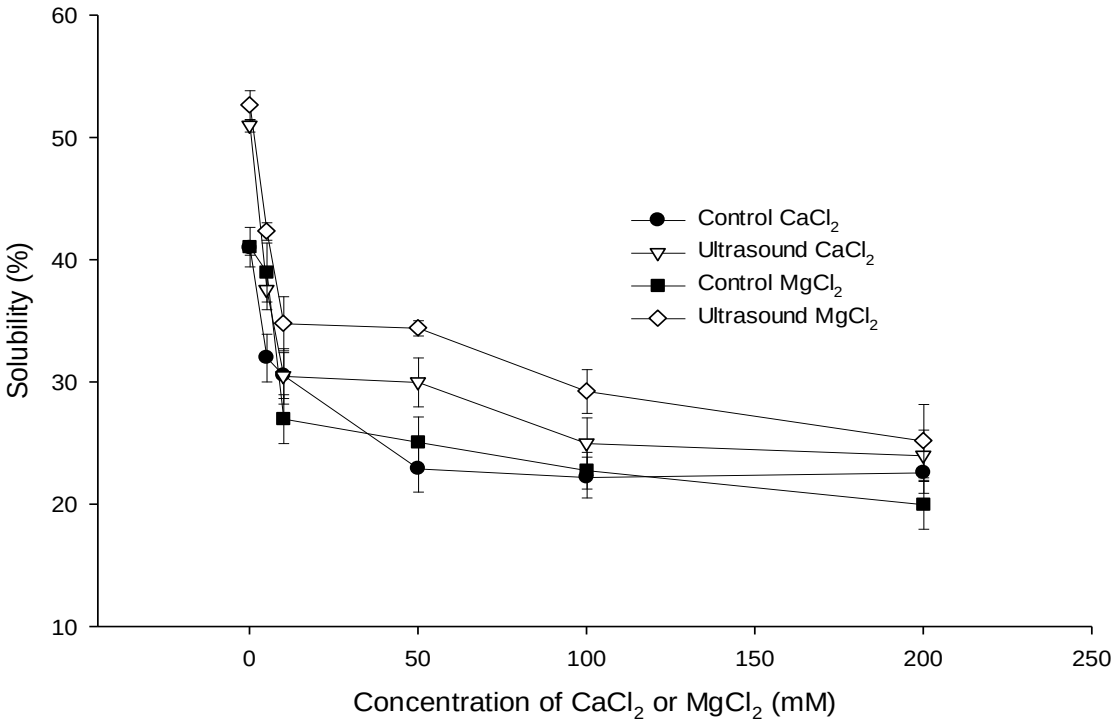

Figure 4.6. Solubility of pea protein suspensions (0.5 mg/mL protein, pH 7.0) treated with different concentrations of divalent salts.

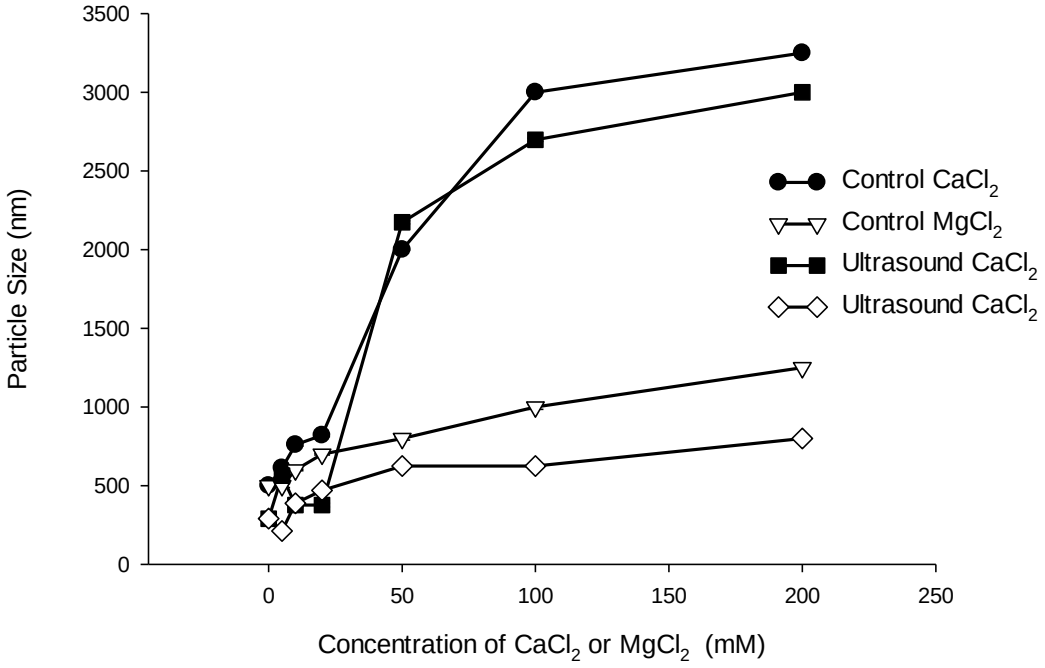

Figure 4.7. Particle sizes of pea protein of pea protein suspensions (0.5 mg/mL protein, pH 7.0) treated with different concentrations of divalent salts.

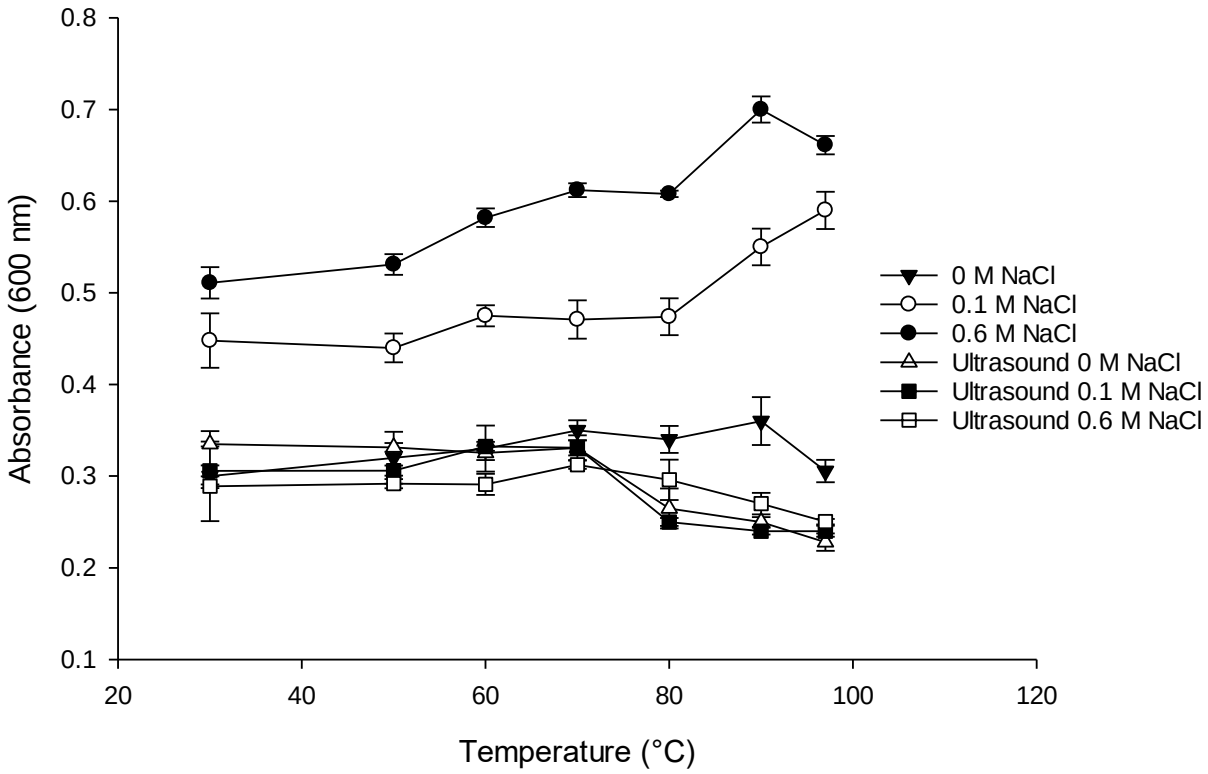

Figure 4.8. Heat-induced turbidity change of pea protein suspensions (0.5 mg/mL protein, pH 7.0) treated with different concentrations of NaCl.

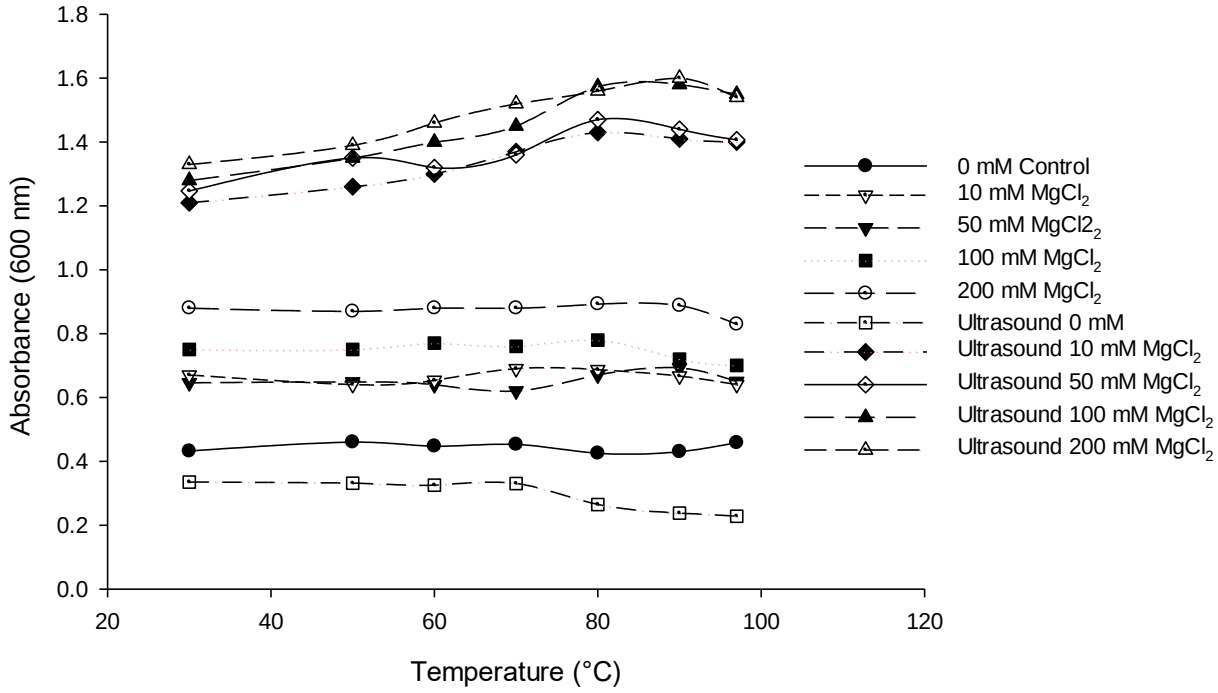

Figure. 4.9. Heat-induced turbidity change of pea protein suspensions (0.5 mg/mL protein, pH 7.0) treated with various concentrations of $MgCl_2$.

