The Optimization and Scale-Up of Pea Protein Extractions and Impact on Structural and Functional Properties

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Dedication

This thesis is dedicated to my parents for supporting me every day of my life, even when they did not know what “food science” was.

Abstract

As the demand for plant proteins continues to grow, there is a need to develop alternative sources of protein other than soy protein, which is limited by being sourced from a GMO crop and a "Big Eight" allergen. Yellow field peas (*Pisum sativum* L. subsp. *arvense*) are similar to soybeans in their agricultural benefits and protein profiles but are non-GMO and of low allergenicity. While soy protein has undergone decades of research to optimize extraction conditions and evaluate structural and functional properties, pea protein is less researched. Currently, pea protein is only mass produced by alkaline solubilization with isoelectric precipitation, which may cause damage to the protein by altering its native structure, thus reducing protein functionality in food applications. Therefore, in order to make pea protein competitive with soy protein, there is a need to optimize both the conditions used for pea protein extractions, as well as the methods of extraction, to produce pea protein isolates (PPI) with high protein purity and preserved structural properties. Additionally, the scalability of optimized extraction methods must be evaluated to determine industrial feasibility.

Therefore, the objectives of this study were: (1) optimize pea protein extraction conditions to maximize protein purity and yield following an alkaline solubilization with isoelectric precipitation and a salt solubilization coupled with membrane filtration; (2) characterize the impact of the two extraction methods on the protein structure and relate structure to functionality; (3) produce pea protein isolates on a pilot scale following the optimized benchtop extraction methods and evaluate the impact of a larger-scale production on protein structure and functionality.

Extraction conditions including solubilization pH, isoelectric precipitation pH, solubilization duration, number of solubilizations, and use of dialysis were optimized for PPI production by alkaline solubilization coupled with isoelectric precipitation (pH-extraction), based on protein purity and yield as well as industrial feasibility. Similarly, purification conditions including use of ultrafiltration (UF) and dialysis, individually or combined, were optimized for PPI production by salt

solubilization coupled with membrane filtration (salt-extraction). Optimized benchtop methods were then scaled-up to pilot plant production. The scaled-up (SU) protein extractions had some notable differences compared to the benchtop counterparts. Differences included overnight solubilization, use of diafiltration instead of dialysis, pasteurization, homogenization, and spray drying. The structural characteristics of the benchtop and SU pH- and salt- extracted PPIs were compared by determining the protein profile using SDS-PAGE, protein denaturation by DSC, surface charge by measuring zeta potential, and surface hydrophobicity as measured by a spectrophotometric method. Additionally, the functional properties of the PPIs were compared by measuring protein solubility, gelation, emulsification, and foaming properties.

The optimized pH extraction conditions were double solubilization for one hour at pH 7.5, followed by isoelectric precipitation at pH 4.5, and dialysis of the neutralized extract. The optimized purification conditions for salt extraction were ultrafiltration followed by dialysis. The benchtop pH-PPI and salt-PPI had protein purity of 87.6% and 92.8%, and protein yield of 64.7% and 72.0%, respectively. The SU-pH and SU-salt PPIs had comparable protein purity, at 88.7% and 92.4%, while protein yields were not determined due to sampling throughout the protein extractions, as well as the recovery of only the high solids retentate from UF.

Protein profiles were generally similar for all PPIs, except that the salt-extracted PPIs contained albumin proteins, while the pH-PPIs did not. Salt-PPIs, therefore, had a slightly higher isoelectric point than pH-PPIs, leading to lower surface charge at pH 7. Because of the presence of albumins, salt-PPI had slightly lower surface hydrophobicity than pH-PPI. Compared to benchtop PPIs, the protein in SU PPIs were partially denatured, had higher surface hydrophobicity, and were more aggregated.

Differences in structural characteristics led to observed differences in functionality. Salt-PPIs had slightly lower protein solubility at pH 7, and comparable or higher solubility at pH 3.4. Though the benchtop salt-PPI had higher gel strength than the pH-PPI, the gel strength of the SU-salt PPI was comparable to that of the

SU-pH PPI due to similar surface charges and levels of denaturation and aggregation. Emulsification capacity (EC) of the benchtop and SU PPIs was similar. Due to differences in relative amounts of globulins and albumins, the pH-PPIs had higher emulsion stability (ES), yet lower EAI, than the salt-PPIs. Similarly, higher albumin content in salt-PPIs potentially contributed to higher foaming capacity (FC) and foaming stability (FS) than pH-PPIs.

In comparison to SU PPIs, commercially available SPI and PPI (cSPI and cPPI, respectively) were completely denatured and extensively aggregated. Compared to cSPI, the SU PPIs had superior solubility at pH 3.4. However, cSPI had superior gelation and emulsification properties. On the other hand, both SU-pH and SU-salt PPIs had superior functional properties, in general, compared to cPPI.

Overall, this study demonstrated successful optimization and scalability of two pea protein extraction methods: alkaline solubilization with isoelectric precipitation and salt solubilization coupled with membrane filtration. Both optimized benchtop methods achieved high protein purity and yield, while using relatively nondenaturing conditions. Scaled-up extractions achieved similar protein purity to the benchtop counterparts. Further work investigating complete recovery of all fractions, while monitoring levels of protein denaturation, is needed to determine scaled-up extraction yields. The slight differences in structural and functional properties between benchtop and SU PPIs were mostly due to the thermal treatment the SU PPIs received that caused partial denaturation and aggregation. However, compared to cPPI, SU PPIs were less denatured, resulting in generally superior functionality that should be considered advantageous to industry. This study is significant in demonstrating that PPI, with superior functionality to commercially available PPI, can be produced on a large scale through both the traditional pH extraction and the novel salt extraction coupled with membrane filtration, under optimized conditions.

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Chapter 1: Review of Literature

1.1 Introduction

Protein continues to be in high demand throughout the world. Protein is widely regarded for its nutritional benefits including its role in muscle growth and maintenance, promoting satiety, and maintaining a healthy weight (Henchion *et al.*, 2017; Roberts, 2017). Additionally, as income levels rise, people look to consume more protein as it is seen as a sign of social status (Henchion *et al.*, 2017; De Boer and Aiking, 2018). Within the protein category, there is rapidly increasing interest in plant proteins as an alternative to meat and animal-based proteins due to the growing population of vegetarians, vegans, and flexitarians as well as concerns regarding the sustainability of consuming animal-based proteins.

For the past few decades, soy protein has been the only plant protein widely accepted and consumed. Soy protein has undergone many years of research and development to reach its current level of use. It is readily available, widely recognized, and has excellent nutritional quality and generally good functional properties in foods. However, soy protein is not without limitations. Soybeans are a GMO crop, are considered a “Big Eight” allergen in the United States, and are viewed with increasing skepticism by consumers (Formanski, 2019). A 2017 study by Mintel found that 24 percent of consumers actively avoided soy, with one-fourth of them doing so for allergy reasons and the remaining three-fourths avoiding soy for health or diet reasons (Formanski, 2019). With the increasing demand for plant proteins and the limitations of soy, there is a need for alternative sources of plant protein.

Yellow field peas (*Pisum sativum* L. subsp. *arvense*) have been utilized for animal feed for decades but are currently underutilized for human protein consumption (Rawal and Navarro, 2019). Field peas contain appreciable amounts of protein (20-30 percent), which can be extracted and concentrated following processes similar to those used for soy protein extraction (Tian *et al.*, 1999; Tulbek

et al., 2016). Pea protein ingredients have potential to compete with soy protein ingredients as they contain similar protein profiles and offer comparable benefits. Additionally, pea protein is of interest because it is produced from an environmentally friendly crop that is non-GMO and currently has low allergenicity (USA Pulses, 2016). Overall, pea protein has potential to be as widely used as soy protein.

To be competitive with other proteins, pea protein must have desirable nutritional properties. With the exception of soy protein, most plant proteins are nutritionally inferior to animal proteins. While animal proteins typically contain adequate amounts of all essential amino acids, plant protein are often limiting in at least one essential amino acid (Henchion *et al.*, 2017). Additionally, plant proteins may contain anti-nutritional factors such as phytic acid, lectins (hemagglutenins), amylase inhibitors, saponins, and trypsin inhibitors that have defense mechanisms in the plant, but reduce protein digestibility and may impair absorption of other nutrients (Panyam and Kilara, 1996; Carbonaro *et al.*, 2015; Thrane *et al.*, 2016; Tulbek *et al.*, 2016; Nosworthy and House, 2017). Pea protein is no exception as it is limiting in the amino acid methionine and contains anti-nutritional factors (trypsin inhibitors, lectins, saponins, and phytic acid) that lead to overall decreased nutritional quality (Sosulski and Holt, 1980; Elkowicz and Sosulski, 1982; Gueguen, 1983; Bhatty and Christison, 1984; Leterme *et al.*, 1990). However, improvements in protein processing and supplementation of limiting amino acids can improve the quality of pea protein to make it nutritionally competitive with other protein sources (FAO/WHO Expert Consultation, 1991).

In addition to requiring good nutritional quality, pea protein must offer useful functionality in food systems in order to gain prominence. The functional properties of a protein are dictated primarily by the protein's structure. Because the structure of the proteins from field peas are similar to the proteins from soybeans, it may be speculated that their functionality would also be similar (Tzitzikas *et al.*, 2006). However, the protein distribution and profile (i.e. content of different proteins) differ between field peas and soybeans (Fukushima, 2011). This leads to differences in

functionality, which in turn impacts the applications each protein is best suited for. Additionally, while soy protein was the subject of many years of research and processing development to enhance functionality, pea protein is comparatively new and under-researched.

The functionality of pea protein is not only affected by its protein profile, but also by the extraction and processing conditions during the protein isolation process. There are three main techniques used to concentrate protein from ground pea flour: air classification, alkaline solubilization with isoelectric precipitation, and salt extraction ("salting in, salting out"). Air classification has been used to produce pea protein concentrates (PPC), while alkaline solubilization with isoelectric precipitation is the most common method for producing pea protein isolates (PPI) (Arntfield and Maskus, 2011; Taherian *et al.*, 2011; Pelgrom, 2015). Salt extractions have been used to produce soy protein isolates, including a salt solubilization step followed by a "salting out" step to concentrate the protein, resulting in high waste streams and salty protein isolates that require several washes and diafiltration (Murray *et al.*, 1981). Alternative concentration techniques such as membrane filtration may replace salting out, which could make this extraction method more feasible.

Each of these extraction methods will result in different protein profile and structural characteristics, which will ultimately impact functionality. With each extraction method, the conditions employed during protein solubilization (pH, ionic strength, duration, temperature) and during protein precipitation/concentration (precipitation pH, salt concentration, membrane filtration) have a major influence on the structure of the extracted protein (Kinsella, 1979; Hoang, 2012). Extraction conditions that maximize protein purity and yield, while preserving native protein structure, are favored because the native protein structure tends to have better solubility and generally good functionality compared to a denatured counterpart (Shand *et al.*, 2007; Taherian *et al.*, 2012). Research on the impact of various extraction/concentration conditions on the protein's structural and functional properties is limited. On the other hand, process scalability and industrial feasibility

are important considerations when optimizing extraction conditions, while maintaining good yield, purity, and functionality.

1.2 Hypotheses and Objectives

Protein extraction methods should be selected and optimized based on their efficiency in extracting protein from the starting flour. Extraction efficiency can be determined by tracking protein purity and yield. Additionally, various extraction conditions have different impacts on the protein structure, resulting in different protein functionalities. Harsh extraction conditions can result in protein denaturation and/or aggregation, which decreases overall functionality and limits use in a food system. Therefore, mild and feasible extraction conditions that produce pea protein isolates of high protein purity and yield and acceptable functionality are desired.

It is hypothesized that the structural and functional characteristics of PPI produced by alkaline solubilization with isoelectric precipitation (pH-extraction) will differ from the those of PPI produced by salt solubilization coupled with membrane filtration (salt-extraction). Furthermore, it is believed that the salt-extraction will produce PPI that is less denatured and thus has superior functional properties compared to that produced by the pH-extraction. Additionally, it is hypothesized that the optimized pH- and salt-extractions can be scaled up to pilot plant production with no significant differences seen in structural and functional properties of the protein compared to that produced on the benchtop scale. Therefore, the main objectives of this research project were to:

1. Optimize pea protein extraction conditions to maximize protein purity and yield following A) an alkaline solubilization with isoelectric precipitation and B) a salt solubilization coupled with membrane filtration.
2. Characterize the impact of the two extraction methods on the protein structure and relate structure to functionality.

3. Produce pea protein isolates on a pilot scale following the optimized benchtop extraction methods and evaluate the impact of a larger-scale production on protein structure and functionality.

1.3 Growing Demand for Plant Proteins

Over the last several years, there has been a large shift in dietary patterns worldwide with people looking to consume more protein. In 2017, the global protein ingredients market was valued at over $36 billion in revenue and is projected to reach over $51 billion by 2022 (Frost & Sullivan, 2018). In 2017, with 7.3 billion people globally, the demand for protein was 202 million tons per year (Henchion *et al.*, 2017). With the rising population, the demand for protein will also increase. In 2018, the plant protein market made up nearly 25 percent of the protein market (Frost & Sullivan, 2018), and is projected to continue to grow.

Plant proteins provide various benefits that consumers are looking for. First of all, plant proteins are a more sustainable source of protein than animal-based proteins. Growing crops directly for consumer consumption instead of animal feed requires much lower inputs of resources per pound of protein produced (Sabaté *et al.*, 2014; Thrane *et al.*, 2016). It is estimated that 6 kg of plant protein are need to produce 1 kg of meat protein (De Boer and Aiking, 2011). Moreover, producing one pound of protein from pulses requires about 5 times less water than producing one pound of meat (Roberts, 2017; Pulses, 2020). In addition to high demands for water, meat production requires intensive energy, fertilizer, and land use (Henchion *et al.*, 2017). The extensive use of resources by meat production combined with the low protein conversion efficiency and the huge global demand for protein lead to concerningly high greenhouse gas emissions (Henchion *et al.*, 2017). The harmful impacts of meat production on the environment and increasing demand for protein prompt the need for alternatives to meat.

In addition to being more sustainable than meat, consumers generally view plant proteins as healthier than animal proteins. A 2019 Mintel survey found that consumers' top reason for avoiding animal proteins was health (Formanski, 2019).

Many people are limiting their consumption of meat because of it being high in saturated fat and cholesterol (Roberts, 2017). Sources of plant proteins, on the other hand, have no cholesterol, are low in fat, and can contain appreciable amounts of antioxidants (Tulbek *et al.*, 2016; Rawal and Navarro, 2019).

The environmental sustainability and health benefits of plant proteins compared to animal proteins are just two reasons behind the growth of the plant protein market. There is an increasing number of people following a vegetarian, vegan, or flexitarian diet that limits or eliminates consumption of proteins from animal sources (Henchion *et al.*, 2017). In addition to dietary preferences, some religious and ethnic groups do not allow consumption of certain animals (Eliasi and Dwyer, 2002; Reddy and Anitha, 2015). Plant proteins provide an alternative to fit the protein demands of these groups.

Moreover, food allergies and sensitivities affect a significant portion of the population. In the United States alone, it is estimated that 32 million people, about 10 percent of the population, have at least one food allergy (Food Allergy Research & Education, 2020). The "Big Eight" (milk, eggs, fish, Crustacean shellfish, tree nuts, peanuts, wheat, and soybeans) account for 90 percent of all food allergies, preventing millions of people from consuming them (FDA, 2004). As the only treatment for food allergies is complete avoidance, there is a large need for hypoallergenic protein sources.

Lastly, as younger generations enter the buying market, they are open to a larger variety of proteins and are interested in trying new products. Specifically, young consumers are looking for foods that are environmentally sustainable, ethically produced, align with their idea of health, and are "free from" certain triggers like allergens and GMOs (Roberts, 2017; Formanski, 2019). As millennial and Gen Z consumers begin to outnumber older generations, it is important to consider how they differ in preferences. For all these reasons and more, the demand for plant proteins is expected to continue expanding.

Traditionally, soy protein has been the most prominent plant protein. This is largely due to the widespread adoption of growing soybeans by farmers, as well

as industry efforts to isolate and develop soy protein into a nutritious, functional ingredient. However, soy protein faces some limitations. Namely, it is one of the "Big Eight" allergens, which prevents nearly 2 million people in the U.S. from being able to consume any product containing soy (Food Allergy Research & Education, 2020). Additionally, while 43 percent of consumers are looking to avoid GMO products, 94 percent of the acreage in the United States used for soybeans was genetically modified in 2019 (Formanski, 2019; National Agricultural Statistics Service, 2019). With many people unable to or choosing not to consume soy, there is a need for other plant proteins.

In more recent years, pea protein has been gaining traction for a number of reasons. First of all, unlike soybeans, field peas are currently of low allergenicity and are not genetically modified. Additionally, field peas have environmental benefits such as reduced needs of fertilizers and contribution to soil health. Furthermore, field peas have a short growing season and can be used in a crop rotation, further contributing to improved nutrient levels in the soil (Sustainable Agriculture Research & Education, 2012; Food and Agriculture Organization of the United Nations, 2015). Moreover, field peas are well suited for fractionation into different valuable ingredients, including pea starch, pea fiber, and pea protein (Arntfield and Maskus, 2011). Unlike soybeans, field peas are low in fat and do not require fat extraction prior to protein isolation, which reduces the time and cost of protein processing and allows for organic production (Sumner *et al.*, 1981; Tian *et al.*, 1999; Singh *et al.*, 2008). Although pea protein is growing in popularity, it is still far less established than soy protein, and is trailing in its nutritional and functional properties.

1.4 Soy Protein

On average, soybeans typically contain about 35-40 percent protein, which is much higher than other legumes averaging 18-25 percent protein (Tharanathan and Mahadevamma, 2003; Thrane *et al.*, 2016). The high protein content of soy is part of its appeal as a source of plant protein. Soy has been consumed worldwide

for centuries but was not introduced to North America until that start of the 20th century, when it was first used for its oil. The high protein meal was utilized for animal feed, before being redirected for human consumption (Thrane *et al.*, 2016). Human food use of soy protein in the United States did not take off until the 1930s, when soy processing exploded (Wolf, 1970). Now, soy protein ingredients are widely used in many food applications.

1.4.1 Ingredients

There are three major classifications of soy protein ingredients, classified by their protein contents: defatted soy flour (50-60 percent protein), soy protein concentrate (SPC; 65-80 percent protein), and soy protein isolate (SPI; >90 percent protein) (Wolf, 1970; FAO/WHO, 2007; Thrane *et al.*, 2016). Defatted soy flour is produced by milling defatted soy flakes. Soybeans are cleaned, dehulled, flaked, and then defatted by hexane (Deak *et al.*, 2008; Thrane *et al.*, 2016). Soy flour carries off-putting beany/grassy flavors that limit inclusion amounts in food applications.