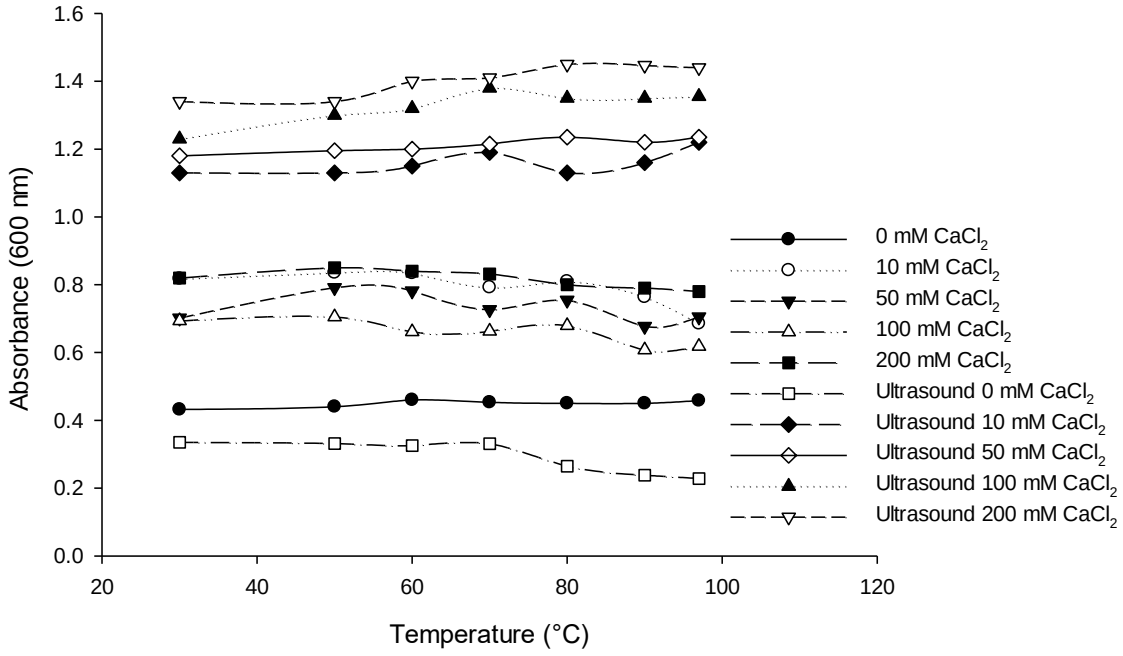

Figure 4.10. Heat-induced turbidity change of pea protein suspensions (0.5 mg/mL protein, pH 7.0) treated with various concentrations of $CaCl_2$.

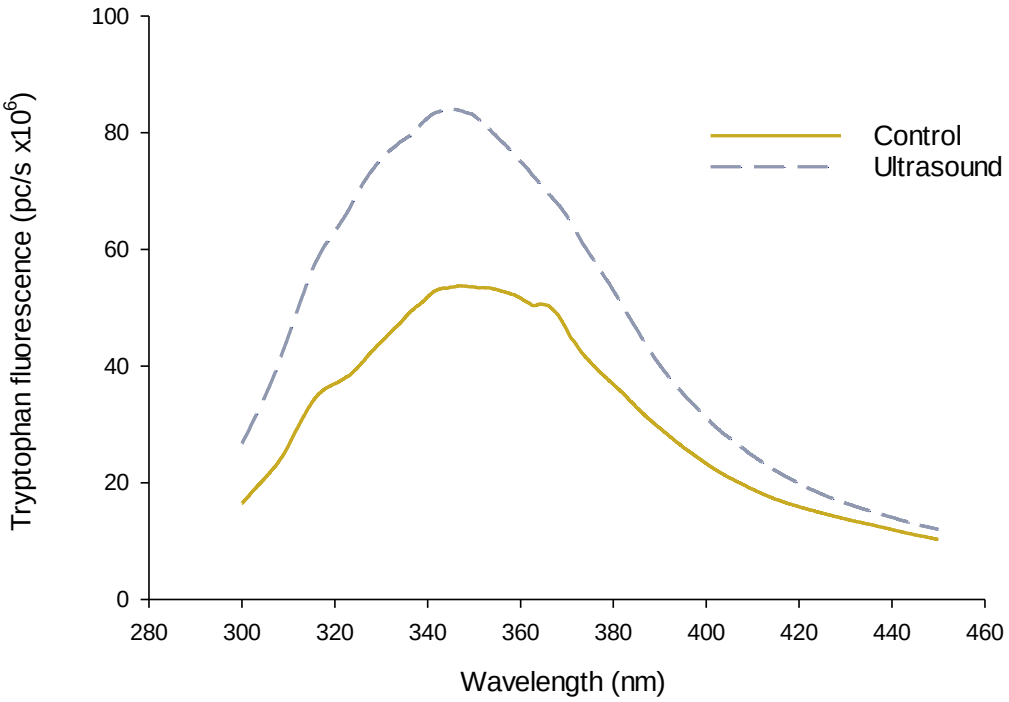

Figure. 4.11. Tryptophan fluorescence emission spectra (excitation wavelength at 295 nm) of pea protein suspensions (1.0 mg/mL protein, pH 7.0).

Table 4.1. Structural parameters of control and ultrasound treated pea protein isolate (PPI)*.

Characteristic	Control PPI	Ultrasound PPI
Surface hydrophobicity (H_o)	93 ± 7.0^{B}	206 ± 13^{A}
Surface SH groups (μmol/g soluble protein)	23.8 ± 2.4^{B}	43.9 ± 3.3^{A}
S-S bonds (μmol/g soluble protein)	5.61 ± 1.9^{A}	5.92 ± 1.3^{A}
Zeta potential ζ (pH 7.0)	-24.2 ± 2.4^{A}	-31.4 ± 2.5^{B}
Particle size (nm)	568 ± 35^{A}	220 ± 17^{B}

*Means ± SD having a common superscript letter in the same row are not significantly different ($P < 0.05$).

CHAPTER 5

Foaming and Application Properties of Pea Protein after High Intensity Ultrasound Treatment

Summary

The ultrasonic effect on the foaming and application properties of pea protein was investigated. Suspensions of pea protein isolate (PPI, 5.0% w/v) were processed at ~60 W cm^{-2} (50 % amplitude, 20 kHz) for 3 min (pulse duration: on 5 s; off 5 s). The surface tension was measured on a tensiometer. The foaming properties were measured as foaming capacity and stability. The application functionality was evaluated in angel food cakes. The ultrasound treatment resulted in an increase in PPI's ability to reduce surface tension. Ultrasound treated PPI resulted in a foaming capacity of 202% compared with 133% for control PPI. Ultrasound treated foams had no visible drainage under various ingredient conditions (0-5% sucrose or 0-0.6 M NaCl) while control drained (25 ± 5 %) across all conditions. Angel food cakes were formulated with egg white, control PPI, and ultrasound treated PPI to test the functionality in a model food system. Egg white and ultrasound treated PPI formulations had similar texture profiles but differed in color and loaf volume (10.1 and 8.1 cm, respectfully). Control PPI formulations were different and inferior in all physical characteristics. The results showed that ultrasound treatment could promote the application of pea protein in food products that require stable foams.

5.1. Introduction

Plant proteins are increasingly utilized as ingredients due to their nutritional value and low cost (Awad, Moharram, Shaltout, Asker, & Yourssef, 2012). The food industry has been driven to find alternatives to traditional production methods and ingredients while retaining functionality (Asioli, Aschemann-Witzel, Caputo, Vecchino, Neas, & Varela, 2017). Interest in using plant sourced protein has increased due to the reduced impact on the environment, ethical concerns, and lower relative cost (Yildiz, Ding, Andrade, Engeseth, & Feng, 2018). This has led to the development of a variety of animal free products (e.g. milk alternatives, mayonnaise, pastas, and baked goods). Currently products made with pea protein lag behind other plant proteins (soy, almond, rice). Pea protein isolate (PPI) in particular has a well-balanced amino acid profile, and low allergenicity, but its utilization in food applications is limited (Sanchez-Monge, Lopez-Torrejón, Pascual, Varela, Martin-Esteban, & Salcedo, 2004). As shown in Chapter 4, it is possible to improve PPI's functionality through ultrasound processing. Ultrasound proved to be an efficient and quick method to improve the solubility and modify surface properties of PPI.

Eggs contain excellent functional, nutritional, and sensory properties (McWatters 1992). Eggs are the second most common food that triggers allergic reactions in adults, and the CDC displayed that children are more susceptible to egg allergies (Branum & Lukacs, 2008). Egg allergies are often believed to disappear after puberty, but recent studies displayed that a significant amount of children keep an egg allergy throughout adulthood (Pablos-Tanarro, Lozano-Ojalvo, Molina, & López-Fandiño, 2018). There is little available allergen data on the prevalence of allergy to peas. Food allergies in the

United States impact 2% of adults and 4-8% of children, of these allergies, pea allergies are estimated to be less than 1% (Branum & Lukacs, 2009; Goldstein & Goldstein, 2009). The growing demand for egg free products has resulted in the need for functional replacements. In order for pea protein to successfully replace animal proteins, they must be able to mimic not only functional properties but have acceptable sensory and nutritional properties as well.

The replacement of egg protein by ultrasound treated PPI has not been investigated. If PPI is to function in the place of traditional proteins, improvements and understanding of its functionality are critical. In this study, the effect of ultrasound on the foaming properties of pea proteins was investigated and determined how conformational changes related to foaming properties. In particular, PPI was treated at 60 W cm^{-2} (50 % amplitude, 20 kHz) for 3 min (pulse duration: on 5 s; off 5 s). Samples subjected to this treatment were analyzed for foaming properties (capacity and stability). The application of modified PPI was subsequently evaluated in angel food cakes.

5.2. Materials and Methods

5.2.1. Materials

Pea protein isolates (PPI, NUTRALYS® S85F, 80% pea protein based on dry basis) in powder form was provided by Roquette America Inc. (Geneva, IL, USA), and was produced using a wet extraction process from dry yellow peas. The protein isolate was not further purified but used as is. The PPI was stored in a refrigerator at 4 °C before use. All other reagents and chemicals were purchased from VWR (Radnor, PA, USA),

Sigma-Aldrich (St. Louis, MO, USA), or Fisher Scientific (Pittsburgh, PA, USA) and were of analytical or higher grade.

5.2.2. Ultrasound treatment

Pea protein suspensions (5% w/v) were obtained by dissolving PPI in deionized water or corresponding buffer with stirring at 25°C for 1 hour. Ultrasound treatment was applied to 25 mL of suspension in 30 mL beakers using a Q700 Sonicator (Qsonica Sonicators, Inc., Newtown, CT, USA) at 50% amplitude, 5 second pulsed cycle for a total of 3 minutes of sonication. An ultrasonic probe of 1/2" (12 mm) diameter was used to deliver acoustic energy into the sample, and the acoustic power density (APD) was controlled at approx 60 W/cm^{-2} (approx 11,000 per replication). The probe was inserted into the solutions at a depth of 1-inch. Heat produced by ultrasonication may cause protein denaturation (Kent & Doherty, 2014). In order to avoid overheating, an iced water bath was used to cool the samples. An integrated temperature probe was also used to monitor the samples with a programmed shutdown of 50 °C.

5.2.3. Surface tension

Surface tension was measured using a Fisher Surface Tensiometer, Model 20 (du Noüy ring method) (Fisher Scientific International, Inc, Hampton, NH, USA) at room temperature using protein solutions as described above diluted 0.1 % (w/v) with deionized water. An aliquot of 30 mL of protein solution was used for each measurement. The platinum ring was flamed before each run, and the surface tension of water and 25% methanol in water were used to calibrate daily.

5.2.4. Foam preparation

Foaming properties of control and ultrasound treated PPI samples were evaluated by the high speed agitation method described by Motoi, Fukudome, and Urabe with modifications (2004). Specifically, test samples (4.0% w/v protein) were prepared in deionized water adjusted to pH 5.0 with 1.0 M HCl, and combined with 0, 0.1, or 0.6 M NaCl or 1.0% or 5.0% sucrose (w/v) (representing high and low salt and sugar levels in usual food systems). An aliquot of 20 mL of protein solution in a 100-mL plastic graduated cylinder was blended with a Kinematica Polytron homogenizer (Model PT 10/35 GT blender equipped with a PTA-20SM generator) (Brinkmann Instruments Inc., Westbury, NY, USA) at setting "5" (approximately 12,825 rpm) for 1 min at 20 °C. The head of the homogenizer was 1.5 cm from the bottom of cylinder.

5.2.5. Foaming properties

The total volume of foam in the graduated cylinder was measured at time zero and used to represent foaming capacity. The foam was allowed to stand undisturbed at room temperature. The volume of liquid (mL) drained from the foam was measured every minute for 10 minutes and was reported as foaming stability.

5.2.6.1. Formulation

Three formulations of angel food cakes were prepared using different proteins as the foaming agent: freshly shelled egg white, control PPI, and ultrasound treated PPI (Table 1). All-purpose flour, AA-grade fresh whole eggs, vanilla extract, salt, cream of tartar, and cane sugar were purchased from a local grocery. PPI was dispersed in water to form a protein solution of 5 g protein per 100 mL. The pH of all protein solutions was adjusted to pH 5.0 based on the optimal foaming capacity and stability data obtained from preliminary tests. All protein solutions were gently stirred for 5 min before use.

5.2.6.2 Baking procedure

Protein solutions were whipped for 30 s at max speed with a hand mixer (Oster 2500, Inspire 240-Watt, 5-Speed; Sunbeam Products, Inc., Boca Raton, FL, USA) equipped with a wire whisk attachment. Salt, cream of tartar, and vanilla were added during continuous mixing for 45 s. Sugar was added in three additions while mixing at max speed for 1 min. Flour was manually mixed in four separate additions. Batters were then transferred into non-stick tube pans (20 cm x 16 cm x 9.14 cm) and baked at 190 °C for 25 min for egg white and 35 min for PPI formulations. Doneness was visually evaluated prior to removal. After baking, the cakes were left to cool at room temperature for 1.5 h. Cakes were then removed from the pans, wrapped carefully in plastic wrap, and stored in a plastic container at room temperature for up to 72 h. Angel food cakes from the same batter were used for textural evaluation and physical measurements. The pH of the angel food cakes was measured by homogenizing triplicate 2 g samples with 50 mL deionized water for 30 sec.

5.2.6.3. Proximate analysis

Proximate analysis was performed on angel food cakes to determine their specific composition (protein, fat, and moisture) using AOAC methods (2012).

5.2.6.4. Textural analysis

Texture was measured on angel food cake slice samples at room temperature. Trapezoid samples were cut to have a 2.5 cm interior and exterior 5 cm. Two parallel plates of an Instron Universal Testing Machine (Intron Corp., Canton, MA, USA) were used to compress each sample to 20% of the sample's height at a test speed of 50 mm/s. Using a two-cycle compression, hardness, deformability, cohesiveness, and gumminess

were calculated (Xiong, Noel, and Moody, 1999). Hardness was defined as the peak of the first compression (peak A force). Using the reduction of force during the second compression (peak B force), deformability was calculated ((peak A force - peak B force) / (peak A force))*100. Peak B height^2 / Peak A height^2 was used to calculate cohesiveness. Gumminess was calculated as cohesiveness multiplied by the hardness (Bourne, 1978). Texture analysis was done on at least six samples per treatment. Samples were vertically cut through the center, and the height measured. The baking loss (%) was determined by weighing the cooked cake and the uncooked batter and calculated as:

Baking loss (%) = [(Initial batter weight–Cake weight)/ Initial batter weight] *100

5.2.6.5. Color measurement

Trapezoid samples were cut to have a 2.5 cm interior and exterior 5 cm, the crust was reformed and the crumb was placed over the aperture. *L** (lightness), *a** (redness), and *b** (yellowness) values were measured in triplicate on the interior crumb using a HunterLab MiniScan 45 LAV (Hunter Associates Laboratory, VA, USA) equipped with a D65 light source, 2.5 cm aperture, and illuminant A (average incandescent, tungsten-filament lighting). The comprehensive numerical total color difference, ΔE was calculated from *L*, *a** and *b** in the equation below with egg white angel food cake being used as the reference values. Whiteness (%) was calculated according to Lu et al. (2005).

$\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$

Whiteness (%) = $100 - \sqrt{(100 - L)^2 + a^2 + b^2}$

5.2.7. Statistical analysis

Data with at least three independent trials (n = 3) each with freshly prepared protein solution was analyzed using the general linear model procedures of the Statistix

10 software package (Analytical Software Inc., St. Paul, MN, USA). Analysis of variance (ANOVA) was performed to determine treatment effect. When significant treatment effects ($P < 0.05$) were found, their means were separated by Tukey's honest significance test.

5.3. Results and Discussion

5.3.1. Surface tension

Successful foams depend on protein adsorption to the air-water interface, reduction of interfacial tension. Smaller particles have been shown to rapidly diffuse to the air-water interface (Foegeding, Luck, & Davis, 2006). Ultrasound treatment significantly decreased particle size with increasing time (Fig. 4.1). The surface tension of control and ultrasound treated PPI was measured and displayed values of 61.9 ± 1.7 and 51.6 ± 1.1 dynes/cm, respectively (Table 5.2). Increased hydrophobic patches in plant proteins have been shown to be correlated with a decrease in surface tension (Liao et al., 2010). The decreased particle size, increased H_o, exposed SH, and ζ-potential combined could explain the decrease in surface tension by weakening the hydrogen bonding between water molecules (Table 5.2).