SPC is prepared from the defatted flakes or flour through extraction by isoelectric precipitation, aqueous ethanol, or heating to precipitate the proteins and remove soluble carbohydrates (Kinsella, 1979; Thrane *et al.*, 2016). Today, SPC is most often prepared by ethanol extraction because it results in the least flavored product (Deak *et al.*, 2008). Alcohol extraction is highly denaturing, so SPC prepared this way has limited functionality, but can be utilized as the starting material to produce texturized soy products (Barac *et al.*, 2006; Singh *et al.*, 2008). No matter the extraction method, SPC has reduced off-flavors compared to soy flour, thus can be used in higher inclusion amount in similar food applications (Wolf, 1970).

To produce SPI, proteins from the defatted soy flakes or flour are extracted in order to remove both the soluble carbohydrates and insoluble carbohydrates (fibers) (Deak *et al.*, 2008; Thrane *et al.*, 2016). Commonly, soy flakes or flour is solubilized at an alkaline pH between 7.5 and 9, which creates two fractions: the

protein and soluble carbohydrates in the aqueous phase and the insoluble carbohydrates in the solid phase (Singh *et al.*, 2008; Thrane *et al.*, 2016). The aqueous phase is then adjusted to the isoelectric point (pI) of soy proteins (between pH 4 and 5), where the proteins have minimal solubility and settle out of solution with centrifugation, leaving the low molecular weight carbohydrates and other solubles in the supernatant (Deak *et al.*, 2008; Thrane *et al.*, 2016). The protein fraction is neutralized and may undergo further modification if desired, or go straight to spray drying to form SPI powder (Kinsella, 1979; Deak *et al.*, 2008; Thrane *et al.*, 2016). Isolates produced in this way tend to have good functional properties, thus can be used in a variety of food applications (Kinsella, 1979).

Soy protein isolates can be hydrolyzed to produce soy protein hydrolysates (SPH). Hydrolysis can be done by both chemical and enzymatic methods, though enzymatic hydrolysis is preferred (Kinsella and Melachouris, 1976; Barac *et al.*, 2006; Damodaran, 2017). A variety of GRAS enzymes can be used to produce hydrolysates that are safer and of a higher nutritional value compared to chemically hydrolyzed counterparts (Panyam and Kilara, 1996). The protein content of the hydrolysate is equivalent to the starting SPI, but the hydrolysate is made up of shorter peptide chains than the starting SPI protein. Therefore, the structural and functional properties of the hydrolysate are altered. It is typically desired to limit the degree of hydrolysis (DH), as extensive hydrolysis can cause the release of bitter peptides and may reduce protein functionality (Panyam and Kilara, 1996; Deak *et al.*, 2008; Barac *et al.*, 2012). Enzymes used and hydrolysis conditions are typically optimized for the desired end application (Panyam and Kilara, 1996). Enzymatic hydrolysis of soy proteins has been popular in industry for over 20 years, and continues to be an effective way to target specific functionalities by selectively altering the protein structure (Barac *et al.*, 2012). In general, the smaller molecular weight peptides of SPH tend to have improved solubility and enhanced emulsification and foaming capacities. However, emulsion and foam stabilities as well as gelling ability of SPH can be lower than those of the intact protein (Damodaran, 2017). To enhance gelation, specific enzymes are used to produce

peptides that are aggregate promoters and can induce polymerization (Panyam and Kilara, 1996). SPH may also contain bioactive peptides with several physiological benefits including antihypertension, immunomodulatory, and satiety promotion activity (Roy *et al.*, 2010; Dahl *et al.*, 2012; Carbonaro *et al.*, 2015).

1.4.2. Nutritional and Physiological Quality

Protein nutritional quality is determined by digestibility and the amino acid composition of the protein, specifically the amount of essential amino acids (United Soybean, 2020). Therefore, the WHO, FAO, and U.S. FDA use the protein digestibility-corrected amino acid score (PDCAAS) as the official method for evaluating protein quality (FAO/WHO Expert Consultation, 1991; Fukushima, 2011). PDCAAS is calculated by multiplying the first limiting amino acid score, which is the milligrams of the first limiting amino acid in 1 gram of test protein relative to the milligrams of the same amino acid in 1 gram of reference protein, by the digestibility of the protein as determined by an *in vivo* rat model (FAO/WHO Expert Consultation, 1991). Harsh processing conditions can alter the amino acid composition of a protein and may reduce its bioavailability and digestibility (Nosworthy and House, 2017). However, processing can also help improve protein digestibility by removing or inactivating antinutritional factors, thus resulting in a higher PDCAAS (Singh *et al.*, 2008; Thrane *et al.*, 2016). Soy protein is considered a complete protein because it contains all essential amino acids in adequate amounts. SPC and SPI have a PDCAAS of 0.92-1.0, depending on the extraction and processing conditions, which may affect amino acid composition and protein digestibility (U.S. Soybean Expert Council, n.d.; Fukushima, 2011; Rawal and Navarro, 2019).

In addition to being a high quality protein source, soy protein has other health benefits. In 1999, the FDA approved an authorized health claim for soy protein and its role in reducing risk of heart disease as part of a diet low in saturated fats. Since then, some studies have seen results inconsistent with this claim, leading the FDA to propose to revoke it in 2017 (Kotz, 2017; Frost & Sullivan,

2018). However, even if revoked, companies would be allowed to use a qualified health claim that requires the wording on products to indicate the limited evidence on soy protein's role in heart health (Kotz, 2017). In addition to the potential to reduce heart disease, it is believed that soy protein, like other plant proteins, can lower LDL cholesterol by activating LDL receptors in the liver (Friedman and Brandon, 2001; Fukushima, 2011). Along with its potential role in heart health and managing cholesterol, soy protein has been shown to help with weight loss and maintenance (Deak *et al.*, 2008; Singh *et al.*, 2008; Thrane *et al.*, 2016).

The contribution of soy protein to nutritional and physiological benefits is dependent on the protein profile and structure. Isolation and processing conditions may result in changes to the protein profile and structure, impacting both the quality and functionality of the protein. The following sections will provide details on soy protein structure as it relates to functionality.

1.4.3 Profile and Structure

Along with considering the nutritional value of soy protein, the structure and composition of soy proteins is important to review. Protein structure determines how the protein interacts with its environment and functions in a food system (Singh *et al.*, 2008; Taherian *et al.*, 2012). It is dependent on both internal and external factors. Intrinsic factors that influence protein structure include protein composition, amino acid sequence, and protein conformation (Kinsella, 1979). Extrinsic factors that affect protein structure include temperature, pH, and ionic strength (Kinsella, 1979). The combined effects of the intrinsic and extrinsic factors on shaping the protein's structure ultimately determines how it functions in a food system.

The structure of soy protein is complex, consisting of many interactions and bonds between amino acids, both intramolecularly and intermolecularly. In a single protein, there are four levels of structure to consider: primary, secondary, tertiary, and quaternary. The primary structure is simply the sequence of amino acids. Interactions between side chains of amino acids within the primary sequence

causes bending, folding, and bonding that dictates the other levels of structure (Kinsella and Melachouris, 1976; "Protein Structure," 2019). Secondary structure refers to the degree of hydrogen bonding between neighboring amino acid residues to form α-helices, β-sheets, or β-turns. Tertiary structure is the 3D-folding of the protein resulting from hydrogen bonding, van der Waals bonds, electrostatic interactions, hydrophobic interactions, and disulfide bonding between amino acids throughout the peptide chain (Kinsella and Melachouris, 1976). The last level of structure is quaternary structure, formed upon the interaction of protein subunits via similar bonding mechanisms stated above. Soy protein consists mainly of two globular proteins, glycinin and β-conglycinin, that have extensive quaternary structures (Kinsella, 1979; Barac *et al.*, 2006), to be discussed in detail in following paragraphs.

Osborne classification is commonly used to categorize proteins into four groups (globulins, albumins, prolamins, and glutenins) based on differences in solubility (Lusas and Riaz, 1995; Tulbek *et al.*, 2016). Approximately 65-90 percent of the proteins in soy are globulin proteins (Kinsella, 1979; Murphy, 2008; Fukushima, 2011; Thrane *et al.*, 2016). Soy also contains low amounts (up to 10 percent of total protein) of albumin proteins, which generally have limited functionality (Gueguen, 1983; Lusas and Riaz, 1995). Globulins are soluble in salt solutions, making them preferentially extracted in alkaline solution, while albumins are water soluble and are largely eliminated by alkaline solubilization (Osborne and Campbell, 1898; Lusas and Riaz, 1995; Deak *et al.*, 2008; Thrane *et al.*, 2016). In addition to being grouped by Osborne classification, proteins can be categorized based on their Svedberg sedimentation coefficient, which groups proteins based on the centrifugal force (in Svedberg units; S) needed to cause sedimentation (Murphy, 2008; Mojica *et al.*, 2015). In soy, there are two main classes of globulin proteins: 7S and 11S, though other minor fractions are also present (Murphy and Resurreccion, 1984; Mojica *et al.*, 2015).

The 11S fraction contains glycinin, and represents the largest protein fraction in soybeans at up to 52 percent of total proteins (Murphy and

Resurreccion, 1984; Deak *et al.*, 2008). It has a molecular weight of 300-380 kDa, composed of two trimers stacked on top of each other and held together by electrostatic and hydrogen bonding to form a hexametric protein, as shown in Figure 1 (Murphy, 2008; Mojica *et al.*, 2015). Each trimer is comprised of three monomers associated by hydrophobic interactions (Plietz *et al.*, 1987; Schwenke, 2001; Adachi *et al.*, 2003). Each monomer is made up of an acidic (~40 kDa) and a basic (~20 kDa) subunit linked by a disulfide bond (Kinsella, 1979; O'Kane *et al.*, 2004a; Boye *et al.*, 2010a). Composition of subunits can vary slightly among soybean varieties, with the acidic subunits ranging from 31 to 51 kDa, while the basic subunit is typically between 17 to 24 kDa (Gueguen and Cerletti, 1994; L'Hocine and Boye, 2007; Mession *et al.*, 2015). Among legumes, the structure of glycinin is conserved in the gene family of legumin proteins (Murphy, 2008).

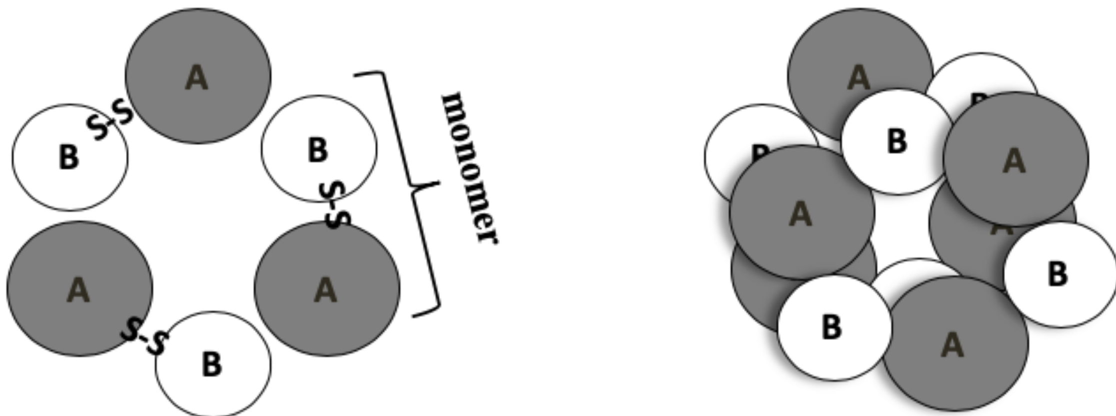

Figure 1. Schematic representation of glycinin trimer (left) and glycinin hexamer (right) (Gillman, 2014).

Five different glycinin monomers have been identified and categorized into two groups based on amino acid sequence homology, with about 90 percent homology within each group and 50-60 percent homology between groups (Nielsen, 1985). Group I ($A_{1a}B_2$, $A_{1b}B_{1b}$, and A_2B_{1a}) is relatively high in methionine, contains two cysteine and three cystine (dimer of cysteine) residues, and has molecular weight of 58 kDa (Nielsen, 1985; Mojica *et al.*, 2015). Group II (A_3B_4 and $A_5A_4B_3$) is lower in methionine, contains two cysteine and two cystine residues, and has a larger molecular weight of 62-69 kDa (Nielsen, 1985; Mojica *et al.*,

2015). The acidic and basic subunits are named so based on their amino acid composition and resulting isoelectric point (pI of α-subunit between 4.5-5.5, pI of β-subunit between 6.5-8.5) (Nielsen, 1985; Mojica *et al.*, 2015). Overall, the isoelectric point of glycinin is around pH 4.7 (Koshiyama, 1972; Derbyshire *et al.*, 1976).

Approximately 25-35 percent of soy proteins are in the 7S fraction, with β-conglycinin accounting for 85 percent of the proteins in this fraction (Murphy and Resurreccion, 1984; Deak *et al.*, 2008; Mojica *et al.*, 2015). The other proteins in this fraction include γ-conglycinin and the basic 7S globulin (Sato *et al.*, 1987). β-conglycinin has a molecular weight of approximately 175-180 kDa, is a trimeric protein made up of three glycoprotein subunits that associate by hydrophobic and hydrogen bonding, and can vary in composition among soybean varieties (Kinsella, 1979; Boye *et al.*, 2010a; Thrane *et al.*, 2016). The structure of β-conglycinin is conserved among legumes in the gene family of vicilin proteins (Murphy, 2008).

The β-conglycinin trimer can consist of the α (68-71 kDa), α' (72-82 kDa), and β (48-52 kDa) subunits in seven known combinations (α_3, $\alpha_2\alpha'$, $\alpha\alpha'\beta$, $\alpha_2\beta$, $\alpha\beta_2$, $\alpha'\beta_2$, β_3), depicted in Figure 2 (Utsumi and Kinsella, 1985; Sathe *et al.*, 1987; Fukushima, 2011). All three subunits are glycosylated on their asparagine residues during post-translational modification, increasing the surface hydrophilicity of β-conglycinin (Utsumi and Kinsella, 1985; Kimura *et al.*, 2008; Murphy, 2008). Unlike glycinin, β-conglycinin is deficient in sulfhydryl groups, with zero to one cysteine residues per subunit and no cystine residues (Wolf, 1970; Croy *et al.*, 1980; Tandang-Silvas *et al.*, 2010). This grants β-conglycinin greater molecular flexibility than glycinin, as it contains no disulfide linkages. Compared to glycinin, β-conglycinin has a slightly higher isoelectric point at pH 4.9-5.0 (Koshiyama, 1972; Gatehouse *et al.*, 1981).

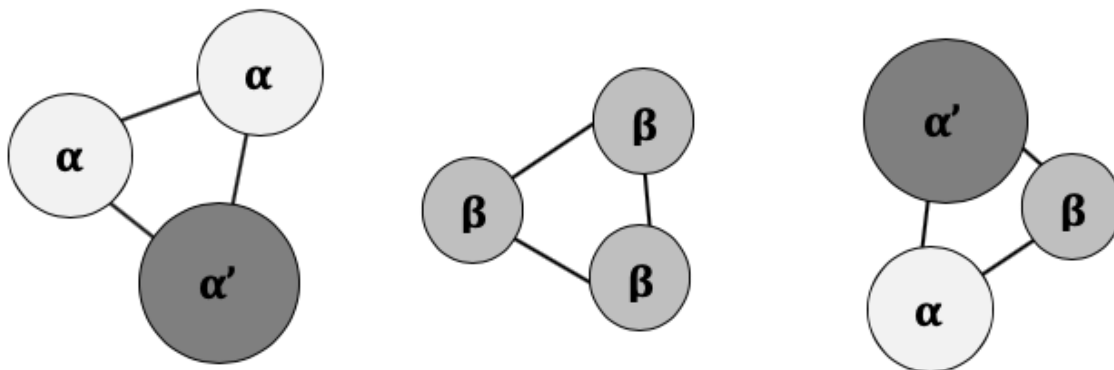

Figure 2. Schematic representation of β-conglycinin trimer structures (Gillman, 2014).

Other minor fractions of proteins also exist in soy protein. The 2S fraction is commonly reported but accounts for a much lower proportion of total extractable proteins, at just 8-22 percent (Deak *et al.*, 2008). Primarily, it is made up of protease inhibitors and enzymes (Kinsella, 1979; Deak *et al.*, 2008; Murphy, 2008). Some researchers report the 15S fraction of soy proteins, which may comprise up to 11 percent of the total proteins (L'Hocine and Boye, 2007; Deak *et al.*, 2008). The 15S fraction is suspected to be the association of several soy protein subunits (Derbyshire *et al.*, 1976; L'Hocine and Boye, 2007; Murphy, 2008).

Of all proteins present in legumes, the 7S and 11S fractions are of the most interest because they contribute the most to protein functionality. While all legumes contain 7S and 11S proteins, the ratio of 7S:11S differs between species (Fukushima, 2011). The ratio of 7S:11S determines the functionality of a protein because of the differences in compositions and structure of the 7S versus 11S proteins. Soy protein contains more glycinin than β-conglycinin, though the exact ratio can vary between cultivars and growing conditions (Tzitzikas *et al.*, 2006).

Compared to β-conglycinin, glycinin contains disulfide bonds and therefore is more thermally stable (Rickert *et al.*, 2004; Murphy, 2008; Fukushima, 2011). Additionally, the presence of disulfide bonds gives glycinin the ability to form a stable and strong heat-induced protein gels (Wolf, 1970; Shand *et al.*, 2007). β-conglycinin, however, has superior emulsifying abilities compared to glycinin. β-conglycinin is smaller in size and is deficient in disulfide bonds, so it can more

easily orient itself at the water:oil interface (Rickert *et al.*, 2004; Fukushima, 2011). Additionally, β-conglycinin has a better surface hydrophobicity to hydrophilicity balance compared to glycinin (Fukushima, 2011; Liang and Tang, 2013a). The differences in the structures of 7S and 11S proteins as well as the amounts of each fraction dictates how the protein functions and interacts with its environment.

1.4.4 Functionality

Protein functionality refers to the physiochemical properties of proteins that affect how they behave within a food matrix upon processing and over shelf life (Kinsella, 1979). The functionality of soy proteins is determined by the protein profile and structural integrity, which is largely dependent on the extraction and processing conditions used to produce the soy protein ingredient (Kinsella, 1979; Rickert *et al.*, 2004; Fukushima, 2011). Differences in solubilization pH and duration, ionic strength, the temperature of the extraction, time left at the protein's isoelectric point, purification/filtration, and drying method impact the structure and profile of the proteins extracted (Kinsella, 1979; Nichols and Cheryan, 1981; Gueguen, 1983; Rickert *et al.*, 2004). Generally, it is desired to select extraction conditions that are nondenaturing to the protein, as denatured proteins tend to aggregate and become less soluble (Kinsella and Melachouris, 1976; Nakai, 1983; Schutyser and Van Der Goot, 2011). For instance, Shand et al. (2007) reported that native SPI had significantly higher protein solubility at all pH values compared to a commercial SPI, which was completely denatured due to high heat during processing. By using mild extraction conditions, native globular protein structure can be preserved in the final soy protein isolate, leading to better overall functionality.