5.3.2. Foaming properties

5.3.2.1. Foaming capacity

Foaming capacity (FC) is the volume of foam that can be generated from a known amount of solution. The FC of PPI under various ingredient conditions and ultrasound treatment can be found in Fig. 5.1. After ultrasound treatment, PPI foams at all ingredient concentrations increased from 133% (control) to 202%. This observed 1.5 fold increase

could be explained by the rapid adsorption of ultrasound treated PPI to the air-water interface at pH 5.0. The reduction in surface tension as a result of the structural changes and particle size reduction can partially explain the increase in FC. Previous studies have shown that FC was correlated with surface hydrophobicity and partial denaturation (Damodaran, 2008). Electrostatic interactions play a significant role in both protein adsorption and interfacial rheology. Foaming properties have been reported as optimal for a range of proteins near their isoelectric points (pI). At the pI protein net charge is zero, reducing protein-protein repulsion, allowing for rapid absorption to the air-water interface (Davis, Foegeding, & Hansen, 2004; Hammershoj, Prins, & Qvist, 1999; Phillips, Schulman, & Kinsella, 1990; Zhu & Damodaran, 1994).

5.3.2.2 Foam stability

Foam stability (FS) is the ability of a protein to resist stress over time (Awad, Moharram, Shaltout, Asker, & Youssef, 2012). To determine FS, liquid drainage was monitored and volume recorded every minute for 10 minutes. Destabilization of protein foams is attributed to disproportionation, bubble coalescence, and drainage (Hammershj, Prins, & Qvist, 1999). Ultrasound treated PPI displayed little to no visible drainage under all conditions (0 ± 1%), while significant drainage was observed for control (25 ± 5% depending on treatment) (Fig. 5.2). Previous studies have shown that sucrose and NaCl have been used to improve FS of PPI by increasing viscosity and limiting drainage but at the depression of FC (Damodaran, 2008; Koocheki, Taherian, & Bostan, 2013). High concentrations of NaCl or sucrose depressed the FC slightly but had no effect on FS in ultrasound treated PPI foams. The improved FS at high ionic strength or high sugar concentration indicated the significant effect of ultrasound on the integrity of PPI foams.

The improved FS results from the rapid diffusion and development of a stable cohesive matrix by the smaller partially unfolded PPI aggregates.

5.3.3. Angel food cakes

The acceptability and physicochemical composition of bakery products such as cakes, muffins, and meringues rely on the foaming properties of their raw ingredients. The texture of cakes is defined by the ability of the protein to generate foam and coagulate into an ordered matrix. Eggs are known to contribute color, aroma, water holding capacity, and textural properties in baked goods. The functionality of egg proteins makes it the gold standard for emulsification, foaming, and gelation (Corke, De Leyn, Nip, & Cross, 2008). Based on the satisfactory results of the PPI foaming experiment, evaluation as total egg white replacement in angel food cakes was conducted

5.3.3.1. Proximate analysis and pH

All three angel food cakes were analyzed for proximate composition. The results of proximate analysis are displayed in Table 5.3. There were no detectable lipids in any of the cakes. Protein solutions were adjusted to target pH 5.0 before cooking based on the improved foaming properties observed at the pI of PPI. The actual pH values of 5.2 ± 0.05 as a batter. Cream of tartar was used to adjust the pH of the egg white formulation. The pH of all three formulations decreased after baking ($P > 0.05$). Previous studies have shown that some imidazole groups, which are located in the interior of native proteins, become titratable upon denaturation (Álvarez, Xiong, Castillo, Payne, & Garrido, 2012). The buffering capacity of PPI is reduced near the pI and could help explain the increase.

5.3.3.2. Baking loss

Baking loss is tabulated in Table 5.3. Baking loss is important in the final weight and consumer acceptance of baked goods and has been shown to impact consumer acceptance. However, there was no significant difference in baking loss.

5.3.3.3. Loaf volume

Loaf volume is reported in Table 5.3. During the baking process, batters expand and set into foam structures. The final cake volume is directly related to the expansion and resistance to collapse (Arunepanlop, Morr, Karleskind, & Laye, 1996; DeVilbiss, Holsinger, Posati, & Pallansch, 1974; Pernell et al., 2002). The ultrasound treated PPI angel food cakes had a loaf volume more similar to egg white than control PPI. This may suggest an ability to form a more cohesive network, entrapping more air and thus preventing significant collapse that was observed in the control. This cohesive network is observable in the cross-sectional images (Fig. 5.3). Control PPI cakes underwent collapse and drainage upon heating and visible gelation is visible. These results agree with the FS and FC results (Fig. 5.1, Fig. 5.2).

5.3.3.4. Textural profile analysis (TPA)

The hardness, cohesiveness, deformability, and gumminess were tested and presented in Table 5.3. Hardness (N) is a measure of maximum force to compress an object by a pre-defined length at a specific rate. The hardness was 3.2, 5.3, and 3.4 N for the egg white, control PPI, and ultrasound treated PPI, respectively. It has been shown that legume addition to baked goods increases hardness, attributed to increased density of the matrix (Majzoobi, Ghiasi, Habibi, Hedayati, & Farahnaky, 2014; Shevkani & Singh, 2014). Cake donuts with black bean and navy bean protein isolate (30% replacement for

egg) generated tougher and darker colored donuts (Vongsumran, Ratphitagsanti, Chompreeda, & Haruthaitanasan, 2014). Cohesiveness has been shown to be linked to the formation of an elastic network; the lack of network will result in less cohesive and elastic texture (Jarpa-Parra, Wong, Wismer, Temlli, Han, Huang, Eckhart, Tian, Shi, Sun, and Chen, 2017). Control PPI cakes had lower cohesiveness values compared to ultrasound treated and egg white cakes. The lower cohesiveness values of control cakes indicate a lower mechanical resistance, which is indicative of a weak protein network. Gumminess is a measurement of the energy required to chew something so it can be swallowed. Egg white and ultrasound treated PPI samples were not significantly different in gumminess but control PPI exhibited significant increase in gumminess. This difference attributed to the gelation layer observed (Fig. 5.3). Deformability was significant in the control cakes, compressing and not returning to the original volume, this could be because of the lack of a cohesive network and gelation layer observed.

5.3.3.5. Cake color

Consumer acceptance of foods is influenced heavily by the appearance. The crumb color is primarily affected by the ingredients used (Majzoobi et al., 2014; Majzoobi, Imani, Sharifi, & Farahnaky, 2018). The crumb color can be found in Table 5.3. ΔE (total color changes) and whiteness values were computed to determine if a visible difference was perceivable in the different formulations. Both control and ultrasound treated PPI cakes had ΔE values above 3, indicating that color differences were obvious to the human eye. Both pea formulations exhibited lower L* values (more black), much higher a* values (more red), and higher b* values (more yellow). The dominant pigments in peas are typically xanthophylls, with low concentrations of

dihydroxy pigments and carotenes (Reichert and MacKenzie, 1982) PPI is rich in lysine, which reacts with reducing sugars during baking resulting in a darker color. Pea products are naturally darker than egg white, so the results were not surprising. Previous studies have shown similar color changes in cakes, donuts, bread, spaghetti, and cookies which used soy, gluten, black bean, green pea, and chickpea proteins to replace traditional proteins (Majzoobi et al., 2014; Singh & Mohamed, 2007; Vongsumran et al., 2014; Zhao, Manthey, Chang, Hou, & Yuan, 2005). The color differences between control and ultrasound treated PPI are attributable to the structural changes and particle size reduction.

5.4. Conclusions

Application of ultrasound was shown to decrease PPI aggregate size by dispersing large protein aggregates and disrupting quaternary and tertiary structures. This disruption induced partial unfolding and rearrangement, exposing buried hydrophobic residues and SH groups, resulting in an increase in solubility and ζ-potential. This increased solubility combined with the other structural changes allowed for ultrasound treated pea protein to display a lower surface tension. Ultrasound induced physiochemical changes improved the interfacial characteristics, resulting in greater foaming ability and stabilization under different ingredient conditions. Angel food cakes made with ultrasound treated PPI had significant differences in color and loaf volume but were comparable in cohesiveness, hardness, deformability, and gumminess to cakes formulated with egg whites. While differences exist, angel food cakes made without eggs are appealing to certain

demographics. Complete replacement of egg white by pea protein would benefit from research on sensory impacts and formulation refinement is required.

Table 5.1. Angel food cake formulations

Ingredients (g)	Egg white	Control PPI	Ultrasound PPI
Flour	30	30	30
Sugar	60	60	60
Protein source	42	100	100
Water	48	0	0
Vanilla extract	1.25	1.25	1.25
Cream of tartar	0.5	0.5	0.5
Total (g)	192	192	192

*Egg white: freshly shelled whole white (12% protein content); PPI: pea protein isolate suspension (5% protein content).

Table 5.2. Surface and chemical characteristics of control and ultrasound treated pea protein isolate (PPI)*

Characteristic	Control PPI	Ultrasound PPI
Surface hydrophobicity (H_o)	93 ± 7.1^{B}	206 ± 13^{A}
Surface SH groups (μmol/g soluble protein)	23.8 ± 2.4^{B}	43.9 ± 3.3^{A}
Disulfide bonds (μmol/g soluble protein)	5.61 ± 1.9^{A}	5.92 ± 1.3^{A}
Zeta potential ζ (pH 7.0)	-24.2 ± 2.4^{B}	-31.4 ± 2.5^{A}
Particle size (nm)	568 ± 35^{A}	220 ± 17^{B}
Surface tension γ (dynes/cm)	61.9 ± 1.7^{A}	52.6 ± 1.1^{B}

*Means ± SD having a common superscript letter in the same row are not significantly different ($P < 0.05$).

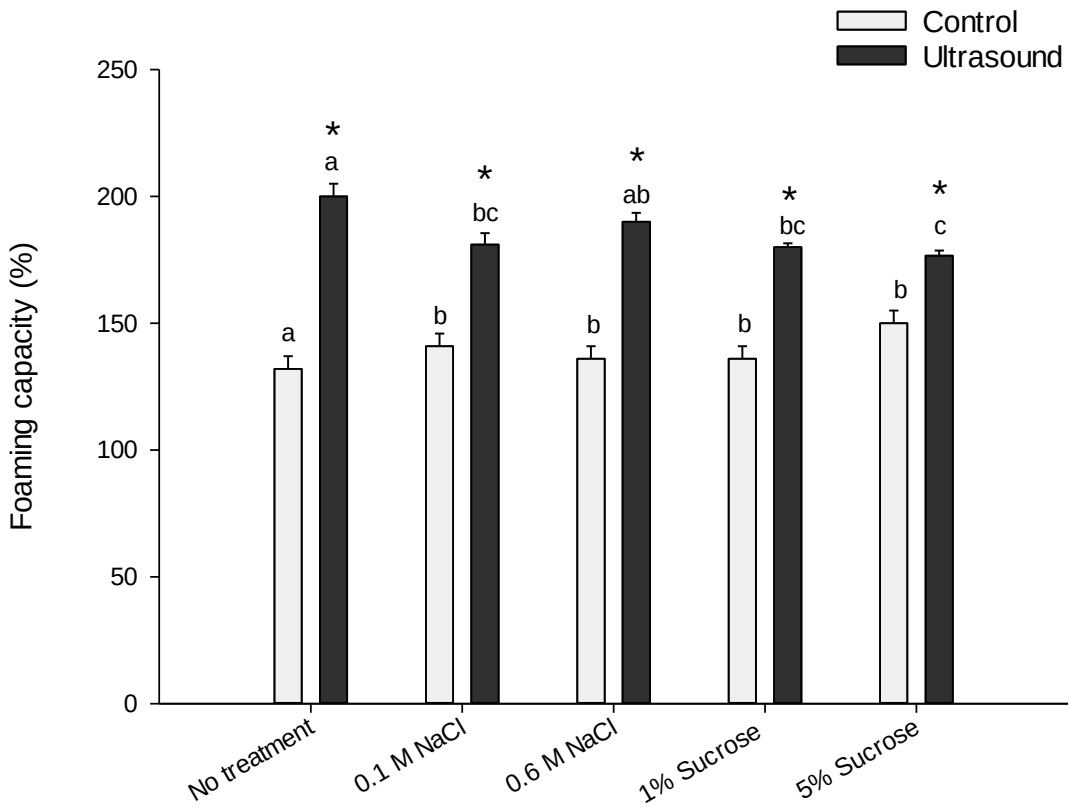

Figure 5.12. Foaming capacity of pea protein (4% w/v protein, pH 5.0) treated with different concentrations of NaCl or sucrose.* Denotes significant difference ($P < 0.05$) between treatments; a-c denote significant differences ($P < 0.05$) within the same treatment.

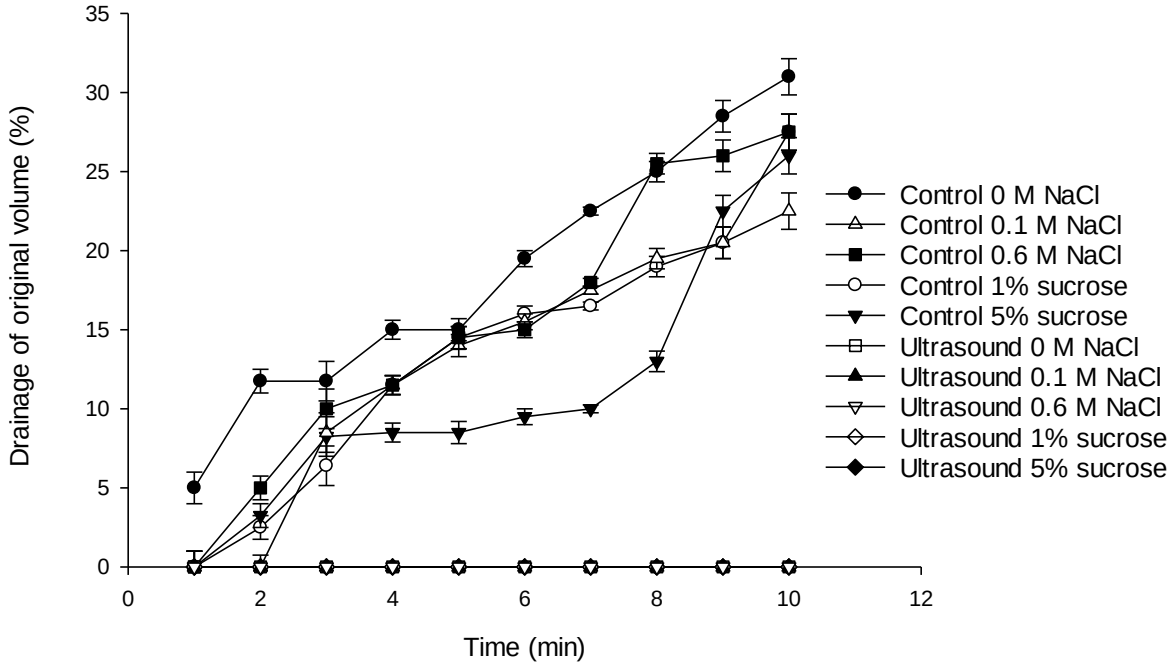

Figure 5.13. Foam drainage of pea protein (4% w/v protein, pH 5.0) treated with different concentrations of NaCl or sucrose.

Table 5.3. Proximate composition, textural, and color measurements of angel food cakes formulated with egg whites, control PPI, and ultrasound treated PPI*

Property	Egg white	Control PPI	Ultrasound PPI
Protein content (wt. %)	85.8	84.1	84.3
Fat (wt. %)	<0.1	0	0
Carbohydrate (wt. %)	0	3.02	3.01
Ash (wt. %)	4.11	4.05	4.04
Moisture content cake (%)	36.0 ± 0.4^{A}	35.1 ± 0.6^{A}	35.5 ± 1.20^{A}
Baking loss (%)	17.2 ± 1.9^{A}	19.1 ± 0.4^{A}	18.2 ± 0.6^{A}
Loaf volume (cm)	10.1 ± 0.3^{A}	3.94 ± 0.8^{C}	8.1 ± 0.8^{B}
Peak A (N)	3.2 ± 0.2^{B}	5.3 ± 0.4^{A}	3.4 ± 0.6^{B}
Peak B (N)	3.1 ± 0.1^{B}	4.8 ± 0.4^{A}	3.2 ± 0.8^{B}
Cohesiveness	0.97 ± 0.01^{A}	0.90 ± 0.01^{B}	0.96 ± 0.02^{A}
Deformability	3.3 ± 1.2^{B}	9.8 ± 1.0^{A}	3.3 ± 1.5^{B}
Gumminess	3.1 ± 0.1^{B}	4.7 ± 0.3^{A}	3.3 ± 0.5^{B}
*L**Crumb	75.56^{A}	53.9^{C}	69.4^{B}
*a**Crumb	0.27^{C}	3.97^{A}	3.43^{B}
*b**Crumb	14.5^{C}	16.14^{B}	19.1^{A}
ΔE Crumb	-	22.1^{A}	8.61^{B}
Whiteness	71.6 ± 2.7^{A}	51.0 ± 5.1^{C}	63.7 ± 1.6^{B}
pH Cake	5.66	5.05	5.16

*Means ± SD having a common superscript letter in the same row are not significantly different ($P < 0.05$). PPI: pea protein isolate.

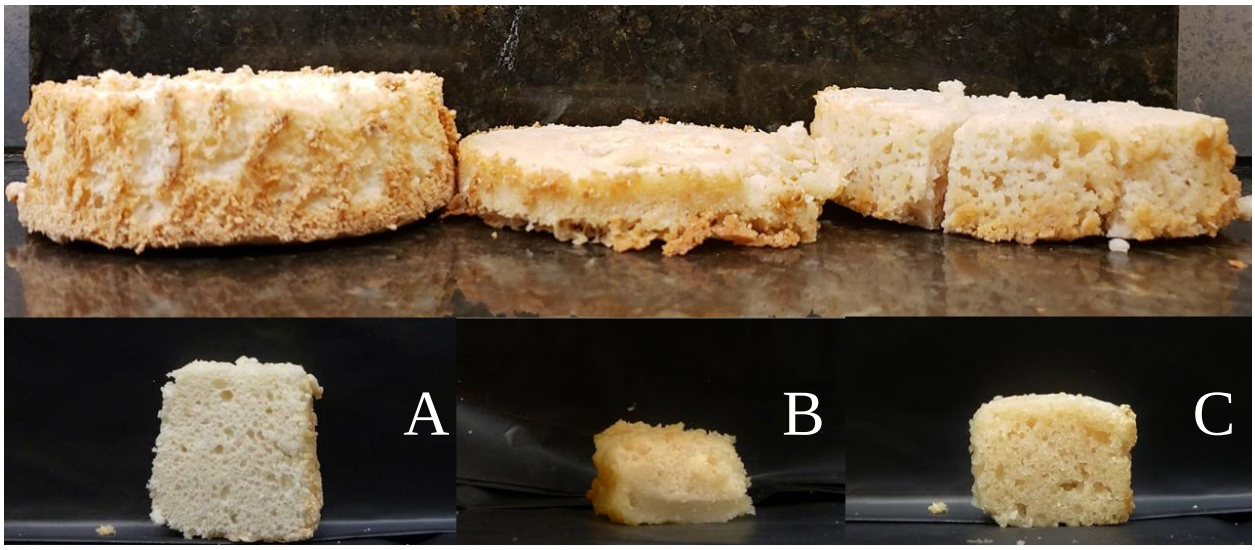

Figure 5.14. Angel food cakes formulated with egg whites (A), control PPI (B), ultrasound treated PPI (C). Upper panel: loaf volume; lower panel: cross section. PPI: pea protein isolate

Chapter 6

A Comparative Study of Ultrasound Treatment on the Physicochemical, Structural, and Emulsification Properties of Pea Protein Isolate

Summary

The ultrasonic effect on the emulsifying properties of pea protein isolate (PPI) was investigated. Proteins solutions were treated at an acoustic intensity of 60 W cm^{-2} (50 % amplitude, 20 kHz) for 3 min (pulse duration: on 5 s; off 5 s). Emulsion capacity was measured in terms of conductivity. Emulsions were prepared with control or ultrasound treated PPI by homogenization with sunflower oil (25% v/v). The emulsifying performance was analyzed in terms of particle size, creaming index (%), emulsion stability index, and emulsion ability index. Oxidative stability was evaluated over the course of two weeks by measuring TBARS. Ultrasound treated PPI had significant increases in solubility, surface hydrophobicity, sulfhydryl groups, zeta potential ($P < 0.05$). The amount of oil encapsulated per gram of protein increased from 0.98 to 1.35 after ultrasound treatment. Emulsions prepared with control PPI and ultrasound-PPI yielded significantly smaller emulsion droplets. Control PPI particles aggregated at a greater rate over the 14 day trial, which was associated with an increase in creaming index (%) when compared to ultrasound treated emulsions. On day 7 and 14, the ultrasound treated PPI emulsions had lower TBARS values than control. These improvements could support further formulation of plant-based beverages.