In addition to inadvertent modifications caused by extraction, the protein structure can be intentionally modified in order to target enhanced functionality (Barac *et al.*, 2006; Deak *et al.*, 2008). Proteins can be modified through chemical, enzymatic, or thermal treatments (Kinsella and Melachouris, 1976; Panyam and Kilara, 1996). Enzymatic hydrolysis is a common and widely accepted modification

technique that can improve protein functionality when controlled. The resulting hydrolyzed protein has a decreased molecular size as well as more ionizable groups and exposed hydrophobic groups, which were previously shielded, increasing the potential for reactions (Panyam and Kilara, 1996). Barac et al. (2006) reported that enzyme hydrolysis of soy protein improved emulsion activity index due to the decreased size of the proteins, allowing easier migration to the water:oil interface, as well as the increased surface hydrophobicity facilitating interactions between the protein and the oil. Protein modifications, in general, can contribute to improved solubility and surface activity, thus altering interactions between the protein and its surrounding environment (Damodaran, 2017).

Along with being influenced by intrinsic factors such as the amino acid composition and sequence, molecular size, conformation, charge distribution, surface hydrophobicity, and extent of inter- and intra-molecular bonding, protein functionality is affected by extrinsic factors as well (Utsumi and Kinsella, 1985; Damodaran, 2017). The temperature, pH, and ionic strength of the environment can promote or prevent interactions between proteins or between proteins and other food components (Kinsella, 1979). For instance, heating commercial soy protein isolates at 50°C improved protein solubility compared to solubilizing at 20°C (Lee *et al.*, 2003). However, extensive heating causes protein denaturation, which decreases protein solubility due to increased exposure of hydrophobic group and subsequent protein aggregation by hydrophobic interactions (Rickert *et al.*, 2004). The environmental conditions as well as the protein’s structural characteristics dictate how it interacts with water, air, lipids, carbohydrates, and/or other proteins (Kinsella and Melachouris, 1976). This, in turn, determines how the protein can be used in a product, or the modifications needed to make the protein more functional in a specific application (Arntfield and Maskus, 2011).

While extraction, processing, and environmental conditions influence soy protein’s structural and functional properties, the inherent nature of the proteins in soy make it naturally suited for certain functions. Soy is naturally high in glycinin which contains cysteine residues that can participate in sulfhydryl-disulfide

interchange (Wolf, 1970). Because of this ability to readily form disulfide bonds, soy protein is known for its gelling abilities (Rickert *et al.*, 2004). In a gel, disulfide bonds can form intermolecularly, providing stability to the system (Wolf, 1970). Increasing the number of cross-links between cysteine residues in SPI led to stronger, more elastic gels (Shand *et al.*, 2007).

In addition to gelation, soy protein ingredients can provide a wide range of functionalities in foods, including solubility, emulsification, water binding, foaming, and viscosity (Kinsella, 1979; Thrane *et al.*, 2016). However, the functionality of the soy protein ingredient will depend on how it is processed and prepared, and no single ingredient can possess all of these functionalities (Deak *et al.*, 2008; Thrane *et al.*, 2016). The conditions and efficiency of the protein extraction will impact the end product’s functionality. For instance, SPI extracted with mild heating (45°C) had higher foaming capacity than SPI extracted without the use of heat (Rickert *et al.*, 2004). The partial denaturation from heating enhanced protein interactions at the water:air interface. However, extensive heating causing increased denaturation reduces foaming capacity due to the formation of protein aggregates incapable of reducing tension of the water:air interface (Kinsella, 1981; Singh *et al.*, 2008). Extraction conditions and modification techniques should be selected with an intended functionality in mind.

1.4.5 Applications

Soy protein ingredients are widely used in products because of their nutritional quality and ability to provide the same functions as animal proteins, alone or in combination with, for a lower cost (Deak *et al.*, 2008). Because of this, soy protein ingredients have found a diverse range of applications across many different food categories.

Soy flour is often used in bakery products. This is due to its antioxidant activity that can naturally bleach carotenoids in flour, inhibit lipid oxidation, and strengthen gluten forming proteins (Kinsella, 1979; Lusas and Riaz, 1995). Additionally, soy flour is utilized because of its ability to absorb water, a property

that improves dough handling (Wolf, 1970; Deak *et al.*, 2008). Soy flour may also help create smoother batters, evenly distributed air cells, and a more uniform texture in finished products (Lusas and Riaz, 1995). However, inclusion amounts are limited by poor sensory qualities. Treatments such as toasting and steaming soy flour can be done to inactivate lipoxygenase enzymes that are known to cause off-flavors, though the thermal treatment may decrease protein functionality and antioxidant activity (Rackis *et al.*, 1979; Robinson *et al.*, 1995).

Soy protein concentrate is also used in bakery products, but can be used in higher amounts because SPC carries less flavor (Kinsella, 1979). SPC is most commonly used in meat products to cut costs while providing high quality protein and functionality (Thrane *et al.*, 2016). Because of its excellent emulsifying, gelling, and thickening abilities, SPC is useful in comminuted meat products (Kinsella, 1979). Additionally, SPC has high water retention and can absorb up to four times their weight in water, which contributes moisture and juiciness to meat products (Kinsella, 1979; Lusas and Riaz, 1995). In the United States, SPC is commonly used as a processing aid to emulsify fat and water in processed meats, in marinades for restructured meats, to increase weight of meat muscle tissues, to help retain juices and prevent cooking losses, and as textured meat extenders or meat analogs (Lusas and Riaz, 1995; Deak *et al.*, 2008; Singh *et al.*, 2008).

Soy protein isolate is used in similar applications as SPC, as SPI provides many of the same functional properties including high solubility, emulsification, water and fat retention, and gelation (Singh *et al.*, 2008). Because of their comparable functional properties, SPI is suitable for use in the types of meat products detailed above for SPC. Additionally, SPI is the preferred soy protein ingredient for use in dairy products because it has the mildest flavor and is highly dispersible (Singh *et al.*, 2008). SPI is also widely used in nutritional beverages and supplements because of its health benefits (Gilani and Lee, 2003).

Soy protein hydrolysates are typically prepared to target an improvement to a specific functionality, and so they can have varied applications. The enzyme(s) used and final degree of hydrolysis will determine how the structure of the protein

is modified, therefore making the protein more suitable for specific functionalities. For instance, soy protein that has been heavily hydrolyzed will likely have better solubility than the starting protein because of the smaller MW peptide chains and increased number of ionizable groups (Panyam and Kilara, 1996). Additionally, hydrolysis improves digestibility, therefore making SPH useful for nutritional supplementation and infant formula (Damodaran, 2017). However, extensive hydrolysis may cause bitterness due to the release of hydrophobic peptides (Panyam and Kilara, 1996; Damodaran, 2017). Excessive hydrolysis can also decrease functional properties such as gelation and emulsification (Barac *et al.*, 2012).

Soy protein ingredients have a wide range of applications with each ingredient having its specific strengths. With the versatility and functional and nutritional benefits of soy protein, it is not surprising that it is so widely used in foods. However, soy protein still faces some limitations.

1.4.6 Limitations of Soy Protein

Soy is one of the “Big Eight” allergens, which prevents many people from consuming it. It is estimated that about 0.6% of the U.S. population is allergic to soy (Food Allergy Research & Education, 2020). A soy allergy is about one-third as common as a peanut allergy, though those with severe peanut allergies often avoid soy as well due to concerns about cross-reactivity among legume proteins (The Scientific Panel on Dietetic Products, Nutrition and Allergies, 2004; Food Allergy Research & Education, 2020). The high consumption of soy containing products may be partially responsible for increasing sensitization among the population (Verma *et al.*, 2013). There are processes being investigated that can reduce allergenicity, but none that can completely inactivate the many soy protein antigens, except for extensive hydrolysis and breaking of over 90 percent of the peptide bonds (Panyam and Kilara, 1996; L’Hocine and Boye, 2007; Verma *et al.*, 2013). However, such extensive hydrolysis renders soy protein nonfunctional and very bitter. Currently, the only method of treatment for soy allergy is complete

avoidance, preventing millions of consumers worldwide from eating soy protein products (The Scientific Panel on Dietetic Products, Nutrition and Allergies, 2004; Food Allergy Research & Education, 2020).

Another limitation of soy is its beany flavor that can be off-putting and hard to mask. In the whole bean, the oil is kept separate from the enzymes in spheresomes, but the flaking process ruptures cell walls and allows lipoxygenase to act on the oil, leading to the formation of off-flavors such as beany and grassy notes (Lusas and Riaz, 1995). These off-flavors limit consumer acceptability of soy protein (Deak *et al.*, 2008; Murphy, 2008). Though breeding efforts are underway to reduce lipoxygenase levels to counteract this flavor development, it continues to be a problem for soy (L'Hocine and Boye, 2007; Thrane *et al.*, 2016). Of all plants, soybeans have the highest level of lipoxygenase activity, and are also high in polyunsaturated fats, making them prone to both enzymatic and nonenzymatic oxidation (Deak *et al.*, 2008; Murphy, 2008). These off-flavors are reduced through the protein concentration process, so SPC and SPI carry less off-flavor than soy flour and therefore can be used in relatively higher inclusion amounts (Singh *et al.*, 2008). Additionally, soy proteins may be bitter or astringent, which is attributed to their saponin content (Murphy, 2008; Tulbek *et al.*, 2016). Flavor issues can prevent high inclusion amounts in formulations and limit the products soy proteins are used in.

Many legumes contain anti-nutritional factors that may be toxic, inhibit nutrient absorption, or inhibit digestibility (Tharanathan and Mahadevamma, 2003; Carbonaro *et al.*, 2015). Trypsin inhibitors and chymotrypsin inhibitors are found naturally in soy and decrease protein digestibility (Friedman and Brandon, 2001; Thrane *et al.*, 2016). Soy flour typically is heat treated to deactivate these inhibitors; however, most commercial flours still contain about 5-20 percent of the original level of inhibitors because the level of heat treatment required for complete inactivation may also destroy essential amino acids such as methionine, cysteine, and lysine (Friedman and Brandon, 2001). The thermal treatment required to

completely inactivate trypsin inhibitors may negatively impact protein structure and thus functionality (Kinsella, 1979).

Genetic modification is yet another limitation of soy protein. The majority of soybeans were genetically engineered to have herbicide tolerance and insect resistance, making them easier for farmers to grow. Additionally, soybeans are being genetically modified to target physiological and physicochemical changes by altering the 3D structure of soy proteins (Fukushima, 2011). Kim et al., for example, modified the $A_{1a}B_{1b}$ subunit of glycinin to increase its surface hydrophobicity, which resulted in enhanced emulsification (Kim *et al.*, 1990). Despite these seeming improvements, since 1996 consumers have been becoming wary of GMO crops and many are now looking to avoid products containing them (Thrane *et al.*, 2016). There are still identity preserved (IP) soybean seeds available, but GMO soybeans have become the dominant variety, with 94 percent of soybeans grown in the United States in 2019 being genetically modified (Thrane *et al.*, 2016; National Agricultural Statistics Service, 2019). The use of GMOs or solvent-assisted extraction of oil from seeds is not allowed for organic products, eliminating the potential of soy protein to enter this market (Singh *et al.*, 2008; Rubio *et al.*, 2014). This points out the need for a non-genetically modified plant protein source.

Because of these limitations and in the efforts to meet the increasing demand for protein globally, other sources of protein must be developed. Peas, in particular, are of interest for reasons to be explained in the upcoming sections.

1.5 Yellow Field Peas

There are several species of peas, though yellow field peas *(Pisum sativum* L. subsp. *arvense*), also called split peas or dry peas, are the type used for protein fractionation (Lasekan *et al.*, 1995; Arntfield and Maskus, 2011). Field peas can be yellow or green in color, with yellow being the genetically dominant trait and the preferred color for producing pea protein ingredients (Sustainable Agriculture Research & Education, 2012; USA Pulses, 2016). Field peas, like soybeans, belong to the legume family. However, while soybeans are considered an oil

legume, peas are classified as a grain legume and are recognized as a pulse (Pelgrom, 2015; Rawal and Navarro, 2019). The FAO defines a pulse as a leguminous crop harvested solely for its dry grain (Food and Agriculture Organization of the United Nations, 2015). Crops harvested green, such as green beans and green peas, are considered vegetables and are excluded, as are crops harvested for their oil, such as soybeans (Food and Agriculture Organization of the United Nations, 2015). Yellow field peas are grown and harvested for their dry seeds, which can then be used in a variety of applications.

1.5.1 Growth and Production

Field peas can be traced back to the Middle East over 11,000 years ago (USA Pulses, 2016). Today, they are harvested all over the world, with Canada, Russia, and China producing over 60 percent of yellow field peas (Frost & Sullivan, 2015). In 2014, Canada was the largest exporter of peas, while India was the largest importer (Frost & Sullivan, 2015). In the U.S., North Dakota, Montana and the Pacific Northwest region are the top producers of field peas, with the majority (70 percent) of field peas currently being exported (Endres *et al.*, 2016; Rawal and Navarro, 2019).

Yellow field peas are a cool-season crop commonly planted after cereal crops like winter wheat or spring barley (Endres *et al.*, 2016). Ideally, planting should occur between mid-March and mid-May, once soil temperature has reached at least 40°F (4°C) (USA Pulses, 2016). Field peas can be grown in a variety of soils, from light sand to heavy clay and in many different locations around the world if the temperatures are cool enough (Endres *et al.*, 2016). Additionally, field peas require moist soil, although they cannot grow in heavily water-logged fields (USA Pulses, 2016). Maturation typically occurs 80-100 days later, depending on growing location and how warm the end of the growing season gets (USA Pulses, 2016).

Field peas do not ripen as uniformly as other crops, and can be considered ready to harvest when the majority of pods appear tan at the bottom, tannish-

yellow in the middle, and yellow-green near the top (Endres *et al.*, 2016). Peas ripen from the bottom of the pod upwards, turning from green to yellow (Endres *et al.*, 2016; Saskatchewan Pulse Growers, 2020). Peas are considered dry at 16 percent moisture, but it is recommended they be combined with a seed moisture content of between 17 and 20 percent to reduce risk of seed shattering (Endres *et al.*, 2016; USA Pulses, 2016; Saskatchewan Pulse Growers, 2020). Once peas are at 25-30 percent moisture, they can be desiccated to help decrease moisture content, thus quickening harvest time and reducing their potential for weather damage in the field (Manitoba Pulse & Soybean Growers, 2018; Saskatchewan Pulse Growers, 2020). To maximize shelf life, field peas should be aerated and stored at similar moisture contents and temperatures as wheat or corn (Endres *et al.*, 2016; Hall, 2017). Field peas stored at 14 percent moisture and 40°F have a shelf life of approximately one year, which can be extended with lower moisture content and/or lower temperature (Endres *et al.*, 2016). Along with having a short growing season and being a relatively tolerable crop, growing field peas offers some environmental benefits to farmers.

1.5.2 Environmental and Agricultural Benefits

Growing field peas has some environmental benefits. First of all, as a legume, they are able to fix nitrogen from the atmosphere and soil bacteria (Kissinger, 2016; Rawal and Navarro, 2019; Pulses, 2020). *Rhizobium* bacteria in the soil attach to the roots of pea plants, forming nodules (Endres *et al.*, 2016; Powers *et al.*, 2019). It is a symbiotic relationship, where the plant provides nutrients and food for the bacteria and the bacteria produces nitrogen that the plants use to grow (Rawal and Navarro, 2019). Peas are among the most efficient crops at fixing nitrogen and may obtain up to 80 percent of its total requirement from nitrogen fixation (Endres *et al.*, 2016). Additionally, pea plants can help preserve nitrogen in the soil for future crops, reducing the inputs of nitrogen fertilizer required. When pea plants die and decompose, they release nitrogen into the soil, improving soil health for the next crop (USA Pulses, 2016; Rawal and

Navarro, 2019). This is important environmentally because legumes like peas can reduce the production of nitrous oxide, a greenhouse gas that is 300 times more potent than carbon dioxide (Schwarzer *et al.*, 2012). The agriculture sector is the largest contributor to nitrous oxide, largely from nitrogen fertilizer application (Stagnari *et al.*, 2017). It is estimated that 70 percent of nonrenewable energy used in Western Canadian cropping systems is due to fertilizer use, particularly nitrogen fertilizer (Kissinger, 2016). The nitrogen fixing ability of peas decreases the need for fertilizer usage and helps decrease agriculture's environmental footprint.

Additionally, peas have great potential for sustainable use in crop rotation systems. Peas can contribute to soil health by increasing availability of nitrogen and phosphorus (Food and Agriculture Organization of the United Nations, 2015). After harvest, the crop residue of the pea plant releases nutrients into the soil that the next crop can utilize, reducing the need for fertilizer application (Kissinger, 2016; Pulses, 2020). Additionally, peas have relatively shallow root systems and are efficient users of water so they do not extract water from deeper soil, making them especially suitable for rotation with small grains (Sustainable Agriculture Research & Education, 2012; Endres *et al.*, 2016).

Peas can be used as a cover crop before cereal rotations or summer cash crops (Sustainable Agriculture Research & Education, 2012). Cereal crops grown after pulses have seen increased yields and protein contents (Kissinger, 2016). When used with a small grain such as oats, protein concentration of the grain increased by 2-4 percent (Endres *et al.*, 2016). Additionally, growing peas before a summer crop can prevent weeds from taking over the field (Sustainable Agriculture Research & Education, 2012). Moreover, using peas as a green manure instead of leaving the field bare restores nutrients to the soil and protects against erosion and water loss (Endres *et al.*, 2016; USA Pulses, 2016). Compared to more popularly grown crops, peas require lower inputs and can improve soil health and yields of the crops grown following.

1.5.3 Seed Composition

Legumes store their energy either as starch or oil. Soybeans, for example, are a legume that stores energy as oil, whereas peas are considered a grain legume and so are naturally low in oil but high in starch. Dehulled raw yellow peas are typically about 60-70 percent carbohydrate (45-48 percent starch, 14-18 percent dietary fiber, and 2-5 percent soluble sugars), 15-30 percent protein, 1-3 percent fat, 2-3 percent ash, and 8-10 percent moisture (Tian *et al.*, 1999; Tzitzikas *et al.*, 2006; Pulse Canada, 2014; Hall, 2017; Wang, 2019). Because of their naturally high starch and protein contents, peas are well-suited for fractionation to produce pea starch and pea protein ingredients (Arntfield and Maskus, 2011). While there is growing interest in fractionation, this currently represents a small portion of how field peas are used.

1.5.4 Current Uses

Historically, pulses have long been consumed by developing countries like India, Pakistan, and Thailand (Boye *et al.*, 2010a; USA Pulses, 2016). Recently, there has been an interest by developed countries of incorporating more pulses in their diets for a number of reasons related to health, expanding diet variety, changing preferences, changing demographics, and allergies to other proteins (Boye *et al.*, 2010a).