6.1. Introduction

Milk is a household staple for a majority of the population, but total dairy sales are decreasing. Milk is being replaced by non-dairy alternatives with 56% of consumers switching to plant-based milk products (Mintel, 2018). The chief reason for this switch is primarily consumers seeking lactose-free products. Pea based milk products are underrepresented on grocery shelves compared to other plant proteins (soy, rice, oat) (Mäkinen, Wanhalinna, Zannini, & Arendt, 2016). Improvement to the functional properties of pea protein in beverage systems could increase its marketability.

Interest in using plant based protein in emulsions has increased because of the reduced impact on the environment, low allergenicity, and decreased cost (Yildiz, Ding, Andrade, Engeseth, & Feng, 2018). Pea protein has emerged as a potential replacement for traditional protein sources. Its main advantages are having low allergen potential, high antioxidant potential, and similar nutritional profiles to other legume proteins (Sanchez-Monge, Lopez-Torrejón, Pascual, Varela, Martin-Esteban, & Salcedo, 2004). The preparation of both the pea protein and emulsion has a large impact on the final stability. Protein purity, pH, viscosity, solubility, particle size, oil to protein ratio, and co-solutes all can impact the stability of a pea protein emulsion (McWatters & Cherry, 1977).

Low frequency (X <100 kHz) ultrasound treatment is often used for a variety of reasons but has been shown in the past to affect the physicochemical properties of many proteins (O'Sullivan et al., 2017). Physical and chemical modifications can generally influence the microstructure of proteins. The impact of ultrasound upon the structure of food molecules is attributed to ultrasonic cavitations, micro-thermal events, and pressure

differentials. Ultrasonic processing has been shown to reduce protein aggregate size and increase surface activity in a variety of proteins (gelatin, egg white, pea, soy, and rice) (Arzeni, Martínez, Zema, Pérez, & Pilosof, 2012; Jambrak, Mason, Lelas, Herceg, & Herceg, 2008; Karki, Lamsal, Grewell, Pometto,Van Leeuwen, Khanal, & Jung, 2009; O'sullivan et al., 2017). Pea protein has been studied before but limited information is available on the impact of high intensity ultrasound treatment on the stability of pea protein emulsions.

The objective of this study was to test the hypothesis that structural modification by ultrasound treatment would improve PPI interfacial adsorption and biophysical behavior in O/W emulsions resulting in increased stability over time and fewer tendencies for oxidation.

6.2. Materials and Methods

6.2.1 Materials

Pea protein isolates (PPI, NUTRALYS® S85F, 80% pea protein based on dry basis) in powder form was provided by Roquette America Inc. (Geneva, IL, USA), and was produced using a wet extraction process from dry yellow peas. The protein isolate was not further purified but used as is. The PPI was stored in a refrigerator at 4 °C before use. All other reagents and chemicals were purchased from VWR (Radnor, PA, USA), Sigma-Aldrich (St. Louis, MO, USA), or Fisher Scientific (Pittsburgh, PA, USA) and were of analytical or higher grade. Sunflower oil was purchased at a local market.

6.2.2. Ultrasound treatment

Pea protein (5% w/v) was obtained by dissolving powder in deionized water with stirring at 25°C for 1 hour. Ultrasound treatment was applied to 25 mL of PP in 30 mL beakers using a Q700 Sonicator (Qsonica Sonicators, Inc., Newtown, CT, USA) at 50% amplitude, 5 second pulsed cycle for a total of 3 minutes of sonication. An ultrasonic probe of 1/2" (12 mm) diameter was used to deliver acoustic energy into the sample, and the acoustic power density (APD) was controlled at approximately 60 W/cm^{-2} (11,000 per replication). The probe was inserted into the solutions at a depth of 1-inch. Heat produced by ultrasonication may cause protein denaturation (Kent & Doherty, 2014). In order to avoid overheating, an iced water bath was used to cool the samples. An integrated temperature probe was also used to monitor the samples with a programmed shutdown of 50 °C.

6.2.3. Emulsion preparation

Emulsion formulations were based on commercially available plant milks made with pea protein (*Ripple, Bolthouse Farms, and Silk*). Oil-in-water (O/W) emulsions were prepared with 25% (v/v) sunflower oil and 75% (v/ v) Control or ultrasound treated PPI (10 mg/mL protein adjusted to pH 7.0 by titration). Initial dispersion was with a Kinematica Polytron PT 10-35 GT with PT-DA 12/2 EC-B154 generator (Brinkmann Instruments, Inc., Westbury, NY, USA) at 13,500 rpm for three bursts of 40 s each. Samples were then introduced into a high-pressure homogenizer for two parallel flow cycles (70 MPa) (NanoDeBee, B.E.E. International Inc., Easton, MA, USA). Emulsions were immediately transferred to an iced water bath to cool and then stored at 2 °C.

6.2.4. Emulsion properties

6.2.4.1. Emulsifying activity and emulsion stability

Aliquots (20 μL each) of samples were taken 0.5 cm from the bottom of the beaker at designated post-homogenization times and dispersed into 7 mL of 0.1% sodium dodecyl sulfate solution (SDS). Absorbance at 500 nm was read. Emulsifying activity index (EAI) and emulsion stability index (ESI) were calculated as (Pearce & Kinsella, 1978). Where A_0 and A_{30} represent the absorbance (500 nm) immediately after emulsification (time 0) and after 30 min at room temperature

$EAI\ (m^2/g) = ((4.606) / (C \times (1-\varphi) \times 10^4)) \times A_0 \times N$

$ESI\ (\%) = (A_{30} / A_0) \times 100$

Where C is the protein concentration (1 g/mL), and φ is the volume fraction (v/v = 0.25) of oil, N is the dilution factor.

6.2.4.2. Emulsifying capacity (EC)

The emulsion capacity was verified by using an YSI Professional Plus portable temp/DO/CND/salt/pH/ORP meter (Yellow Springs Instrument Company, Yellow Springs, OH, USA) to measure the conductivity of the emulsion (Hung & Zayas, 1991). Protein solutions of 0.25% (w/v) were continuously mixed while sunflower oil was added at a rate of 0.5 mL/s. The steep drop in conductivity was taken to be an indicator of protein overwhelming. Capacity is expressed as g of sunflower oil per g of protein before inversion. Conductivity measurement electrodes were calibrated daily with YSI 3167 conductivity calibrator (potassium chloride 0.053%).

6.2.4.3. Creaming Index

Oil-in-water emulsions were prepared by homogenization (as previously described). Emulsions were then transferred into 25 mL sealed graduated glass cylinders (inner diameter = 10.5 mm; height = 160 mm) immediately after preparation. The stability of the emulsions (Demetriades, Coupland, & McClements, 1997) was monitored by observing the separation of a cream layer after 1 h of storage at room temperature, then after 1,7, and 14 days in storage at 2 °C. Overtime emulsions began to separate into an optically opaque darker cream layer (top), and a turbid layer at the bottom with a similar appearance to the original emulsion. Creaming Index *(*CI*)* was expressed as:

$CI\ (\%) = H_t / H_e \times 100$

Where H_t is height of the top layer and H_e is the total emulsion height.

6.2.5. Oxidative stability (TBARS)

For TBARS (Sinnhuber, 1977), 2 g of sample emulsion was taken on days 0, 1, 7, and 14 from storage at 2 °C and was mixed with trichloroacetic acid (TCA) and thiobarbituric acid (TBA) followed by boiling for 30 min. The sample was cooled to room temperature then centrifuged at 5,000g for 15 min. 5 mL of sample supernatant were transferred to a glass screw-top test tube and 2 mL of chloroform added to extract any lipids. After centrifugation at approximately 2,000g for 10 min, absorbance (532 nm) of the upper phase was recorded and TBARS content calculated using the molar extinction coefficient of 152,000 M cm-1 (Witte, Krause, & Bailey, 1970).

6.2.8. Statistical analysis

Data with at least three independent trials (n = 3) each with freshly prepared protein solution was analyzed using the general linear model procedures of the Statistix

10 software package (Analytical Software Inc., St. Paul, MN, USA). Analysis of variance (ANOVA) was performed to determine treatment effect. When significant treatment effects ($P < 0.05$) were found, their means were separated by Tukey's honest significance test.

6.3. Results and Discussion

6.3.1. Emulsifying activity

An emulsion consists of 2 immiscible phases; oil and water are the most common in food systems. Emulsions are unstable and will undergo coalescence and creaming when destabilized. Proteins stabilize emulsions by forming an elastic film which slows down coalescence and creaming/flocculation. The rapid diffusion to the oil-water interface is critical for emulsion ability (O'Sullivan et al., 2016).

Emulsifying activity index (EAI) is a measurement of the total interfacial areas stabilized by a given amount of protein. EAI was measured for both emulsions immediately after generation and is reported in Table 6.1. The ultrasound treatment improved EAI (133.9 ± 9.6) over control EAI (91.8 ± 3.6). Improvements to EAI by ultrasound treated are attributed to accelerated diffusion and film formation at the oil-water interface. Globular protein unfolding at oil-water interface induces exposure of non-polar groups and sulfhydryl groups (McClements, 2004). As shown in chapter 4, the ultrasound treated samples had increased exposed hydrophobic groups exposures as a result, the reactivity of the globular proteins increased due to hydrophobic interaction with oil droplets or other proteins molecules (Table 4.1). Smaller particle size and exposed SH groups on the surface have been shown to improve emulsifying properties

(Wouters, Rombouts, Fierens, Brijs, & Delcour, 2016). Surface hydrophobicity has also been linked to the initial anchoring of a protein to the oil–water interface (Kato & Nakai, 1980).

6.3.2. Emulsifying capacity (EC)

Conductivity can be indicative of the emulsion type. High conductivity values indicate that water is the continuous phase and oil is the dispersed phase in an O/W emulsion. In contrast, the conductivity values will be low for a W/O emulsion (Züge, Haminiuk, Maciel, Silveira, & de Paula Scheer, 2013). Phase inversion is an instability mechanism, thus factors which change the stability of an emulsion impact the inversion boundary (McClements, 2015). The conductivity started at 50 mA and as the emulsion broke, the conductivity dropped to 20 mA or lower. The moment of sudden drop was take as the emulsion capacity. The properties of the emulsifier, its concentration, and processing conditions will modify the boundary. Increasing the amount of oil entrapped per gram of protein could allow for less protein to be used in potential food systems. It should be noted that the concentration of the emulsifier in question (protein) will impact the amount of oil entrapped. The oil source (soy, canola, olive) will also play a significant role on the emulsion capacity (Binks & Lumsdon, 2000).

For control PPI, an EC value of 0.98 ± 0.1 g oil/g protein was observed (Table 6.1). Ultrasound treated PPI exhibited an EC of 1.4 ± 0.1 g oil/g protein, a 1.4 fold increase. These values are similar to previous studies on pulses. The EC values of native fava, soy, chickpea, and lentil proteins were found to be 1.0, 1.3, 2.08, and 1.6 g oil/g protein, respectively (Karaca, Low, & Nickerson, 2011; McWatters & Cherry, 1977). Protein acts as a surface active agent, which reduces the surface tension between the two

phases. The modification of PPI's structural properties, specifically an increase in hydrophobic patches (Table 4.1) and reduction in particle size (Fig. 4.2) by ultrasound could explain the increased EC. These results are further strengthened by the observed decrease in surface tension (Table 5.2). Previous studies (Zayas & Lin, 1989) have shown that as protein solubility increased, the amount of oil emulsified increased, which agrees with previously presented data (Fig. 4.3).

6.3.3. Emulsion stability

The emulsion stability index (ESI) of control and ultrasound treated PPI emulsions was measured (Table 6.1). ESI is a measurement of an emulsion's ability to resist changes to its physicochemical properties over time. There was no significant difference in ESI between the control or ultrasound treatment. The lack of difference is primarily a result of the 30 min window of the experiment probably not being enough time for a detectable difference. It has been reported that stable O/W emulsions contain small droplets (< 300 nm) (Walker, Decker, & McClements, 2015). Control and ultrasound treated PPI had particle sizes below 300 nm immediately after emulsion generation, a result of the high pressure homogenization process. Destabilization was observed later in the experiment but was undetectable in the first 24 hour. EAI and ESI do not provide information on microstructure or the mechanism of destabilization and thus other techniques must be utilized.

The stability of emulsions was also tested in terms of creaming index (%), which is a measurement of the percentage of oil that aggregates and separates from an emulsion. The ability to resist creaming depends on particle size, surface charge, density, and viscosity of the emulsion. Emulsions with smaller particles, similar densities, and high

viscosity are the most stable (McClements, 2007). It has been shown that creaming stability correlated with higher absolute surface charge, smaller particles, and solubility (Karaca, Low, & Nickerson, 2011). Ultrasound treated PPI exhibited an increased surface hydrophobicity, zeta potential, sulfhydryl exposure, and reduced particle size (Table 4.1). The destabilization of PPI-sunflower oil emulsions is associated with an increase in particle size (Fig. 6.1). No differences were visible in the creaming index between ultrasound treated and control PPI samples on day 0 or 1 (Fig. 6.2). Although the emulsions were different in particle size and EAI, the impacts on the cream layer were too small for a significant difference to be observed during day 0 and 1. The cream layer developed further on day 7, and 14. Significant creaming indicated that the emulsions were destabilizing with age. Similar studies agree with these results, systems with soy and milk proteins have shown a link between particle size and creaming index (Lethuaut, Métro, & Genot, 2002; Loi, Eyres, & Birch, 2019).

6.3.4. Oxidative stability (TBARS)

The oxidative stability of control and ultrasound treated PPI sunflower oil emulsions were determined by measurement of TBARS over two weeks (Fig. 6.3). On day 0 and 1, the TBARS values were not different between the two emulsions. On day 7 and 14, the ultrasound treated PPI had decreased TBARS values than control ($P < 0.05$). Ultrasound treatments exposed more reactive sulfhydryl groups and hydrophobic groups (Table 4.1). It is possible that the rearrangement of reactive groups is responsible for the increased antioxidant activity as methionine, histidine, and lysine amino acids have been shown to inhibit lipid oxidation in model systems (Marcuse, 1960). It has also been reported that smaller particle emulsions are more resistant to oxidation than emulsions

with larger particles (Nakaya, Ushio, Matsykawa, Shimuzu, & Ohshima, 2005; Jiang, Zhu, Liu, & Xiong, 2014). Protein structural unfolding has been shown to result in increased reactive groups capable of reacting with radicals (Peña Ramos, and Xiong, 2002; Tong, Sasaki, McClements, & Decker, 2000; Zhang, Xiong, Chen, & Zhou, 2013).

6.4. Conclusions

Ultrasonic processing of PPI increased the solubility, hydrophobic group exposure, exposed sulfhydryl groups, and zeta potential. These structural changes yielded decreased particle size and surface tension. These structural changes promoted ultrasound treated PPI's interaction with sunflower oil as evidenced by the improved EC. Emulsions with ultrasound treated PPI had significant improvements in emulsifying activity and emulsion stability (resistance to creaming, inhibition of oxidation). This study shows that ultrasonic processing is an effective method for enhancing the functionality of PPI and shows potential for application to beverage systems. Further research on the relationship with co-solutes such as sugar, flavors, and stabilizers merits investigation.

Table 6.1. Emulsion properties of control and ultrasound treated pea protein isolate (PPI)*

Measurement	Control PPI	Ultrasound PPI
Emulsifying Activity Index EAI (m^2/g)	91.8 ± 3.6^{B}	133.9 ± 9.6^{A}
Emulsion Stability Index ESI (%)	92.6 ± 2.7^{A}	95.5 ± 1.4^{A}
Emulsifying Capacity EC (g of oil per 1 g of protein)	0.98 ± 0.1^{B}	1.4 ± 0.1^{A}

*Means ± SD having a common superscript letter in the same column are not significantly different ($P < 0.05$).

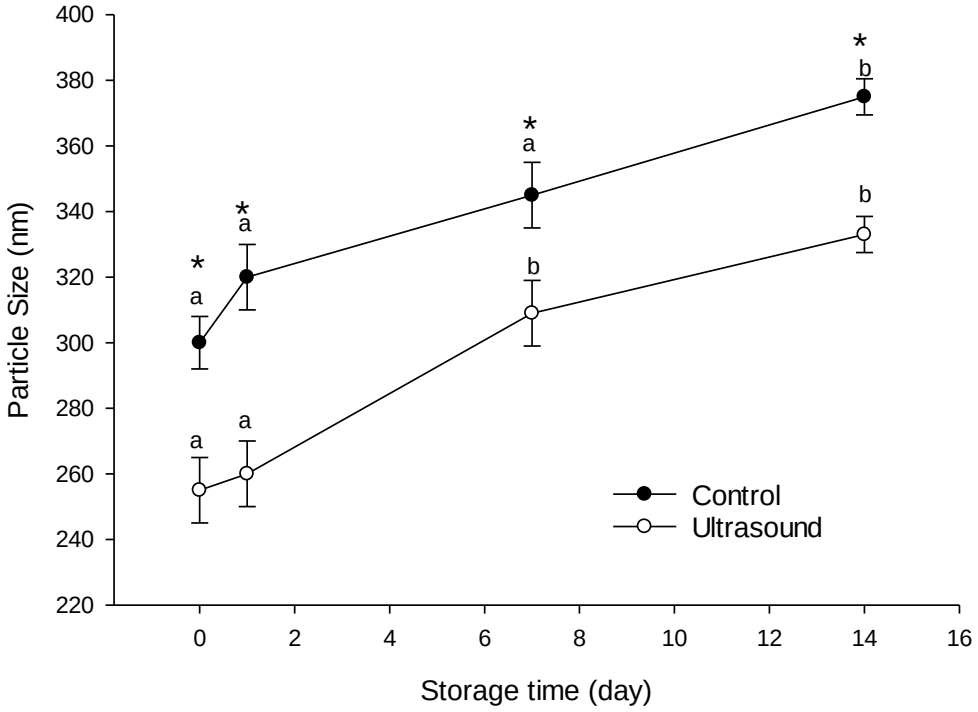

Figure 6.1. Particle size of pea protein-sunflower oil emulsions (10 mg/mL protein, pH 7.0) during storage at 4 ^{o}C. * Denotes significant difference ($P < 0.05$) between treatments; a-c denote significant differences ($P < 0.05$) within the same treatment.