In the more recent past, yellow field peas have largely been produced for use in animal feed to provide protein. In developed countries, specifically, yellow field peas continue to be used primarily for animal feed (Rawal and Navarro, 2019). As peas are limiting in methionine and cysteine, they may be supplemented with these amino acids or used in combination with grains to provide a good feed for livestock (Powers *et al.*, 2019). Though soy is also widely used to supplement protein in animal feed, peas have about 5-20 percent lower content of trypsin inhibitors adjusted per gram of protein, and therefore can be used directly for feed without requiring heat treatment beforehand (Reinkensmeier *et al.*, 2015; Endres *et al.*, 2016). Markets for peas in animal feed are readily available and have limited

restrictions. On the other hand, markets for human consumption are stricter and require the farmer meet certain specifications. To determine the proper market, field peas can be sent to the Federal Grain Inspection Service to be graded on factors such as seed size and shape, moisture at harvest, handling during storage, and damage to the seed such as splitting or color bleaching (Endres *et al.*, 2016).

For human consumption, there are a few ways yellow field peas are used. Whole peas and split peas are an important source of food globally, as they offer a relatively cheap source of vegan protein that also provides fiber and micronutrients like iron and zinc (Powers *et al.*, 2019). The large majority of yellow field peas produced in North America are exported, with India and China being the largest importers (Rawal and Navarro, 2019). Whole peas are widely available and may be cooked, split, or milled. For whole peas, important quality criteria include size, color, shape, and overall uniformity (Tulbek *et al.*, 2016). Split peas are also fairly popular and used often in soups or in global food aid (Tulbek *et al.*, 2016). Other popular uses for whole and split yellow peas include roasting them into a snack food or cooking them in a soup or curry (AGT Foods, 2019; Powers *et al.*, 2019). Additionally, dry yellow peas are utilized to produce pea protein ingredients with numerous applications, to be discussed in the following sections.

1.6 Pea Protein

Although yellow field peas have been used primarily for livestock feed in the past, they have great potential as another source of plant protein to compete with soy protein. While pea protein is relatively similar to soy protein in terms of its production methods, protein quality, structure, and functionality, there are key differences that distinguish pea protein from soy protein.

1.6.1 Ingredients

Unlike soy protein ingredients, there are no official definitions for the terms pea flour, pea protein concentrate (PPC), and pea protein isolate (PPI). However,

there are loose guidelines for each category based on protein levels that are commonly used in industry. Additionally, the way each ingredient is produced largely determines its end protein content, and therefore the classification of protein ingredient (Arntfield and Maskus, 2011). Pea flour is typically 20-30 percent protein, depending on the starting protein content of the whole peas (Tian *et al.*, 1999; Tulbek *et al.*, 2016). PPC is produced mostly by dry milling and can be up to 60 percent protein, and PPI is produced by wet milling and is usually at least 80 percent protein (Arntfield and Maskus, 2011; Pelgrom, 2015).

Pea flour is produced similarly to soy flour with one main difference. Unlike soybeans, field peas are naturally low in fat, thus a defatting step is not necessary (Gwiazda *et al.*, 1979). To start, whole yellow peas are cleaned to remove any impurities such as dirt or soil and eliminate potential contamination from other crops (Tulbek *et al.*, 2016). After cleaning, the peas are dehulled, a relatively easy process compared to the dehulling of some other legumes (Arntfield and Maskus, 2011). The dehulled peas are then split and milled into a flour of the desired particle size (Tulbek *et al.*, 2016). Pea flour can be used as an ingredient on its own or processed further to concentrate the protein.

PPC is produced from pea flour following either dry- or wet-milling, though dry-milling is more common. These processes will be discussed in more detail in following sections. Briefly, the pea flour is subjected to air classification, which separates the protein from the starch based on differences in particle density (Boye *et al.*, 2010a; Pelgrom, 2015). This process concentrates the protein to more than double that of the starting pea flour, typically within the range of 50-60 percent protein (Gwiazda *et al.*, 1979; Pelgrom, 2015). Air classification is insufficient to concentrate protein to a higher degree, so any pea flour that undergoes air classification is considered a PPC.

PPI is the third major ingredient. As mentioned, it requires wet milling techniques to concentrate the protein further (Arntfield and Maskus, 2011). There are a number of wet milling techniques that can be performed, which are detailed in Section 1.7. The techniques used will impact the structure of the protein and the

resulting functionality and applications. PPI is often used in products for the nutritional and functional properties it provides. Furthermore, PPI has a cleaner taste than pea flour or PPC, making it easier to incorporate in foods (Tulbek *et al.*, 2016).

Similar to SPI, PPI can be modified. Enzymatic hydrolysis of PPI has been investigated to improve protein functionality. Pea protein hydrolysates (PPH) can be quite different than the starting material in terms of their physicochemical properties because of the changes made to the protein structure (Barac *et al.*, 2012). Furthermore, hydrolysates can be very different from each other due to the different enzymes and hydrolysis conditions used, and the degree of hydrolysis achieved (Panyam and Kilara, 1996). Because of the countless possible outcomes, enzymatic hydrolysis remains a popular protein modification technique for targeting specific functionality improvements (Barac *et al.*, 2012). While pea protein ingredients are versatile, their usefulness in the food industry is largely determined by the protein's nutritional quality.

1.6.2 Nutritional and Physiological Quality

Pea protein contains high levels of lysine, but it is limiting in methionine (Sosulski and Holt, 1980; Bhatty and Christison, 1984; Leterme *et al.*, 1990). Because of this, pea protein is commonly paired with cereals, which are high in methionine but are limiting in lysine (Stone *et al.*, 2015; Nosworthy and House, 2017). Cereal and legume proteins are complementary, meaning that consuming them together supplies sufficient amounts of all essential amino acids required for humans (Arntfield and Maskus, 2011). In addition to being limited in methionine, some researchers have reported pea protein to be limiting in cysteine (Sosulski and Holt, 1980; Bhatty and Christison, 1984; Leterme *et al.*, 1990). Methionine, however, is agreed to be the first limiting amino acid in pea protein, giving it an amino acid score (AAS) of 0.79 (FAO/WHO Expert Consultation, 1991).

The nutritional quality of a protein depends not only on its amino acid composition, but also on how well it can be digested, as illustrated by the PDCAAS.

PDCAAS is dependent not only on the source of the protein, but also on processing conditions. PDCAAS for pea protein has been reported to be between 0.54-0.82, depending on cultivar, extraction protocol, and processing conditions (Pulse Canada, n.d.; U.S. Soybean Expert Council, n.d.; Arntfield and Maskus, 2011; Nosworthy and House, 2017; Rawal and Navarro, 2019). Continued research on pea protein isolation and processing should help increase the PDCAAS of pea protein by selecting conditions that preserve essential amino acids and enhance protein digestibility.

Although pea protein is lagging behind soy protein in terms of PDCAAS, it has physiological benefits similar to those of soy protein. Pea protein is believed to help lower cholesterol. Specifically, in a rat study, pea protein stimulated the excretion of bile acids in feces, leading to a reduced concentration of cholesterol in the liver and consequently reduced secretion of cholesterol by VLDL (Spielmann *et al.*, 2008). In another study, it was reported that rats fed a diet of pea protein had lower hepatic cholesterol, as well as reduced plasma cholesterol and triglycerides compared to rats fed casein (Lasekan *et al.*, 1995). It is believed that pea protein may regulate cholesterol metabolism through a mechanism similar to that of soy protein (Spielmann *et al.*, 2008).

Additionally, pea protein may have a beneficial effect on athletic performance. Traditionally, many athletes have consumed whey protein after exercise because whey protein contains a high amount of branched-chain amino acids (BCAAs; leucine, isoleucine, and valine), which are important for building and regenerating muscles (Babault *et al.*, 2015; Banaszek *et al.*, 2019; Lu *et al.*, 2019). Pea protein is relatively high in BCAAs, making it a potential alternative to whey protein (Babault *et al.*, 2015; Banaszek *et al.*, 2019). In a randomized, double-blind clinical study, young men consuming 50 g of pea protein a day had significantly higher bicep muscle thickness than men consuming a maltodextrin placebo after 12 weeks of strength training. Furthermore, there was no significant difference between the group of men consuming pea protein versus the group consuming whey protein (Babault *et al.*, 2015). In another study on the

performance of high-intensity functional training, there were no significant differences in physical performance, strength, or body composition in athletes consuming whey protein or pea protein over 8 weeks (Banaszek *et al.*, 2019). These findings suggest that pea protein may be a suitable plant-based alternative to animal proteins traditionally consumed by athletes.

Though pea protein shows promise for its nutritional and health benefits, it must first be successfully incorporated into foods for those benefits to be realized. The successful incorporation of pea protein into foods will depend on its interactions with other components of the product, and those interactions are determined by the profile and structural properties of the protein.

1.6.3 Profile and Structure

The profile and structure of the proteins in field peas determine their nutritional and functional properties, thus they have been widely studied. Pea protein consists primarily of salt-soluble globulins, and secondly of water-soluble albumins (Gueguen and Barbot, 1988; Crévieu *et al.*, 1996; Boye *et al.*, 2010a; Tulbek *et al.*, 2016). Of all extractable protein, globulins account for 65-80 percent of total protein, while albumins are typically 15-35 percent (Schroeder, 1982; Gueguen and Barbot, 1988; Crévieu *et al.*, 1996; Boye *et al.*, 2010a). The globulins are the main interest when concentrating protein, as they are largely responsible for desirable functionality. The albumin fraction, on the other hand, contains the majority of undesirable components such as enzymes, lectins, protease inhibitors, and trypsin inhibitors (Crévieu *et al.*, 1996; Boye *et al.*, 2010a).

Among legumes, the structure of the main globular storage proteins is highly conserved between species (Danielsson, 1949). Like in soy protein, pea protein consists of four fractions (2S, 7S, 11S, and 15S), with 7S and 11S being the dominant fractions (Derbyshire *et al.*, 1976). Though the names and proportions of each protein differ between pea and soy proteins, the 7S and 11S proteins in soybeans have similar structure and composition as the 7S and 11S proteins in field peas (Tzitzikas *et al.*, 2006). While β-conglycinin is the major 7S protein in

soy, vicilin and convicilin are analogous proteins in peas. Similarly, glycinin in the 11S fraction of soy protein is comparable to legumin in the 11S fraction of pea protein (Tzitzikas *et al.*, 2006).

While glycinin (legumin) is the dominant protein in soy, legumin comprises a smaller portion of total protein in peas at about 6-25 percent (Tzitzikas *et al.*, 2006). Legumin has similar molecular weight, amino acids, and subunit structures as glycinin (Derbyshire *et al.*, 1976). Like glycinin, legumin is a hexamer comprised of two trimers stacked on top of each other. Each trimer is made up of three monomers, and each monomer consists of a pair of acidic (~40 kDa) and basic (~20 kDa) subunits linked by disulfide bonds (Boulter, 1983; Arntfield and Maskus, 2011). The monomers (~60 kDa) associate by hydrophobic interactions to form trimers, and the two trimers interact by hydrogen bonding and electrostatic interactions to form the hexamer (Murphy, 2008; Mojica *et al.*, 2015). Of the three main proteins in peas, legumin has the largest molecular weight at 300-400 kDa, which, along with its disulfide bonds, causes legumin to denature at a higher temperature than vicilin and convicilin (Danielsson, 1949; Derbyshire *et al.*, 1976). Like glycinin, legumin's cysteine residues enable it to form a strong gel. However, pea legumin contains fewer cysteine residues per subunit than soy glycinin (O'Kane *et al.*, 2004a).

The 7S fraction of pea protein consists of two proteins: vicilin and convicilin. Vicilin is typically 26-52 percent of total protein in field peas and is the major protein in the 7S fraction (Tzitzikas *et al.*, 2006). Vicilin is most similar to β-conglycinin in soy, with comparable molecular weight, amino acid composition, and subunit structures (Derbyshire *et al.*, 1976). However, vicilin comprises a larger proportion of pea protein than β-conglycinin comprises of soy protein (Tzitzikas *et al.*, 2006) Of the three main proteins in field peas, vicilin has the lowest molecular weight at 150-170 kDa (Gatehouse *et al.*, 1981; Boye *et al.*, 2010a). The subunits of the trimeric vicilin are quite heterogeneous, with molecular weights ranging between 12.5 and 50 kDa (O'Kane *et al.*, 2004b). It is believed that all subunits start at 50 kDa and then may undergo post-translational proteolysis, producing smaller

polypeptides (Gatehouse *et al.*, 1982; O'Kane *et al.*, 2004b). Additionally, post-translational modifications commonly includes glycosylation, which helps improve vicilin's solubility (Boulter, 1983; Tzitzikas *et al.*, 2006; Stone *et al.*, 2015). As with β-conglycinin, vicilin is known for its surface-activity, as it contains a good balance of hydrophobic and hydrophilic amino acids on its surface and has a relatively small and flexible structure (Dagorn-Scaviner *et al.*, 1986; Taherian *et al.*, 2012; Liang and Tang, 2013b).

Convicilin as a third separate storage protein was discovered later than vicilin and legumin. It comprises the smallest portion of the three proteins, at about 4-8 percent of total pea protein (Tzitzikas *et al.*, 2006). It was originally thought to be equivalent to vicilin, until Croy et al. (1980) proved it was distinct. Compared to vicilin, convicilin contains very little carbohydrate conjugates and is overall much larger in size at about 280-290 kDa (Boulter, 1983). It is believed to be a tetrameric protein, comprised of four subunits approximately 71 kDa each (Croy *et al.*, 1980; Boye *et al.*, 2010a). Convicilin and vicilin have high amino acid sequence homology (about 80 percent), but convicilin is distinguished by its terminal N sequence that is highly charged and contains few hydrophobic residues (O'Kane *et al.*, 2004b; Lu *et al.*, 2019). The highly charge N-terminus may help with protein solubility, but has been seen to inhibit gel formation by favoring protein-water interactions over protein-protein interactions (O'Kane *et al.*, 2004c). Convicilin is also differentiated from vicilin because convicilin contains one cysteine residue per subunit, whereas vicilin contains none (Croy *et al.*, 1980; Boye *et al.*, 2010a).

Research evaluating 59 lines of peas found that the ratio of 7S to 11S globulins was between 1.2 and 8, while in soy that ratio is between 0.47 and 0.79 (Tzitzikas *et al.*, 2006; Taherian *et al.*, 2011). Though there was quite a range depending on variety tested, in all cases it was shown that peas contain higher contents of vicilin proteins than legumins, while soy contains higher amounts of glycinin compared to β-conglycinin. This is significant in explaining differences in their functional properties.

1.6.4 Functionality

As with soy protein, pea protein functionality is highly dependent on protein structure. Because of the similarities in structure of the soy and pea proteins, it may be expected that they would have similar functionalities. However, the proportions of proteins (i.e. the protein profile) of soy and pea differ. The globulin proteins are responsible for protein functionality in foods, whereas the albumin proteins have limited functionality. Soy protein contains 10 percent albumin proteins at most, whereas pea protein may contain up to 35 percent albumins, therefore diluting the overall amount of functional proteins in pea protein (Gueguen and Barbot, 1988; Boye *et al.*, 2010a).

In addition to the differing contents of globulins versus albumins, soy and pea proteins consist of different ratio of 7S:11S globulin proteins. The differing structures of the globulin proteins make them more suitable for different functionalities. Vicilin is highly surface-active and consequently is useful for foaming and emulsifying (Dagorn-Scaviner *et al.*, 1986; Arntfield and Maskus, 2011). Legumin, on the other hand, is a large protein containing sulfhydryl groups and disulfide bonds, and thus is good for heat-induced gelation (O'Kane *et al.*, 2004a). Pea protein contains more vicilin than legumin, while soy protein is higher in legumin (glycinin) than vicilin (β-conglycinin), which results in differences in functional properties between pea and soy protein (Tzitzikas *et al.*, 2006).

Because of its high vicilin content, pea protein has been reported to be competitive with or superior to soy in emulsification and foaming. The smaller size and absence of covalent disulfide bonds enables vicilin to be a better emulsifying agent than legumin (Dagorn-Scaviner *et al.*, 1986; Arntfield and Maskus, 2011; Tulbek *et al.*, 2016). Vicilin is able to quickly migrate to the interface of two immiscible phases (water:oil or water:air) and orient its hydrophobic residues with the dispersed (oil or air) phase and its hydrophilic residues with the continuous (water) phase (Dagorn-Scaviner *et al.*, 1986; Barac *et al.*, 2012). Additionally, vicilin contains the necessary balance of hydrophilic and hydrophobic amino acids needed to interact with both phases to stabilize emulsions and foams (Arntfield

and Maskus, 2011). Pea protein, containing a high proportion of vicilin, has shown potential in stabilizing emulsions and foams (Koyoro and Powers, 1987; Tzitzikas *et al.*, 2006; Arntfield and Maskus, 2011).

On the other hand, while soy protein is well-known for its gelling abilities, pea protein is a weaker gelling agent (Shand *et al.*, 2007). Soy protein contains a higher proportion of legumin (glycinin) than pea protein, thus is able to form more disulfide intermolecular cross-links that provide strength and elasticity to the gel (Wolf, 1970; Sun and Arntfield, 2011a; Tulbek *et al.*, 2016). Pea protein, on the other hand, contains primarily vicilin proteins, which are not able to form disulfide linkages, thus pea protein gels are weaker (Shand *et al.*, 2007; Tulbek *et al.*, 2016). Compared to soy protein, pea protein requires a higher protein concentration in order to form a gel (O'Kane *et al.*, 2005; Taherian *et al.*, 2011, 2012).

As mentioned previously (Section 1.4.4), the functionality of a protein is dependent not only on the source of the protein, but also the conditions used for extracting and processing, as well as environmental conditions. Testing different extraction and drying techniques, as well as modification by enzymatic hydrolysis, has shown that there is promise to improve pea protein functionality (Tulbek *et al.*, 2016). A limited DH (<10%) can improve functional properties such as solubility, emulsification capacity, and foaming capacity. However, hydrolysates may have lower emulsification stability and foaming stability because the smaller peptides may not form a cohesive film at the interface (Barac *et al.*, 2012; Damodaran, 2017). Evaluating the functional properties of pea protein is essential in order to identify best suited applications.

1.6.5 Applications

As with soy protein, pea protein can provide a range of functional properties, allowing it to be used in several applications. As with soy flour, pea flour is often used in bakery products. Using pea flour in combination with wheat flour improves overall nutritional quality because the proteins are complementary (Tulbek *et al.*, 2016). By combining pea flour and wheat flour in bakery applications, a complete

protein claim can be made, and functionality can be enhanced. Pea protein has excellent water retention and can help with dough handling and control product moisture, while the viscoelastic gluten proteins provide structure (Asgar *et al.*, 2010; Lu *et al.*, 2019). Additionally, similar to soy flour, pea flour naturally contains lipoxygenase, which acts as a natural bleaching agent (Tulbek *et al.*, 2016). However, as with soy flour, lipoxygenase in pea flour contributes to the formation of undesirable flavor compounds, so inclusion amounts are limited (Swanson, 1990; Taherian *et al.*, 2012). The protein concentration process removes some of the compounds responsible for flavor formation, thus PPC and PPI have fewer undesirable flavors (Tulbek *et al.*, 2016).