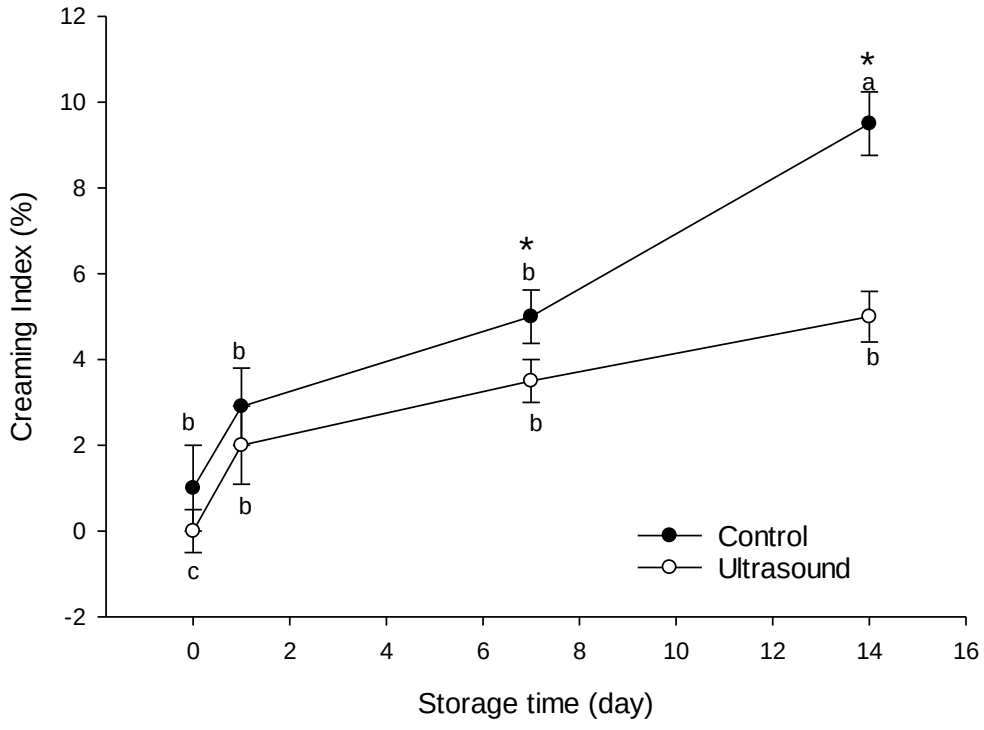

Figure 6.2. Creaming Index (%) of pea protein-sunflower oil emulsions (10 mg/mL protein, pH 7.0) during storage at 4 ^{o}C.*denotes significant difference ($P < 0.05$) between treatments on the same day. a-c denotes significant differences with the same treatment.

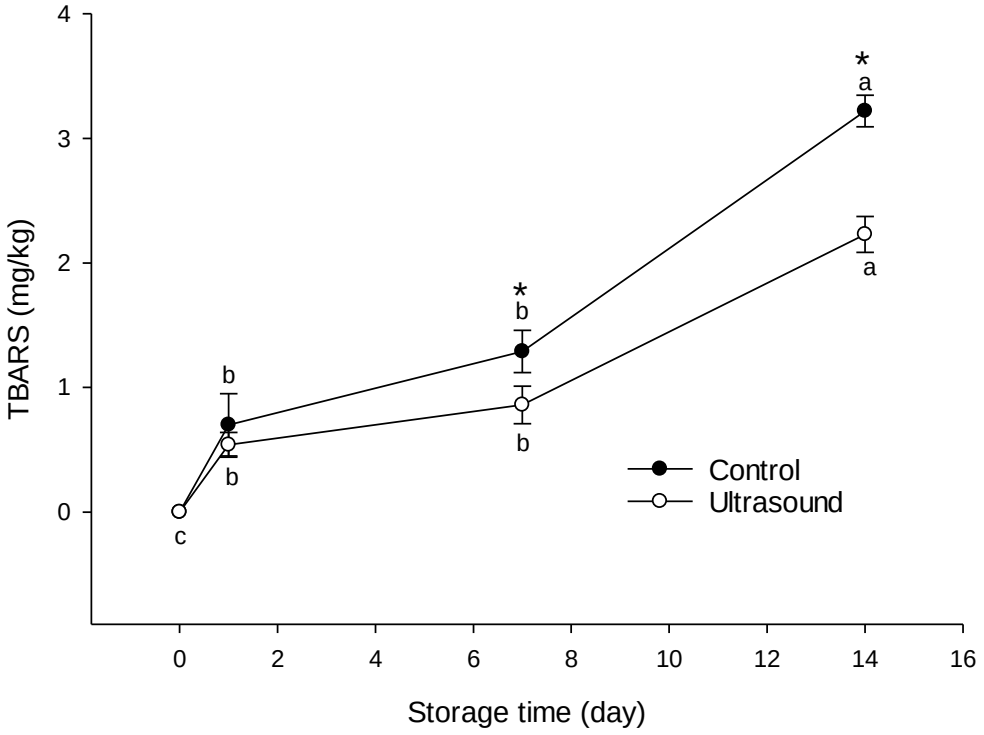

Figure 6.3. Overall treatment means of TBARS values (mg malonaldehyde/kg) of pea protein-sunflower oil emulsions (10 mg/mL protein, pH 7.0) during refrigeration storage at 4 ^{o}C. *denotes significant difference ($P < 0.05$) between treatments on the same day. a-c denotes significant differences with the same treatment.

Chapter 7

Conclusions and Future Work

The overall purpose of this Master's thesis research was to improve the understanding of PPI after physical alteration by ultrasound and under various food ingredient conditions.

The thermal and aggregation characteristics of PPI were evaluated under different pH (2-8), salt types (monovalent, divalent), and salt concentrations. As pH became more alkaline, the thermal stability of PPI decreased. NaCl increased the thermal stability with increasing concentrations, while divalent salt ($CaCl_2$) had the opposite effect on thermal stability. Specifically, $CaCl_2$ addition resulted in protein destabilization, reducing the ΔH with increasing concentrations. The particle size of PPI increased with salt concentration regardless of salt type. Turbidity testing agreed with the particle size data, displaying larger aggregates with increasing salt concentrations.

The evaluation of ultrasound treatment on PPI and the effect on molecular structure and functionality was performed. The 3 min–50% amplitude setting was chosen for significant differences in solubility and for feasibility. With increased sonication time and power, protein solubility increased across the tested range of pH (2-10). Particle size reduced from 560 ± 35 nm to 220 ± 15 nm after 1 min of treatment, and the particle size of ultrasound treated PPI was smaller than the control PPI under all salt concentrations. Divalent salts ($MgCl_2$ and $CaCl_2$) had a negative effect on the solubility of PPI, but ultrasound treated PPI was less susceptible to the destabilization effects possibly due to decreased particle size and modified structures. The turbidity of PPI solution was not

impacted by NaCl concentration, but significant aggregation was observed with $MgCl_2$ and $CaCl_2$. Ultrasound treated PPI demonstrated a higher turbidity with the divalent salts.

Tryptophan fluorescence results were unexpected, showing the fluorescence intensity being enhanced by ultrasound treatment. It is theorized that this is because of the dissociated subunits that rearranged into new patterns which created more hydrophobic pockets for tryptophan, or the larger particle in native protein blocked emission (light scattering). Surface hydrophobicity and exposed sulfhydryl groups were found to increase by ultrasound treatment. The particle size reduction and increased solubility are likely the result of the disruption of protein quaternary structures and original protein aggregates. This superior solubility and structural changes in PPI led to investigation into functional applications.

Ultrasound treated PPI exhibited superior foaming capacity and foam stability under different ingredient conditions (0.6 M NaCl and 5% sucrose, at pH 5.0). The improved interfacial properties are attributed to the particle size reduction and structural rearrangement allowing for cohesive matrixes to be formed. Angel food cakes made with ultrasound treated PPI had significant differences in color and loaf height, but were comparable in texture to egg white containing cakes. Control PPI formulations were unsatisfactory in both texture and appearance characteristics. While differences existed, angel food cakes made without eggs are appealing to certain demographics and these results are indicative of PPI's ability to function as a possible egg replacement.

The ultrasonic effect on the emulsifying properties of PPI was investigated in sunflower oil emulsions similar to milk alternatives. The emulsifying activity and capacity were significantly improved due to the structural changes induced by ultrasound.

The increase in hydrophobic groups and smaller particle size are hypothesized to allow for more effective adsorption of pea protein at the oil-water interface. Compared to the control PPI emulsions, ultrasound treated emulsions had greater oxidative and emulsion stability over the 14-day storage trial. The increase in exposed reactive groups and lower particle aggregation rate are the probable reason for improved stability. For the future development of PPI based milk substitutes, it would be beneficial to understand the impact of co-solutes such as flavors, sweeteners, and stabilizers.

Overall, the findings from this thesis research indicate that thermal characteristics of PPI can be modified by changing pH, salt type, and salt concentration. Additionally, ultrasound is an effective method to enhance the functionality of PPI and its potential application in food systems. While this research demonstrated the potential of ultrasound treatment for PPI, it is necessary to further study the processing conditions to ensure optimal performance in final products. As new food proteins are investigated and new processing techniques are developed, unknown research opportunities will continue to emerge. Rising cost of animal source proteins, risks relating to allergens, ethical concerns, digestibility, and functionality are leading the industry towards alternative proteins. Increasing the functionality of alternative proteins will allow for a reduction in manufacturing costs and dependence, improvement of quality characteristics, and more options for formulators. Research on the scale of application is required, as all experiments have been done at the lab scale. Another key parameter not addressed in this thesis is the impact of ultrasound or salt treatment on the sensory profile of developed foods.

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