Similar to SPC and SPI, PPC and PPI are most commonly used as meat extenders or meat analogs (Arntfield and Maskus, 2011; Tulbek *et al.*, 2016). As with soy protein, pea protein can help with the texture and water retention of meat due to its emulsifying, gelling, and water-holding abilities (Tulbek *et al.*, 2016). Compared to hamburgers made of only beef, beef patties formulated with PPI were more tender and easier to compress (Lu *et al.*, 2019). Pea protein provides a non-allergenic, non-GMO alternative to soy protein to use in meat products, and can also be processed on its own to mimic the texture of meat for vegan protein products (USA Pulses, 2016). Extrusion is commonly done to form texturized vegetable proteins with a fibrous, chewy texture that closely resembles meat (Asgar *et al.*, 2010). Soy protein remains the most common starting material for extrusion, but pea protein isolates can also be used to produce meat-like products through high moisture extrusion cooking (Asgar *et al.*, 2010; Osen *et al.*, 2014). In fact, pea protein is already being utilized by popular vegan meat brands like Beyond Meat (Roberts, 2017).

In addition to meat extenders and analogs, PPC and PPI have several other applications. Along with replacing meat, PPC or PPI is often substituted for other animal proteins or major allergens. For example, pea protein can be used as an egg replacement for vegan consumers or those with egg allergies (Tulbek *et al.*, 2016). Pea protein can provide many of the same functional properties as eggs,

including fat binding, water binding, emulsification, coagulation, and leavening in order to obtain similar texture, density, and elasticity as products made with eggs (USA Pulses, 2016). Additionally, pea protein has been used as a dairy replacement in vegan yogurts and sports nutrition products (Han *et al.*, 2010; Arntfield and Maskus, 2011; Lu *et al.*, 2019).

Because pea protein is compositionally similar to soy protein and has the added benefits of being non-GMO and not a major allergen, there is growing interest in developing pea protein. As has been done for soy protein, optimizing pea protein extractions procedures and processing, as well as modification techniques, can be done to select for certain functional properties desired in the final product (Makri *et al.*, 2005; Boye *et al.*, 2010a; Tulbek *et al.*, 2016). While soy protein has been studied and optimized for decades, pea protein is in an earlier phase of development and still faces some limitations.

1.6.6 Limitations of Pea Protein

One of the biggest drawback of soy protein is that it is a “Big Eight” allergen. Though pea protein is not currently a major allergen in the United States, it should be watched closely. Pea proteins are very similar in structure and composition to soy proteins, so there is a strong chance that individuals that react to soy proteins will have cross-reactivity with pea proteins (Barnett *et al.*, 1987; Boye *et al.*, 2010a; Verma *et al.*, 2013). Additionally, with more exposure to pea protein, it may become a cause for an allergenicity concern in the U.S. (Verma *et al.*, 2013). For instance, individuals from areas like Europe, Asia, and the Mediterranean, where they consume more pulses on average, have already experienced allergic reactions to pea proteins (Boye *et al.*, 2010a). Though pea protein is currently low risk, it may become more allergenic as its utilization increases.

Moreover, like soybeans, peas naturally contain anti-nutritional factors including phytic acid, lectins (hemagglutenins), saponins, and trypsin inhibitors (Panyam and Kilara, 1996; Thrane *et al.*, 2016; Tulbek *et al.*, 2016; Lam *et al.*, 2018). Though amounts of anti-nutritional factors are generally lower in peas than

they are in soybeans, they still must be deactivated by processing or they can inhibit bioavailability and digestibility (Reinkensmeier *et al.*, 2015; Damodaran, 2017).

Like with soy protein, pea protein can be limited by its organoleptic properties. Pea protein may naturally have a grassy/beany taste due to volatile ketones and aldehydes formed by lipoxygenase activity, or a bitter/astringent taste caused by compounds including saponins and polyphenols (Kinsella and Melachouris, 1976; Swanson, 1990; Robinson *et al.*, 1995; Arntfield and Maskus, 2011; Tulbek *et al.*, 2016). Processing can reduce unpleasant flavors, although it is often accompanied by a decrease of protein functionality. Heat treatment such as toasting or steaming flours can inactivate enzymes like lipoxygenase, but may cause protein denaturation (Kinsella and Melachouris, 1976; Rackis *et al.*, 1979; Robinson *et al.*, 1995). Using a polar organic solvent like ethanol for protein extraction can be done to reduce lipoxygenase activity, but at the cost of protein functionality (Kinsella and Melachouris, 1976; Rackis *et al.*, 1979). Along with potential changes to protein functionality, extra processing adds to the cost of the ingredient (Arntfield and Maskus, 2011). Nevertheless, there are some protein concentration processes that naturally remove flavor compounds (Singh *et al.*, 2008). For instance, membrane filtration of pea protein removes flavor- and color-producing compounds, as these tend to associate with smaller proteins that pass through the membrane (Deak *et al.*, 2008). Flavor is consistently considered the most important factor in foods, so in order to be widely utilized, pea protein ingredients will need more work on eliminating off-flavors.

While pea protein may eventually become an allergen of concern, it currently offers an alternative to many major allergens including soy protein. Additionally, extraction and processing conditions can be optimized to eliminate anti-nutritional compounds and off-flavors, and preserve the protein's structure and functionality.

1.7 Pea Protein Extraction and Isolation

The extraction and isolation techniques used for protein concentration are important to consider, as the methods selected determines the profile of proteins extracted and their structure, and therefore have implications on protein functionality and applications. Pea protein can be extracted from pea flour by dry and wet fractionation techniques. Compared to dry fractionation techniques, wet fractionation is a less efficient process because it requires high amounts of water and chemicals, and needs high energy input to dry the final products (Schutyser and Van Der Goot, 2011; Pelgrom, 2015; Kissinger, 2016). Dry fractionation, on the other hand, does not use water or chemicals, but cannot reach the same level of protein purity as wet fractionation techniques (Schutyser and Van Der Goot, 2011; Pelgrom, 2015). Dry fractionation is done by air classification, which is a fairly simple process of separation based on differences in particle density, whereas wet fractionation takes advantage of differences in solubility and has more variability.

In wet fractionation, the basic process is to first suspend the pea flour in a solvent that the proteins are soluble in to separate it from other components such as starch and insoluble fiber (Fredrikson *et al.*, 2001; Thrane *et al.*, 2016). Once the starch and insoluble fibers have been removed, a second step with altered solvent or use of filtration further concentrates the proteins by separating out soluble fibers and small sugars (Tian *et al.*, 1999; Fredrikson *et al.*, 2001; Arntfield and Maskus, 2011). The most common extraction techniques, and the ones that will be focused on in the upcoming sections, are alkaline solubilization with isoelectric precipitation, salt extraction (“salting in, salting out”), and salt solubilization coupled with membrane filtration.

Other less commonly used extraction techniques to briefly note include water extraction, acidic solubilization, and micellarization (Boye *et al.*, 2010a; Sun and Arntfield, 2011a; Reinkensmeier *et al.*, 2015; Lam *et al.*, 2018). Additionally, some modifications to extractions can be done to increase protein solubilization, such as using heat (typically 50-60°C) during the extraction or including reducing

agents such as sulfites to reduce disulfide bonds and prevent protein polymerization (Wolf, 1970; Kinsella, 1979; Boye *et al.*, 2010a). The extraction techniques and conditions used are critical in explaining differing functional properties of pea protein isolates (Stone *et al.*, 2015).

Conditions of the extraction greatly impact the effectiveness of the extraction. The method of extraction, solubilization pH, solubilization duration, number of washes, ionic strength, solvation ratio, temperature, extraction equipment, and filtration/purification techniques are some key variables that affect the efficiency of the extraction as well as the characteristics of the protein isolate (Kinsella, 1979; Boye *et al.*, 2010a; Feyzi *et al.*, 2018). Harsh conditions such as extreme pH, high temperature, and physical abuse have been seen to cause protein denaturation and/or excessive polymerization during extraction that can greatly reduce its functionality (Kinsella and Melachouris, 1976; Schutyser and Van Der Goot, 2011; Taherian *et al.*, 2011). Therefore, mild conditions should be selected to minimize damage to the proteins. Furthermore, it is important to consider feasibility of large-scale production.

1.7.1 Air Classification

Air classification is a dry fractionation technique that can be used to concentrate pea protein. Pea protein can be physically separated from starch based on the different particle sizes, bulk densities, and powder characteristics of proteins and starch granules after milling the pea seeds into a flour (Arntfield and Maskus, 2011; Tulbek *et al.*, 2016). Milling helps separate the smaller protein bodies (<10 μm) from the larger starch granules and fiber components (20-70 μm) (Pelgrom, 2015; Tulbek *et al.*, 2016). The flour is subjected to a stream of spiraling air in the separation chamber of an air classifier. Inside the chamber, there is a classifier wheel with a small slit that allows the fine fraction to float up and be collected, while the heavy/coarse fraction is rejected and kept in the lower portion of the chamber (Boye *et al.*, 2010a; Pelgrom, 2015). This process enables the

recovery of both the concentrated protein (fine) fraction and the starch/fiber (heavy/coarse) fraction.

For optimum separation efficiency, there must be a balance of milling the flour fine enough to separate the protein bodies and starch granules, while avoiding causing damage to the starch granules that would break them into smaller fragments that may enter the fine phase (Boye *et al.*, 2010a). Legumes like peas are well-suited for air classification because their starch granules are fairly large and uniform in size (Pelgrom, 2015). Additionally, field peas are naturally low in fat, unlike soybeans, which are unsuitable for air classification (Elkowicz and Sosulski, 1982; Pelgrom, 2015).

This dry fractionation technique can be used to produce pea protein concentrates of 50-60 percent protein and protein recovery of up to 77 percent (Pelgrom, 2015). However, it is not possible to concentrate proteins much further than 60 percent due to the natural interactions of the protein bodies with starch (Boye *et al.*, 2010a). For higher protein purity, wet fractionation techniques are necessary.

1.7.2 Alkaline Solubilization with Isoelectric Precipitation

While dry fractionation takes advantage of the different particle sizes in pea flour, wet fractionation is based on the different solubilities of components in pea flour. Alkaline solubilization with isoelectric precipitation is the most common method for isolating proteins (Arntfield and Maskus, 2011; Taherian *et al.*, 2012). This method takes advantage of differences in the solubility of pea protein across different pHs. It is well-known that pulse proteins are most soluble at alkaline pHs. Therefore, pea flour is first solubilized at a pH between 7 and 11 to extract proteins into the aqueous phase (Fredrikson *et al.*, 2001; Boye *et al.*, 2010a; Arntfield and Maskus, 2011). The solution is then centrifuged to remove insoluble components including starch and/or insoluble fiber, while the proteins remain in the supernatant.

The supernatant is then adjusted to the isoelectric point of pea proteins, somewhere between pH 4 and 5 (Tulbek *et al.*, 2016; Lam *et al.*, 2018). Around

this pH, globular proteins will have a net charge of zero or close to zero, and hydrophobic interactions between proteins will lead to aggregation and precipitation (Murray *et al.*, 1981; Lam *et al.*, 2018). Typically, the solution is again centrifuged to aid in separation of the two phases. This time, the pellet fraction contains mostly the proteins, while the supernatant is mostly soluble carbohydrates (soluble fiber and small sugars) and may contain some albumin proteins, which remain soluble at pH 4.5 because they have a higher isoelectric point than globulins (Makri *et al.*, 2005; Thrane *et al.*, 2016; Damodaran, 2017). The pellet is sometimes washed to remove salts, followed by neutralization and drying (Boye *et al.*, 2010a).

Several components of this extraction technique can be modified. Solubilization pH between 8 and 10 are most common (Sumner *et al.*, 1981; Makri *et al.*, 2005; Shand *et al.*, 2007; Boye *et al.*, 2010b). Typically, raising solubilization pH increases protein yields, though protein purity may decrease because starch may also get solubilized at highly alkaline pH (Hoang, 2012; Reinkensmeier *et al.*, 2015). Additionally, high alkalinity is damaging to the protein, causing denaturation and polymerization, which in turn cause reduced protein digestibility and nutritional quality (production of lysinoalanine, racemization of amino acids), reduction in organoleptic properties, and loss of protein functionality (Arntfield and Maskus, 2011). Under alkaline pH, cysteine and serine amino acids can be converted into nephrotoxic lysinoalanine compounds, resulting in a loss of essential amino acids (Lam *et al.*, 2018). Additionally, alkalinity can induce cross-linking of proteins, which decreases protein digestibility and biological availability of amino acids involved in or near the cross-links (Damodaran, 2017). Alkaline pH also can cause racemization of amino acids from L- to D-, which are not absorbed as well (Damodaran, 2017). At extremely high pH values, electrostatic repulsion between protein chains is increased, especially with ionization of previously buried carboxyl, phenolic, and sulfhydryl groups, which causes the protein molecule to unfold (Damodaran, 2017). Unfolding of the protein will expose hydrophobic moieties and sulfhydryl groups, leading to cross-linking and aggregation of proteins (Alizadeh-

Pasdar and Li-Chan, 2000). Extraction pH should be selected to ensure efficient protein extraction while minimizing changes in the protein structure.

In addition to pH, other components of the solubilization can be adjusted to optimize extraction efficiency. The duration of solubilization is typically between 30 minutes to 2 hours, with longer times tending to result in better yields (Makri *et al.*, 2005; Taherian *et al.*, 2011; Hoang, 2012). Furthermore, to increase extraction efficiency, multiple solubilizations can be done. After the first solubilization and centrifugation step, the precipitate can be resuspended and solubilized again in effort to ensure all soluble proteins get extracted (Nichols and Cheryan, 1981; Makri *et al.*, 2005). The solvation ratio may also affect extraction efficiency, though it seems to play a lesser role (Hoang, 2012). Generally, flour:water ratios are between 1:5 and 1:20 (Makri *et al.*, 2005). A higher total solids ratio is often preferred to minimize water usage.

Along with modifying the solubilization step, the isoelectric precipitation step can be adjusted. The pH used to precipitate the proteins will impact the final protein profile, as vicilin, convicilin, legumin, and albumin proteins differ in their pI (Derbyshire *et al.*, 1976; Rickert *et al.*, 2004; Rubio *et al.*, 2014; Damodaran, 2017). Additionally, the time proteins spend at their isoelectric point will impact overall functionality. Ideally, proteins should spend as little time as possible at their isoelectric point in order to avoid irreversible protein aggregation and polymerization, which cause permanent loss of solubility and overall functionality (Kinsella, 1979).

After isoelectric precipitation, the precipitated proteins are resuspended and neutralized. At this point, they may be filtered or dialyzed to remove salts and reduce ash content, or they may go directly to drying (Nichols and Cheryan, 1981; Tian *et al.*, 1999; Cutler, 2004). The choice of drying method will also impact the overall protein structural and functional properties. Lyophilization is common for laboratory-scale extractions, where no heat is applied and the protein structure is well preserved (Kinsella and Melachouris, 1976; Zhao *et al.*, 2013; Stone *et al.*, 2015). However, it is an expensive and time-consuming drying method, making it

infeasible for industrial use (Sumner *et al.*, 1981). Instead, industrially produced protein isolates are most commonly dried by spray drying (Zhao *et al.*, 2013). Spray drying parameters will determine how much heat the proteins are subjected to and the moisture content and particle size of the final PPI.

Using alkaline solubilization with isoelectric precipitation and thoughtfully selecting extraction conditions enables the preservation of native protein structure, resulting in high protein solubility and overall functionality (Damodaran, 2017). Furthermore, high protein purity (80-90 percent) and yield (60-70 percent) can be achieved (Swanson, 1990; Owusu-Ansah and McCurdy, 1991; Tian *et al.*, 1999; Boye *et al.*, 2010b; Stone *et al.*, 2015). Alkaline solubilization with isoelectric precipitation continues to be the most widely used method commercially. However, most parameters and conditions utilized in industry have led to protein denaturation and polymerization, resulting in reduced nutritional and functional properties. While there is room for improvement and optimization of extraction/isolation conditions, alternative extraction methods are also being explored.

1.7.3 Salt Extraction (“Salting In, Salting Out”)

In addition to extracting based on differences in solubility across pH, proteins can be extracted based on solubility in different ionic strengths using the principles of “salting in” and “salting out” proteins (Boye *et al.*, 2010a). First, the proteins are solubilized in dilute salt solution. The ions in solution partially shield protein molecules and prevent electrostatic interaction with other protein molecules and enhance protein-water interactions (Duong-Ly and Gabelli, 2014). When salt concentration is increased further, the abundance of salt ions draws water away from the proteins by completely shielding all charges on the protein’s surface, and competing for water interactions (Zhou, 2005; Novák and Havlíček, 2016). Shielding charges will encourage hydrophobic interactions among protein molecules, leading to aggregation and precipitation (Novák and Havlíček, 2016).

Salts have ion specific effects on protein solubility, meaning that some salts have a "salting out" effect at lower concentrations than other salts (Zhou, 2005). The Hofmeister series ranks cations and anions of common salts based on their solvation effects at a constant concentration, and is useful in deciding the type and concentration of salt to use for extracting protein (Zhou, 2005; Damodaran, 2017). However, proteins also differ in their responses to salts based on the amount of hydrophobic amino acid clusters on the protein surface (Damodaran, 2017). For solubilizing pea protein, studies have reporting salting in at concentrations of 0.1-0.5 M NaCl (Gueguen, 1983; Tian *et al.*, 1999; Sun and Arntfield, 2010, 2011a; Stone *et al.*, 2015). For salting out, ammonium sulfate, $(NH_4)_2SO_4$, is commonly used because its cation and anion are known to be highly effective in decreasing protein solubility based on the Hofmeister series (Damodaran, 2017). Additionally, ammonium sulfate has high solubility in water and can be used in concentrations of up to 90 percent, making it possible to precipitate all types of protein (Rangel *et al.*, 2003; Duong-Ly and Gabelli, 2014).

There is low risk of denaturation using salt extraction, so the native structure of pea protein is mostly well preserved (Novák and Havlíček, 2016). In fact, salt-extracted protein isolates tend to be more resistant to denaturation than alkaline extracted protein isolates due to the stabilizing effects of salt on the protein's structure (Hermansson, 1986; Sun and Arntfield, 2011b). Another difference is that the salt extraction produces PPI with a different protein profile than alkaline solubilization. In contrast to alkaline solubilization, salt solubilization extracts both globulins and albumins (Arntfield and Maskus, 2011; Stone *et al.*, 2015). The preserved structure and combination of globulins and albumins in salt-extracted PPI causes noticeable differences in the functional properties of the isolates compared to traditional alkaline extracted PPI.

However, salt extraction results in a final protein isolate that is highly salty, thus a desalting process is necessary (Murray *et al.*, 1981). Most commonly, dialysis is used to remove salts while retaining protein on a lab scale, while diafiltration is done on an industrial scale (Crévieu *et al.*, 1996; Cutler, 2004; Sun

and Arntfield, 2011a). However, dialysis and diafiltration are time-consuming and result in the production of large amounts of salty wash water, which must then be properly disposed of (Lam *et al.*, 2018). Another option to reduce the amount of salt is using membrane filtration instead of "salting out", as will be discussed in the next section.

1.7.4 Salt Solubilization Coupled with Membrane Filtration

Membrane filtration is growing in popularity as a way to concentrate and purify pea protein extracts. Following protein solubilization, membrane filtration can be used to remove small undesired components (salts and sugars) while concentrating the protein (Taherian *et al.*, 2011). Membrane filtration has primarily been used in place of isoelectric precipitation after alkaline solubilization (Makri *et al.*, 2005; Boye *et al.*, 2010b; Damodaran, 2017). However, it can also be used following salt solubilization instead of "salting out" the protein. There is currently only one known study that has performed salt solubilization coupled with membrane filtration (Tian *et al.*, 1999). Membrane filtration deserves further consideration, as it is a mild technique for isolating plant proteins.

To concentrate the protein and remove low molecular weight (MW) constituents, the solubilized protein stream undergoes ultrafiltration (UF). The proteins, being larger than the molecular weight cut-off (MWCO) of the membrane, are held back in the retentate, while salts and small molecules like mono-, di-, and oligosaccharides can pass through the membrane in the permeate (Fredrikson *et al.*, 2001; Taherian *et al.*, 2011). Additionally, some antinutritional factors, such as phytic acid and off-flavor compounds, are small enough to pass through the membrane, resulting in higher protein purity and cleaner flavor (Arntfield and Maskus, 2011; Taherian *et al.*, 2011; Lam *et al.*, 2018).

UF can be used to concentrate components that are 10 to 1,000 kDa in molecular weight. For successful separation by UF, components to be filtered out should be at least one order of magnitude smaller in MW than the protein (Cutler, 2004). Although separation is based mostly on molecular weight, the size and

conformation of the molecule are also important. Generally, it is advised to select a membrane with a MWCO ten times smaller than the MW of the proteins, because sometimes proteins with a MW that would be expected to be retained by the membrane can still pass through if it has a compact tertiary structure (Cutler, 2004). For pea protein extractions, UF membranes with MWCO of 3 to 50 kDa are commonly used (Vose, 1980; Tian *et al.*, 1999; Fredrikson *et al.*, 2001; Makri *et al.*, 2005; Pownall *et al.*, 2010; Taherian *et al.*, 2011; Lu *et al.*, 2019).

UF has some benefits over other protein concentration techniques, the main ones being that it is non denaturing and can achieve high protein recovery. Pea protein isolates produced by UF have been reported to have up to 92 percent protein purity and 60 percent protein yield, which are comparable or superior to other extraction techniques (Gueguen, 1983; Fredrikson *et al.*, 2001; Makri *et al.*, 2005; Boye *et al.*, 2010a; Taherian *et al.*, 2011). Additionally, while isoelectric precipitation is harsh on protein structure, ultrafiltration is not damaging to protein structure (Kinsella and Melachouris, 1976; Lam *et al.*, 2018). Protein isolates prepared by UF tend to have better solubility and overall functionality than isolates prepared by isoelectric precipitation (Vose, 1980; Gueguen, 1983; Boye *et al.*, 2010a; Lam *et al.*, 2018). Lastly, compared to salting out, UF is a more feasible process because it does not require high amounts of salt and water (Taherian *et al.*, 2011). However, it can be expensive, due to the cost of equipment, along with the cost of membranes and maintenance related to membrane fouling.

Membrane filtration has been used in the dairy industry for the production of whey protein ingredients for decades. In the 1970s, it was discovered that ultrafiltration could be used to recover proteins from cheese whey, which led to the production of whey protein concentrate (WPC) (Pouliot, 2008; Duke and Vasiljevic, 2015). Then, new methods were developed to defat the whey before UF, leading to the production of whey protein isolate (WPI) (Pouliot, 2008). UF removes small solutes including lactose and calcium ions, helping to improve protein functionality of whey protein ingredients (Damodaran, 2017). UF remains a widely used process in the dairy industry for its effective concentration and purification of whey proteins

to produce high-value protein ingredients. In addition, UF can be scaled up, another important consideration for protein extractions.

1.8 Scaled-Up Protein Extractions

The preparation and processing of protein ingredients affects their profile and structure, which in turn affects their functionality in the end product (Kinsella, 1979). The extractions reported in research articles are typically done on a laboratory scale, where there is high control over the process. Some procedures may not translate well onto a larger scale or the steps may be too meticulous to be feasible for industry production (Cutler, 2004). Adaptions often must be made in scaling-up that may alter the procedure and therefore the resulting protein isolate (Cutler, 2004). Therefore, it is important to consider how extractions can be scaled up from the laboratory benchtop to a processing plant in terms of the feasibility of the process, the protein purity and yields obtained, and the quality of the protein produced. However, few studies have been done that compare a benchtop process to a scaled-up plant trial.

Crévieu et al. (1996) studied how pea protein fractionation into albumins and globulins compared on benchtop and pilot plant scales. The extraction process differed from what is commonly done for pea protein extractions since it was simultaneously extracting both albumins and globulins, but the study gives some insight into the scale-up process. On a benchtop scale, centrifugation and dialysis were performed, but these techniques do not have direct analogs when scaled up (Cutler, 2004). Instead, desludging centrifugation and diafiltration were used in the pilot plant. Mass balance is often tracked and calculated to account for losses and end yield to determine the efficiency of the process (Hensley and Lawhon, 1979). Compared to the benchtop, Crévieu et al. (1996) saw lower protein recovery on the pilot plant scale because the desludging centrifuge had less efficient separation than the benchtop centrifuge. This observation demonstrates that differences in extraction efficiency can be expected during scale-up, which is important to note because protein is a valuable ingredient and low yields means lost money.

Additionally, another important consideration is how scaling up affects protein functionality.

Though not a direct comparison of scalability, a study evaluating lab prepared PPIs against commercially available PPI found that the commercial PPI was highly denatured and had far inferior functionality compared to the lab produced PPIs (Stone *et al.*, 2015). This indicates a need for an extraction process that is scalable and less harsh than what is currently being done industrially. Processing conditions for commercial food proteins are typically not specified, but highly impact the level of denaturation/aggregation of the protein, and therefore the functionality of the final protein ingredient (Schutyser and Van Der Goot, 2011).

On the other hand, drying procedures can have a significant effect on the protein functionality (Tulbek *et al.*, 2016). On a lab scale, protein extracts are commonly lyophilized, though this is not feasible in mass production (Cutler, 2004). Therefore, the impact of drying on the structure and functionality of pea protein isolates is of interest. Some research done on the impacts of drying has demonstrated differences. Pea protein isolates dried using the drum drier were more denatured and less functional than isolates dried by spray drying or lyophilizing (Gwiazda *et al.*, 1979; Sumner *et al.*, 1981). Lyophilized samples are expected to be less denatured than spray-dried and drum-dried samples because lyophilization does not require or produce heat (Elmore *et al.*, 2007). However, spray drying is the only drying method commonly done in industrial production of protein isolates, so differences in protein quality of benchtop and scaled-up protein extraction processes would be anticipated (Cutler, 2004).

In addition to the use of different drying methods, another big difference between benchtop and industrial protein extractions is that protein isolates made on an industrial scale require pasteurization for food safety (Qi *et al.*, 2015). Pasteurization causes thermal stress to the protein isolates that can alter their structural and functional properties. However, there is very limited research done on investigating how pasteurization affects protein structure. One study looked at the effects of pasteurized, spray-dried soy protein isolates versus “native” SPI that

was lyophilized and not pasteurized and saw that the pasteurized, spray-dried SPI had different secondary structures. Specifically, it contained lower amounts of α-helices and higher amounts of random coil, which indicated thermal effects on the protein structure, namely denaturation (Elmore *et al.*, 2007). These changes correspond to increased surface hydrophobicity, and therefore increase susceptibility to protein aggregation (Elmore *et al.*, 2007). More research is needed on the effects of pasteurization on protein functionality in order to anticipate how processes optimized in the lab will translate into industrial production.

Currently, most commercial protein products for either soy or pea are prepared by alkaline solubilization with isoelectric precipitation. However, an economic evaluation of the costs associated with starting up and operating a plant for membrane filtration of protein isolates concluded that membrane filtration, while relatively more expensive, is industry feasible and eliminates major waste streams (Hensley and Lawhon, 1979). The higher cost may be offset by a higher premium for a more functional protein. This demonstrates that extraction methods other than alkaline solubilization are possible on a large scale, and therefore are worth investigating further.

1.9 Summary and Conclusions

While soy protein has been widely characterized and is now fairly well understood, pea protein is much less researched. Structural similarities between the storage proteins (7S and 11S globulins) of soybeans and field peas suggest that they should be suitable for similar applications. However, the ratio of 7S:11S proteins varies between soy and pea protein, with soy proteins being dominant in 11S globulins whereas pea protein is higher in 7S globulins. 7S and 11S proteins differ in their size and structure, and thus their interactions with their surroundings are different. As a result of differences between globulin proteins and differing amounts of each protein component, pea protein and soy protein are naturally predetermined for different functional properties. However, protein profile and

structure can be altered, intentionally or consequentially, by conditions chosen for extraction and processing.

Alkaline solubilization with isoelectric precipitation is the most common extraction technique for both soy and pea protein. The industry is already familiar with this procedure, although extraction and processing conditions can be highly variable. This variability leads to distinct differences in the functional properties and therefore the food applications of the resulting protein isolates. In order to produce pea protein isolates with good functional properties and widespread applications, it is important to preserve native protein structure, which can be done through careful control of the extraction and processing conditions. Solubilization pH, solubilization duration, number of washes, extraction temperature, isoelectric precipitation conditions, and purification techniques should be optimized to effectively isolate pea proteins with high protein purity and yield, while minimizing protein denaturation and aggregation.

In addition to extractions based on pH, salt can be used to extract proteins by taking advantage of how surface charge changes with differences in ionic strength. Salt extraction methods are less common in industry because the extraction creates a waste stream of salty water, particularly because of the "salting out" precipitation step. However, it may be possible to develop an extraction using dilute salt concentration for solubilization coupled with membrane filtration for salt removal and protein concentration. If an extraction was developed using dilute salt concentrations that produced superior protein to that of the alkaline extraction, salt extraction may be industrially feasible and beneficial.

There is a growing demand for high quality protein ingredients that pea protein can help satisfy. Pea protein is similar to soy and shows promise to reach the same level of widespread usage in food applications, along with offering some unique benefits of being non-GMO and of low allergenicity. However, pea protein is still several years behind soy protein in terms of research and development. A thorough study of pea protein extraction conditions and their impact on the structural and functional properties of the proteins will aid in the development of

pea protein ingredients that are just as successful in food applications as soy protein.

Chapter 2: Materials and Methods

2.1 Materials

Yellow field pea flour was kindly provided by AGT Foods (Regina, SK, Canada). The starting flour had a moisture content of less than 13%, fiber content less than 6%, and protein at least 20%. Commercial whey protein isolate (cWPI, 94.6% protein, 4.10% ash), BiPro®, was kindly provided by Agropur Ingredients (Eden Prairie, MN, USA). Defatted soy flour (7B, 53% protein) and commercial soy protein isolate (cSPI, 90.7% protein, 2.36% ash), ProFam® 974, were kindly provided by Archer Daniels Midland (ADM) (Decatur, IL, USA). Commercial pea protein isolate (cPPI, 81.2% protein, 3.86% ash), PURIS™ Pea Protein, was kindly provided by Puris Foods (Minneapolis, MN, USA). When not in use, the samples were stored at -20°C.

SnakeSkin™ dialysis tubing with 3.5 kDa molecular weight cut off (MWCO) and Sudan Red 7B were purchased from Thermo Fisher Scientific™ (Waltham, MA, USA). Criterion™ TGX™ 4-20% precast gels, Laemmli sample buffer, 10X Tris/Glycine/SDS running buffer, Imperial™ Protein Stain, and Precision Plus molecular weight marker were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Vivaflow® membrane ultrafiltration cross-flow cassettes (3 kDa MWCO) were purchased from Sartorius™ (Gottingen, Germany). Aluminum crucibles (40 µL, with pin) for DSC were purchased from Mettler-Toledo (Columbus, OH, USA). Folded capillary cuvettes for zeta potential were purchased from Malvern (Malvern, UK). Costar® solid opaque black 96-well plates, 8-anilino-1-napthalenesulfonic acid ammonium salt (ANS), electrophoresis grade sodium dodecyl sulfate (SDS), and 2-mercaptoethanol (BME) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pure corn oil (Mazola) was purchased from grocery stores. All other chemical grade reagents were purchased from either Thermo Fisher Scientific or Sigma-Aldrich.

2.2 Production of Native Soy Protein Isolate

Native soy protein isolate (nSPI) was produced following a method described by Margatan et al. (2013), with slight modification. Defatted soy flour was dispersed in distilled deionized water (DDW) at 1:10 w/v and adjusted to pH 7.5 with 2 N NaOH. Solutions were stirred 1 hour on a magnetic stir plate, then the solution was centrifuged in a Beckman floor centrifuge (Beckman J2-21, Beckman Coulter, Brea, CA, USA) at 5000 x g for 30 minutes to separate insoluble components. The supernatant was collected and adjusted to pH 4.5 with 2 N HCl. The solution was stirred 10 minutes and then centrifuged at 5000 x g for 10 minutes to precipitate proteins. The pellet was then resuspended in DDW (1:4 w/v), neutralized with 2 N NaOH, and stirred for two hours to resolubilize the protein.

The protein solution was then dialyzed against deionized (DI) water at 4°C to remove salts, following Thermo Fisher Scientific™ specifications. Briefly, the protein solution was sealed in SnakeSkin™ membrane tubing (3.8 mL sample/cm tubing; 3.5 kDa MWCO) and placed in a 5 L bucket of DI water on a stir plate in the cold room (4°C). The water was stirred gently for 4-8 hours to reach an equilibrium in salt concentrations of the DI water and protein solution within the SnakeSkin™ tubing, at which point the DI water was replaced. A total volume of dialysis water 300 times the volume of the protein solution was used, with DDW being used for the final wash. After dialysis, samples were lyophilized. The protein content was determined to be 99.7% following the Dumas method (AOAC 990.03) using a LECO® FP828 nitrogen analyzer (LECO, St. Joseph, MI, USA) with nitrogen-to-protein conversion factor of 6.25. When not in use, the sample was stored at -20°C.

2.3 Optimization of Pea Protein Extraction Conditions Following an Alkaline Solubilization with Isoelectric Precipitation Method

Pea protein extraction conditions were optimized for alkaline solubilization with isoelectric precipitation (pH extraction), using the method described for native soy protein extraction as a starting point. In order to optimize pea protein extraction

conditions, the impacts of solubilization pH, isoelectric precipitation pH, solubilization duration, number of solubilizations, and dialysis were each evaluated, in turn. The protein purities of each fraction from the extraction (PPI, discarded pellet, and discarded supernatant) were measured by Dumas (N x 6.25). Mass balance was monitored to track the masses of each fraction, which were then used to calculate protein yield using Equations 1, 2, and 3 (sample calculations can be found in Appendix A and illustrated in Table 5).

$$\%\ protein\ yield\ in\ PPI = \frac{PPI\ mass\ (g) \times PPI\ protein\ purity\ (\%)}{pea\ flour\ mass\ (g) \times pea\ flour\ protein\ purity\ (\%)} \quad \text{Eq. 1}$$

$$\%\ protein\ residue\ in\ pellet = \frac{pellet\ mass\ (g) \times pellet\ protein\ purity\ (\%)}{pea\ flour\ mass\ (g) \times pea\ flour\ protein\ purity\ (\%)} \quad \text{Eq. 2}$$

$$\%\ protein\ lost\ in\ supernatant = \frac{sup.\ mass\ (g) \times sup.\ protein\ purity\ (\%)}{pea\ flour\ mass\ (g) \times pea\ flour\ protein\ purity\ (\%)} \quad \text{Eq. 3}$$

Together, protein purities and protein yields were evaluated to determine the effectiveness of extraction conditions. Each extraction was done in triplicate. When not in use, the samples were stored at -20°C.

First, optimal solubilization pH was determined. An aliquot (10 grams) of pea flour was dispersed in DDW (100 mL, 1:10 w/v dispersion) and adjusted to the solubilization pH (pH 7, 7.5, or 8) with 2 N NaOH. Solutions were stirred for 1 hour on a magnetic stir plate, then centrifuged at 5000 x g for 30 minutes to precipitate insoluble materials. The supernatant was adjusted to the isoelectric point (pH 4.5) with 2 N HCl and stirred for 10 minutes. The solution was then centrifuged at 5000 x g for 10 minutes to precipitate the proteins. The pellet was immediately resuspended in DDW (1:4 w/v) and neutralized with 2 N NaOH. Each fraction (the pellet from the first centrifugation step, the supernatant from the second centrifugation step, and the final neutralized PPI fraction) were lyophilized, weighed, and analyzed for protein content by Dumas. Mass balance was tracked and used to determine protein yield of each fraction as state previously. Together, protein purities and protein yields were used to determine the optimal pH for the

extraction. Solubilization at pH 7.5 was considered optimal and was then used in the optimization of other extraction conditions.

The isoelectric precipitation point was examined next. The same extraction procedure detailed above was followed, using the optimized solubilization pH (pH 7.5), 1 hour of solubilization, a single solubilization, and no dialysis. The isoelectric point was tested at both pH 4.5 and pH 5. Again, protein purities and protein yields were determined by Dumas and mass balance, respectively, and used to determine the optimal isoelectric point. The pH of 4.5 was considered optimal for isoelectric precipitation and was then used in the optimization of the other extraction conditions.

Solubilization duration was optimized next. Again, the same extraction procedure was followed, using the optimized solubilization pH (pH 7.5), optimized isoelectric point (pH 4.5), a single solubilization, and no dialysis. The pea flour was solubilized for a duration of either 1 or 2 hours. Protein purities and protein yields were determined and used to select the optimal solubilization duration. A solubilization duration of 1 hour was considered optimal and was then used in the optimization of other extraction conditions.

Next, the number of solubilizations was determined. The extraction procedure mirrored the other extractions to start, with pea flour being solubilized in DDW (1:10 w/v) at the optimized pH (pH 7.5) for the optimized duration of time (1 hour), then centrifuged (5000 x g for 30 minutes). However, after the centrifugation step, the pellet was collected and resolubilized at the optimized pH (pH 7.5) for the optimized duration of time (1 hour), while the supernatant was set aside. The resolubilized pellet was centrifuged again (5000 x g for 30 minutes), and the supernatants from both solubilization steps were combined. The combined supernatants were adjusted to the optimized isoelectric point (pH 4.5), stirred for 10 minutes, and centrifuged (5000 x g for 10 minutes) to precipitate the proteins. The pellet was then resuspended in DDW (1:4 w/v), neutralized, and lyophilized. Again, protein purities and yields were used to determine how the number of solubilizations affected extraction efficiency. It was found that a double

solubilization was superior to a single solubilization, so double solubilization was used in extractions moving forward.

Lastly, the effect of dialyzing the final PPI fraction was investigated. The extraction was performed using the optimized solubilization pH (pH 7.5), optimized isoelectric point (pH 4.5), optimized solubilization duration (1 hour), and optimized number of solubilizations (double solubilization). However, after the PPI fraction was neutralized, it was either left as is or subjected to dialysis, as described in Section 2.2. Following dialysis, the PPI fraction was lyophilized. Protein purities and yields of only the PPI fraction were used to compare the effects of dialysis, because the pellet and supernatant fractions were not affected by dialysis. Additionally, the ash contents of both the dialyzed and non-dialyzed PPI fractions were measured to determine if dialysis effectively removed salts formed during the extraction. Ash content was determined following the official AOAC method (AOAC 942.05). The protein purities, protein yields, and ash contents of the dialyzed and non-dialyzed PPIs were compared to determine the effect of dialysis. The use of dialysis was determined to be optimal.

The final optimized pH extraction protocol entailed solubilization at pH 7.5 for 1 hour, with the initial pellet being resolubilized at pH 7.5 for another hour, combination of the supernatants and isoelectric precipitation of the proteins at pH 4.5, resolubilization of the proteinaceous precipitate at neutral pH, followed by dialysis and lyophilization. The optimized conditions were used to produce pH-PPI to use for structural and functional characterization.

2.4 Optimization of Pea Protein Extraction Conditions Following a Salt Solubilization Coupled with Membrane Filtration Method

Pea protein extraction using salt solubilization coupled with membrane filtration (salt extraction) was also performed as a second technique of extraction. The conditions for membrane filtration and protein purification were optimized by comparing the effects of membrane ultrafiltration (UF), dialysis, or UF followed by dialysis. Traditionally, following a salt solubilization, proteins are precipitated by

increasing the ionic strength of the solvent to “salt out” the proteins. However, this is undesirable because it leads to highly salty wastewater and PPI with high salt content. Instead, membrane filtration and/or dialysis was investigated as an alternative way to concentrate and purify proteins solubilized in dilute salt solution.

All salt extractions followed the same solubilization step. An aliquot (5 grams) of pea flour was solubilized in dilute salt solution (0.5 M NaCl) at 1:20 w/v, and the solution was stirred on a magnetic stir plate at its natural pH (pH 6.4) at room temperature (23°C). After 1 hour of stirring, the solution was centrifuged at 5000 x g for 30 minutes to separate insoluble materials. The supernatant containing the solubilized proteins was collected and neutralized with 2 N NaOH. The neutralized supernatant was then either subjected to cross-flow (tangential) ultrafiltration (UF), dialysis, or UF followed by dialysis to further concentrate the protein and reduce salt content.

For supernatants being ultrafiltered, the benchtop Sartorius Vivaflow® 200 system was used with two Vivaflow® membrane cassettes running in parallel to increase the speed of filtration. The system was set up according to manufacturer instructions, with the protein solution in a feed reservoir and the feed tube connected to a peristaltic pump (Masterflex Easy Load Pump Head- Size 15, Masterflex Economy Drive Peristaltic Pump 230V, Sartorius) to pump the feed solution under pressure (2.5 bars) across the membranes. Components in the solution smaller than the membrane pore size (3 kDa MWCO) passed through as the permeate and were collected in a waste bottle. Components larger than the filter pores were retained and recirculated to the feed reservoir. Using this set up, the volume of the protein solution was first concentrated to 50 mL to reduce the amount of water needed for diafiltration. After concentration, the samples were diafiltered following Sartorius’s instructions, against 6 volumes of DDW (300 mL total) to continually decrease salt concentration and the presence of other low molecular weight (MW) compounds and further concentrate the protein. After all volumes of diafiltration water were added, the solution was concentrated to 25 mL. To end UF, the feed tube was removed from the feed reservoir and a few seconds

of air were pumped through the system in order to remove protein solution from the tubing. The feed tube was then placed in a beaker of DDW, and approximately 25 mL of DDW was pumped through the system in order to flush out any remaining protein solution and help increase protein recovery. After completing UF, the protein solution was lyophilized.

In a second set of extractions, dialysis was tested in place of UF to see if dialysis could efficiently concentrate the protein and remove salt. Proteins were solubilized as described above and centrifuged to remove insoluble materials. The neutralized supernatant was then dialyzed, as described in Section 2.2. Following dialysis, the PPI solution was lyophilized.

As a third option for extraction protocol, the effects of UF followed by dialysis were assessed. Proteins were solubilized, ultrafiltered, and dialyzed as described previously. After ultrafiltration and dialysis, the PPI solution was lyophilized.

For all three methods of salt extraction, protein content of the pellet and PPI fractions were determined by Dumas. Mass balance was tracked and Equations 1 and 2 were used to calculate protein yield (%) for the PPI and protein residue (%) for the pellet fractions, respectively (sample calculations can be found in Appendix B and illustrated in Table 6). Additionally, ash contents of the PPI fractions were measured as described previously. As with the optimization of pH-PPI extractions, protein purities, protein yields, and ash contents were compared amongst extraction procedures to determine the efficiency of protein isolation and the purity of the final salt-PPI. It was determined that the optimized method for salt extraction was solubilization of pea flour in 0.5 M NaCl for 1 hour, neutralization of the supernatant, ultrafiltration, dialysis, and lyophilization. Each extraction was done in triplicate. When not in use, the samples were stored at -20ºC.

2.5 Pilot Plant Scaled-Up Protein Extractions from Pea Flour

The optimized pH and salt extraction methods were scaled up in the Joseph J. Warthesen Food Processing Center at the University of Minnesota to determine how the optimized benchtop extractions translated to larger scale production. The

ultimate goal of optimizing pea protein extraction methods is to produce functional PPI that can be mass produced by the food industry for widespread use. Therefore, it is important to examine how well the optimized procedures translate to a larger scale.

For both the pH- and salt-extractions, there were some unavoidable differences between the benchtop and scaled-up (SU) extractions. Differences were encountered when benchtop equipment did not have a direct analog in the pilot plant. First, the centrifuges used differed noticeably in their separation power. The benchtop Beckman floor centrifuge forms dry, compact pellets and a clear supernatant, while the horizontal decanter centrifuge (Westfalia Separator AG, 1 gal/min, GEA Westfalia Separator Group Gmbh, Oelde, Germany) in the pilot plant cannot achieve the same level of separation. To help improve separation, a desludging disc centrifuge (Westfalia SB7, 1 gal/min, GEA Westfalia Separator Group Gmbh, Oelde, Germany) was used in sequence to clarify the supernatant further. Additionally, large scale dialysis is infeasible, so ultrafiltration/diafiltration was used instead. Total solids of the permeate was constantly measured using a CEM AVC-80 Microwave Moisture/Solids Balance Analyzer (CEM, Charlotte, NC, USA) to monitor salt removal. Furthermore, foods produced in the pilot plant must be food-grade, so the PPI was pasteurized after filtration. Following pasteurization, the PPI was homogenized to help improve ease of drying. Lyophilizing is not commonly done in industry because it is time consuming and costly, so the scaled-up PPI was dried using a spray drier. Another difference to note is that extractions in the pilot plant used deionized (DI) water in place of DDW, because the pilot plant does not have large quantities of DDW available. Lastly, extractions in the pilot plant spanned across two days of processing, so precautionary steps were added to the extraction methods to prevent microbial growth overnight.

2.5.1 Scaled-Up pH-Extraction

The scaled-up pH-extraction followed the method optimized in the laboratory with some unavoidable differences, as mentioned previously. To start,

pea flour (83.4 lbs, 37.85 kg) was solubilized in DI water (100 gallon, 378.5 L) (1:10 w/v) adjusted to pH 7.5 with concentrated NaOH (6.25 N) for 1 hour at room temperature (23°C), and then agitated in a jacketed tank (150 gallon) equipped with an automated mixer (Vektor Series, Lightnin®, Rochester, NY, USA). The solution was then separated using a horizontal decanter centrifuge and clarified with a desludging disc centrifuge. The separated liquid was set aside in a stainless-steel can (10 gallon) in the cold room (6-8°C). The precipitate was weighed and the total solids (%TS) was measured using a CEM AVC-80 Microwave Moisture/Solids Balance Analyzer (CEM, Charlotte, NC, USA). The mass and %TS were used to calculate the volume of DI water needed to resuspend the pellet at 1:10 w/v (sample calculation can be found in Appendix C). The solution was adjusted to pH 7.5 and again agitated in a jacketed tank (150 gallon) for 1 hour. The solution was then passed through the decanter centrifuge and desludging centrifuge again, and the separated liquid was collected and combined with the separated liquid from earlier in a clean jacketed tank (150 gallon) equipped with an automated mixer. The combined solution was then adjusted to pH 4.5 with HCl (4 N) and agitated for 10 minutes. The solution was then passed through the decanter centrifuge again, with the proteinaceous precipitate being collected in a clean plastic can (Rubbermaid BRUTE, 44 gallon, Atlanta, GA, USA). The supernatant was pumped through the desludging centrifuge to ensure that no additional solids precipitated. The precipitate from the decanter was weighed, transferred to a jacketed tank (150 gallon) with automated mixer, and solubilized in DI water at 1:4 w/v. This marked the end of the first day of production. Leaving the protein at its isoelectric point overnight would cause protein denaturation, leading to aggregation and irreversible polymerization, while neutralizing the supernatant could allow for microbial growth overnight. Instead, the solution was adjusted to pH 3 and left in the cold room (6-8°C) overnight.

At the beginning of the second day, the solution was neutralized and agitated for 1 hour at room temperature (23°C). Ultrafiltration/diafiltration was performed in place of dialysis using a UF/RO unit (15-20 psi inlet, 10-15 psi outlet,

PTI Advanced Filtration, PTI Technologies, St. Louis, MO, USA) with tangential (cross) flow and a spiral wound membrane (3 kDa MWCO). The protein solution (22 gallons, 85 L) was diafiltered with a set up similar to the benchtop diafiltration. The feed solution was pumped across the membrane, with the retentate continuously recirculating to the feed tank, while the permeate stream was collected as waste. DI water was added (10 gallons, 40 L at a time) until the % TS of the exiting permeate stream was 0.00%, measured with a CEM AVC-80 Microwave Moisture/Solids Balance Analyzer. In total, 30 gallons of water was added. At the end of ultrafiltration/diafiltration, the concentrated (high solids) retentate (6 gal, 22.5 L) was disconnected from the UF/RO unit and set aside. DI water was then pumped through the membrane to flush out remaining protein solution, which was collected in a separate tank as the low solids retentate. This step was done separately because although combining the rinse low solids retentate with the high solids retentate would increase overall protein yield of the extraction, the %TS would be too low for spray drying. In order to spray dry the solution, an evaporation step would be necessary to increase %TS, but would subject the protein to continued heating at 60-70°C for 30-60 minutes, causing protein denaturation. Instead, only the high solids retentate was used for the remainder of the extraction.

After ultrafiltration/diafiltration, the high solids retentate (6 gallons, 22.5 L) was pasteurized by running the solution through a high temperature short time (HTST; 73°C for 15 seconds) processing system (MicroThermics® Electric Model 25HV Hybrid, 15-45 gal/hr, MicroThermics® Inc., Raleigh, NC, USA), followed by two-stage homogenization (Gaulin 125 L, 2500 psi, 60 gal/hr, Manton-Gaulin Mfg. Co. Inc., Everett, MA, USA). The solution was then spray dried using a SPX Flow Anhydro Spray Dryer (9.5% TS, 180°C inlet, 90°C outlet, 2.4 gal/hr) with a wheel type atomizer (24,500 rpm) (SPX Flow Inc., Charlotte, NC, USA).

The protein yield of the final SU-pH PPI could not be accurately determined for two reasons. First, several samples of solution (~3 lbs, or 0.37 gal, per sample) were pulled at steps throughout the extraction for a separate research project on

flavor development during protein extractions. This decreased the protein yield of the extraction, as each collected sample removed some protein. The samples pulled near the end of extraction after ultrafiltration, when the protein solution was more concentrated, specifically would have decreased the reported protein yield of the process. Secondly, as mentioned, the low solids retentate was not collected from the ultrafiltration/diafiltration step. There is potential to combine high solids and low solids retentates to improve yield, though with a suspected decline in protein functionality. Protein purity and ash content of the final SU-pH PPI were determined by Dumas and dry ashing, respectively, to assess how the pilot plant extraction compared to the benchtop extraction. When not in use, the sample was stored at -20°C.

2.5.2 Scaled-Up Salt-Extraction

The scaled-up salt-extraction followed the method optimized in the laboratory with some slight differences. In addition to the differences listed previously, the SU salt-extraction had two other notable differences. First, the initial precipitate from the decanter was resolubilized at 1:5 w/v and run through the decanter a second time in order to achieve better separation and increase protein yield. The higher w/v ratio was used here because resolubilization was not a part of the original benchtop salt extraction, but in order to pass the pellet through the decanter again, it needed to be resuspended. Additionally, resolubilizing the pellet at 1:20 w/v again would have resulted in high amounts of liquid to process moving forward through the extraction, whereas 1:5 w/v kept the volumes manageable. A second difference between the benchtop and scaled-up salt extraction was that, as previously mentioned, the pilot plant uses ultrafiltration/diafiltration and no dialysis. The benchtop salt extraction involved ultrafiltration followed by dialysis, but in the pilot plant, the solution was ultrafiltered/diafiltered until the %TS reached 0.00%, after which additional filtration would not change composition.

To begin the SU salt-extraction, pea flour (62 lbs, 28 kg) was solubilized in 0.5 M NaCl (150 gal, 560 L) (1:20 w/v) at its natural pH (pH 6.4) and agitated in a

jacketed tank (150 gallon) with automated stirrer for 1 hour at room temperature (23°C). The solution was then separated using a horizontal decanter centrifuge and clarified with a desludging centrifuge. The separated liquid was set aside in a stainless-steel can (50 gallon) in the cold room (6-8°C). The precipitate was weighed and the total solids was measured using a CEM AVC-80 Microwave Moisture/Solids Balance Analyzer. The mass and %TS were used to calculate the volume of 0.5 M NaCl needed to resuspend the precipitate at 1:5 w/v (sample calculation can be found in Appendix D). The solids were resuspended and agitated in a jacketed tank (150 gallon) with automated stirrer for 30 minutes, just enough to resuspend the pellet. The solution was then passed through the decanter centrifuge and desludging centrifuge again, and the separated liquid was collected and combined with the separated liquid from earlier in a jacketed tank (150 gallon) with automated stirrer. The solution was neutralized with concentrated with NaOH (6.25 N) and left stirring in the cold room (6-8°C) overnight. It was assumed the salt content of the solution and the cool temperature would be sufficient to prevent microbial growth.

To start the second day, the protein solution (161 gal, 610 L) was ultrafiltered/diafiltered using the set up described for the scaled-up pH extraction. The feed solution was pumped across the membrane (3 kDa MWCO), with the retentate continuously recirculating and the permeate stream being collected as waste. The protein solution was first concentrated to a volume of about 1/8 the tank's capacity (approximately 20 gal, 76 L). After concentration, DI water was added until the %TS of the exiting permeate stream was 0.00%, with the total volume of water added being about 158 gal (600 L). As with the SU-pH extraction, the high solids retentate (5.3 gal, 20 L) was disconnected from the UF/RO unit. DI water was then pumped through the membrane to flush out remaining protein solution, which was collected in a separate tank as the low solids retentate. Only the high solids retentate was used for the remainder of the extraction.

The high solids retentate was then pasteurized using the high temperature short time (HTST; 73°C for 15 seconds) MicroThermics processing system,

followed by two-stage homogenization, as described in Section 2.5.1. The solution was then spray dried using the same spray dryer used for the scaled-up pH-extraction under the same conditions.

As with the SU pH-extraction, several samples of solution (~3 lbs, or 0.37 gallons, per sample) were pulled at different steps throughout the extraction for a research project on flavor development during protein extractions. Additionally, as with the SU pH-extraction, the low solids retentate was not collected from the ultrafiltration/diafiltration step, which reduced overall yield. Therefore, protein yield of the SU-salt PPI was not determined. Protein purity and ash content of the final SU-salt PPI were determined by Dumas and dry ashing, respectively, to assess how the pilot plant extraction compared to the benchtop extraction. When not in use, the sample was stored at -20°C.

2.6 Structural Characterization of Proteins

2.6.1 Protein Profiling by Gel Electrophoresis

Protein profiling of all isolates was performed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), following the method outlined by Laemmli (1970). Protein samples were first dissolved in DDW (4 mg protein/mL), and then mixed 1:1 (v/v) with Laemmli buffer under reducing and nonreducing conditions. An aliquot (5 µL, delivering approximately 0.05 mg protein) and 10 µL of Precision Plus MW standard were loaded onto a Criterion™ TGX™ 4-20% precast Tris-HCl gradient gel and electrophoresed at 200V. The gel was then stained using Imperial™ Coomassie blue R-250 staining solution and destained with DDW. Molecular Imager Gel Doc XR system (Bio-Rad Laboratories) was used to image the gels, and bands were identified by their molecular weights.

2.6.2 Differential Scanning Calorimetry (DSC)

Thermal denaturation of protein samples was determined using a DSC instrument (DSC 1 STARe System, Mettler Toledo, Columbus, OH, USA),

following the method described by Tang et al. (2007). Samples were solubilized in DDW (20% protein, w/v) and stirred overnight. An aliquot (20 μL, delivering approximately 4 μg protein) was transferred to an aluminum pan and hermetically sealed. An empty sealed pan was run simultaneously as reference. The pans were held at 25°C for 5 minutes, then heated from 25°C to 110°C at a rate of 5°C/min. Thermograms were manually integrated to obtain the peak denaturation temperature and enthalpy of denaturation for each protein using Mettler Toledo's STARe Software version 11.00. Each sample was run in triplicate. Indium was used for calibrating the instrument.

2.6.3 Zeta Potential

Zeta potential was measured using a dynamic light scattering instrument (Malvern Nano Z-S Zetasizer). Protein solutions (5 mL) were prepared in triplicate in DDW (1% protein, w/v), adjusted to pH 7, and stirred for 2 hours. An aliquot (1 mL) of solution was dispensed into a folded capillary cell and inserted into the Zetasizer. After a 30 second equilibration period, electrophoretic mobility was measured by three sub-rep readings taken every 10 seconds for each replicate. Zeta potential was determined using the Smoluchowski model, using Malvern's Zetasizer software (version 7.13).

2.6.4 Surface Hydrophobicity

The surface hydrophobicity of samples was determined spectrophotometrically based on the method by Kato and Nakai (1980), with modifications by Alizadeh-Pasdar and Li-Chan (2000). Protein solutions (20 mL, 0.05% protein w/v) were prepared in 0.017M: 0.165M citric acid:sodium phosphate buffer (pH 7), in triplicate, stirred for 2 hours, and diluted to concentrations between 0.05% and 0.005% protein (w/v). An aliquot (200 μL) of each concentration was dispensed into black 96-well plate, along with 200 μL of the citric acid:sodium phosphate buffer as a blank. The initial relative fluorescence intensity (RFI) was measured at room temperature with excitation and emission wavelengths of

400/30 and 460/40 nm (wavelength/bandwidth), respectively, with gain set to 40, using a microplate reader (BioTek Synergy HT, BioTek Instruments, Winooski, VT, USA). After the initial reading, 20 µL of 8-anilino-1-napthalenesulfonic acid ammonium salt (ANS) probe solution was added to each well on the plate, mixed gently with the pipette tips, and held in the dark for 15 minutes. The RFI was measured again under the same conditions. Net RFI was calculated as described by Alizadeh-Pasdar and Li-Chan (2000). The net RFI was plotted against percent protein concentration and the slope (S_0) was used as an index of protein surface hydrophobicity. Sample calculations for RFI and surface hydrophobicity can be found in Appendix E and illustrated in Figure 10.

2.7. Functional Characterization of Proteins

2.7.1 Protein Solubility

Protein solubility was determined following the method described by Wang and Ismail (2012), with slight modifications. Solutions (5 mL) were prepared at 5% protein (w/v), in triplicate, and adjusted to pH 7 or pH 3.4 using 2 N HCl and 2 N NaOH and an Orion™ ROSS Ultra™ pH Electrode (Thermo Scientific). Solutions were stirred at medium speed on a magnetic stir plate for 2 hours. The initial protein content of a 200 µL aliquot of each solution was determined, in duplicate, using the Dumas method. For non-heated samples, 1 mL aliquots were centrifuged at 15,682 x g for 10 minutes at 23°C to separate insoluble material. To determine the effect of thermal treatment on solubility, 1 mL aliquots were placed in a water bath at 80°C for 30 minutes, followed by centrifugation at 15,682 x g for 10 minutes. The protein content of the supernatants was then measured by Dumas. The percent protein solubility was calculated using Equation 4 (sample calculation can be found in Appendix F).

$$\%\,protein\ solubility = \frac{\%\,supernatant\ protein}{\%\,initial\ protein} \qquad \text{Eq. 4}$$

2.7.2 Gel Strength

Protein solutions (8 mL) were prepared in DDW, in triplicate, at pH 7 and stirred for 2 hours. Aliquots (1 mL) of protein solutions were dispensed into lightly oiled microcentrifuge tubes using a positive displacement pipette. Microcentrifuge tubes were sealed and heated in a water bath at 95°C (± 2°C). Heat-induced protein gels were formed at 15% protein with 10 minutes of heating for SPI samples. Pea protein samples did not gel under these conditions; thus, PPI gels were prepared at 20% protein with 20 minutes of heating. After cooling completely to room temperature, gels were removed from the microcentrifuge tubes by cutting off the tip and using a gentle stream of air to blow the gels out. The strength of the gels was measured by a TA-TX Plus Texture Analyzer (Stable Micro Systems LTD, Surrey, UK) using a 100 mm diameter probe, 5 mm/s test speed, and a target distance of 0.5 mm from the plate. The maximum force measured was considered to be the rupture force of the gel.

2.7.3 Emulsification Capacity

Emulsification capacity (EC) was determined using the method described by Rickert et al. (2004), with some modifications. Protein solutions (20 mL, 1% w/v) were prepared in DDW, adjusted to pH 7, and stirred for 2 hours. A 5 mL aliquot of each protein solution was added into a 250 mL beaker, in triplicate. Corn oil, dyed with 4 µg/mL of Sudan Red 7B, was dispensed at a steady rate 2-3 drops/s into the beaker as the protein solution was blended by a homogenizer (IKA RW 20 Digital, IKA Works Inc., Wilmington, NC, USA) with a 4-blade, 50 mm diameter shaft (IKA R 1342) spinning at 860-870 rpm. Samples were homogenized with continuous addition of oil until a phase inversion was observed where the emulsion lost viscosity. PPI solutions did not form thick emulsions at 1% protein, thus they were tested at 2% protein. EC was expressed as grams of oil emulsified per gram of protein, using Equation 5 (sample calculation can be found in Appendix G).

$$EC = \frac{volume\ of\ oil\ titrated\ (mL) \times density\ of\ oil\ (\frac{g}{mL})}{mass\ of\ protein\ (g)} \qquad \text{Eq. 5}$$

2.7.4 Emulsion Stability and Activity

Emulsion stability (ES) and emulsion activity index (EAI) were determined based on the turbidometric method described by Pearce and Kinsella (1978) and adapted by Tang et al. (2003) and Rickert et al. (2004), with some modifications. Solutions (25 mL) were prepared at 0.1% protein (w/v) and adjusted to pH 7. After 2 hours of stirring, a 5 mL aliquot was added to a beaker (50 mL) containing 1.67 mL of corn oil and immediately homogenized at 10,000 rpm using a Scilogex D500 homogenizer (Rocky Hill, CT, USA), in triplicate. After 1 minute of homogenization, a 50 μL aliquot of the emulsion was added to 5 mL of 0.1% SDS, to prevent flocculation, and vortexed for 5 seconds. The sample was transferred to a cuvette and the initial absorbance (A_0) of the emulsion was read at 500 nm using a UV/VIS spectrophotometer (Beckman Coulter DU 800, Brea, CA, USA). After 10 minutes, another 50 μL aliquot of the emulsion was vortexed with 5 mL of 0.1% SDS and the final absorbance (A_{10}) was measured. The ES was determined using Equation 6, as reported by Rickert et al. (2004), while the EAI was calculated from Equation 7, as reported by Cameron et al. (1991). Sample calculations for ES and EAI can be found in Appendix H.

$$ES\ (\mathrm{min}) = \frac{A_0}{A_0 - A_{10}} \times 10\ min \qquad \text{Eq. 6}$$

$$EAI\ \left(\frac{m^2}{g}\right) = \frac{2T}{(1-\phi)C} \qquad \text{Eq. 7}$$

Where:

A_0 = initial absorbance at 500 nm

A_{10} = final absorbance at 500 nm

C = weight of protein per volume of aqueous phase

ϕ = volume fraction of oil

T = turbidity of oil at 500 nm = $\frac{2.303 \times A_0}{l}$

l = path length of the cuvette

2.7.5 Foaming Capacity and Stability

Foaming capacity and stability were determined based on the method described by Bera and Mukherjee (1989), with modifications. Samples (200 mL) were prepared at 0.5% protein (w/v), adjusted to pH 7, and stirred at low speed to avoid inducing premature foaming. After 2 hours, a 50 mL aliquot was transferred into an 800 mL beaker and blended at 800 rpm with a Sun Beam® hand mixer (Fort Lauderdale, FL, USA) for 2 minutes, in triplicate. Solutions were quickly but carefully transferred into a 250 mL short form graduated cylinder and the initial foam and liquid volumes were recorded. Foaming capacity was determined using Equation 8. After 30 minutes, the foam and liquid levels were measured again and used to calculate foaming stability using Equation 9. Sample calculations for foaming capacity and foaming stability can be found in Appendix I.

$$foaming\ capacity\ \left(\frac{mL\ foam}{g\ protein}\right) = \frac{total\ solution\ volume\ (mL) - liquid\ volume\ (mL)}{mass\ of\ protein\ (g)} \quad \text{Eq. 8}$$

$$foaming\ stability\ (\%) = \frac{total\ solution\ volume_{end} - liquid\ volume_{end}}{total\ solution\ volume_{initial} - liquid\ volume_{initial}} \times 100 \quad \text{Eq. 9}$$

2.8 Statistical Analysis

Analysis of variance (ANOVA) was determined using RStudio software version 1.1.463 for Mac (RStudio, Inc., Boston, MA, USA). Tukey-Kramer Honest Significant Difference (HSD) multiple means comparison test was used to determine significant differences ($P \leq 0.05$) between the means ($n = 3$) of at least three different samples. A student's unpaired t-test was used to test for significant differences ($P \leq 0.05$) between the means ($n = 3$) of two different samples. ANOVA tables can be found in Appendix J (Tables 7-41).

Chapter 3: Results and Discussion

3.1 Optimization of Pea Protein Extraction Following Alkaline Solubilization Coupled with Isoelectric Precipitation

Solubilization pH, isoelectric precipitation pH, solubilization duration, number of solubilizations, and use of dialysis were each evaluated, in turn. With all other conditions constant, the solubilization pH was set at either pH 7, 7.5, or 8. PPI extracted at pH 7 had the lowest protein yield compared to PPI samples extracted at pH 7.5 and 8, and significantly higher protein content and protein residue in the discarded pellet fraction (Table 1), demonstrating that pH 7 was least effective in extracting proteins from pea flour. Solubilizing the protein at pH 8, on the other hand, resulted in significantly higher PPI protein yield compared to PPI samples extracted at pH 7 or 7.5. It is known that higher extraction pH results in higher protein yield, as proteins carry more negative surface charge under higher alkaline pH (Hoang, 2012). Although no significant difference was observed for protein purity, it tends to decrease with high pH, because the higher pH can also coextract other non-protein constituents such as polysaccharides (Hoang, 2012; Reinkensmeier *et al.*, 2015; Feyzi *et al.*, 2018). Additionally, with higher extraction pH, the functionality of the protein can be impaired due to protein denaturation (Lee *et al.*, 2007). Therefore, pH 7.5 was selected as the optimal solubilization pH, because it resulted in acceptable PPI protein purity (> 85% protein) and yield, and potentially could have a lower impact on the protein.

Next, the isoelectric precipitation pH was evaluated. At the isoelectric point, a protein has a net charge of zero, leading to a loss of interaction with water and enhanced hydrophobic protein-protein interactions that cause the protein to precipitate out of solution (Murray *et al.*, 1981; Damodaran, 2017). Because different proteins have different amino acid compositions and structural conformations, they differ in surface charge and consequently differ in their isoelectric point. Globulin proteins commonly have an isoelectric point of between pH 4 and 5 (Gueguen and Cerletti, 1994; Taherian *et al.*, 2012; Lu *et al.*, 2019).

Though there were no significant differences in the final PPI protein purity or yield, the supernatant fraction that was discarded after protein precipitation at pH 5 had a significantly higher protein content and % protein lost than the supernatant discarded after protein precipitation at pH 4.5 (Table 1). More proteins remained soluble at pH 5 than at pH 4.5, indicating that pH 4.5 was the isoelectric point for a larger portion of proteins. pH 4.5, therefore, was selected as the preferred pH for isoelectric precipitation. This finding aligns with previously reported pea protein precipitation pH used during alkaline based extraction (Sumner *et al.*, 1981; Koyoro and Powers, 1987; Tian *et al.*, 1999; Stone *et al.*, 2015; Tulbek *et al.*, 2016).

With regard to solubilization duration, there was a slight decrease in PPI protein yield coupled with a slight increase in protein purity, both not statistically significant, when solubilized for one hour versus two (Table 1). This observation, although not statistically significant, was expected. Longer solubilization periods increase protein yield at the cost of protein purity. While more proteins can be extracted with the longer time, other non-protein constituents are also coextracted (Feyzi *et al.*, 2018). Similar to our finding, Petersen et al. (2020) observed no effects of increasing solubilization time, beyond one hour, on pea protein yield. Similarly, Hoang (2012) concluded that solubilization pH has a greater impact on pea protein yield than solubilization duration of 15, 30, or 45 minutes. Pea flour, however, was solubilized at pH 10, where the proteins have higher solubility and are more readily extracted than solubilizing at pH 7.5, the chosen pH for the present study. Solubilizing at pH 10 would cause complete protein denaturation and potential polymerization, leading to reduced functionality. A milder solubilization pH along with a longer solubilization duration of one hour, therefore, were chosen to ensure acceptable protein yield, while maintaining the protein's structural integrity. One hour of solubilization was selected as optimal since no statistical differences were noted compared to two hours of solubilization. Additionally, one hour of solubilization is more industry feasible compared to two hours.

The benefit of double solubilization was also investigated. A second solubilization could result in the extraction of additional protein from the first pellet fraction, leading to increased protein yield (Deak *et al.*, 2008). In this study, the double solubilization technique was indeed effective in extracting more protein from the first pellet, which ultimately led to increased protein yield of the final PPI (Table 1). There was a significant decrease in % residual protein in the discarded pellet fraction of 10 percent, corresponding to a significant increase in protein yield of the PPI fraction. Other benchtop pea protein extractions prepared by alkaline solubilization with isoelectric precipitation report PPI protein yields of 60-70 percent, so this method with double solubilization would be in line with or superior to what has been observed for this type of protein isolation (Gueguen, 1983; Owusu-Ansah and McCurdy, 1991; Gueguen and Cerletti, 1994; Boye *et al.*, 2010b; Stone *et al.*, 2015). Furthermore, the high yields seen using the double solubilization method were achieved using nondenaturing pH conditions.

Finally, the use of dialysis prior to drying the protein extract was evaluated. Dialysis was tested as a measure to further purify the protein by removing small sugars and salts produced upon pH adjustments. Dialysis caused a decrease in the protein yield due the additional transfer step and incomplete recovery of the protein from the dialysis tubing. However, though the differences were not significant, the dialyzed PPI had 3% higher protein purity and a slightly lower ash content than its non-dialyzed counterpart (Table 1). There are no known reports comparing the effect of dialysis on pea protein extraction. Most researchers do not include dialysis of the final PPI, as it is fairly time-consuming. However, PPI with high protein purity is desirable from a nutritional standpoint and may demonstrate superior functional properties due to the reduced content of other interfering components. Therefore, the use of dialysis was adopted.

Based on these findings, the final optimized extraction conditions were double one-hour solubilization of the pea flour at pH 7.5, protein precipitation at pH 4.5, and the use of dialysis. The final optimized pH-PPI had a protein content of 87.6% and yield of 64.7% (Table 1). This outcome is comparable and in some

instances superior to what has previously been reported for PPI extracted by alkaline solubilization with isoelectric precipitation (Sumner *et al.*, 1981; Gueguen, 1983; Owusu-Ansah and McCurdy, 1991; Boye *et al.*, 2010b; Reinkensmeier *et al.*, 2015).

Furthermore, compared to conditions reported for previous extractions, the conditions used for the optimized extraction in this study were mild and not expected to cause protein denaturation. Previously reported pea protein extraction conditions included a solubilization pH of 8-10 most commonly, which may cause protein denaturation and polymerization, as well as oxidation that may lead to browning, off-flavor, and further protein polymerization (Sumner *et al.*, 1981; O'Kane *et al.*, 2004a; Makri *et al.*, 2005; Shand *et al.*, 2007; Boye *et al.*, 2010b; Barac *et al.*, 2012). Increasing the solubilization pH from 8.5 to 9.5 during pea protein extraction resulted in an increase in protein denaturation, with consequent adverse effects on solubility, and the highest content of off-flavor compounds (Gao *et al.*, 2020). Oxidation, which occurs at higher alkalinity, results in production of off-flavors, which is a known problem for pea protein (Owusu-Ansah and McCurdy, 1991; Tulbek *et al.*, 2016). Similarly, increasing the solubilization pH from 8 to 9.5 during lentil protein extraction increased protein denaturation, resulting in decreased functionality (Lee *et al.*, 2007). Along with a mild solubilization pH, the optimized pH-extraction achieved high protein yields without utilizing heat or reducing agents, which would alter the protein's native structure. In addition to selecting nondenaturing extraction conditions, the optimized conditions were selected to be feasible for scaled-up production.

Table 1. Protein extraction purities (%) and yields (%) of the PPI, pellet, and supernatant fractions from pH extractions under different extraction conditions, as well as ash content (%) of each PPI.

Extraction Treatment					**PPI**[1]			**Discarded Pellet**[2]		**Discarded Supernatant**[3]	
Solubilization pH	Precipitation pH	Solubilization Duration (hr)	Number of Solubilizations	Dialysis of PPI	Protein Purity (%)	Protein Yield (%)	Ash (%)	Protein Purity (%)	Protein Residue (%)	Protein Purity (%)	Protein Lost (%)
7	4.5	1	1	No	89.7^{a}	56.3^{eB}	5.11^{a}	8.08^{aA}	22.1^{aA}	29.5^{b}	18.7^{d}
8	4.5	1	1	No	86.5^{ab}	60.9^{cA}	5.02^{a}	6.76^{abB}	18.9^{aB}	29.5^{b}	19.7^{cd}
7.5	4.5	1	1	No	88.3^{ab}	58.1^{cdeB}	5.03^{a}	6.31^{abcB}	18.2^{aB}	28.7^{bc}	19.3^{cd}
7.5	5	1	1	No	89.4^{a}	57.5^{de}	$4.24^{b\wedge}$	5.82^{bc}	17.1^{a}	$31.5^{a\wedge}$	$23.4^{a\wedge}$
7.5	4.5	2	1	No	85.0^{ab}	60.5^{cd}	5.07^{a}	5.66^{bc}	16.6^{ab}	27.7^{bc}	20.1^{c}
7.5	4.5	1	2	No	84.5^{b}	$69.9^{a\dagger}$	5.19^{a}	$3.00^{d\dagger}$	$8.11^{c\dagger}$	$27.4^{c\dagger}$	$21.8^{b\dagger}$
7.5	4.5	1	2	Yes	87.6^{ab}	64.7^{b*}	4.96^{a}	4.30^{cd}	11.2^{bc}	28.0^{bc}	21.6^{b}

[1]Pea protein isolate. [2]Pellet discarded after alkaline solubilization. [3]Supernatant discarded after isoelectric precipitation. Protein yield (%) represents the amount of protein extracted relative to the total amount of protein in the starting pea flour; Protein residue (%) represents the amount of protein left in the discarded pellet relative to the total amount of protein in the starting pea flour; Protein lost (%) represents the amount of protein lost to the discarded supernatant relative to the total amount of protein in the starting pea flour. Means (n = 3) in each column with different lowercase letters indicate significant differences across extraction treatments according to the Tukey-Kramer multiple means comparison test (*$P < 0.05$*). Means with different capital letters indicate significant differences among different solubilization pHs, according to the Tukey-Kramer multiple means comparison test (*$P < 0.05$*). Means with no capital letters indicate no significant differences. A cross of Lorraine (†) designates a significant difference among corresponding samples solubilized once or twice, an asterisk (*) designates a significant difference among a corresponding dialyzed and non-dialyzed sample, while a carrot (^) designates a significant difference among corresponding samples precipitated at pH 4.5 and 5, as tested by the student's two-sample unpaired t-test ($P < 0.05$). There were no significant differences found between corresponding samples solubilized one versus two hours, as tested by the student's two-sample unpaired t-test ($P < 0.05$).

3.2 Optimization of Pea Protein Extraction Following Salt Solubilization Coupled with Membrane Filtration

Salt extraction of plant proteins is less researched than alkaline extraction and is not currently used in industry for the production of pea protein isolate. A more novel approach to salt extraction rather than the traditional “salting in, salting out” method was investigated. Membrane filtration was used in place of “salting out”. “Salting out” requires the use of excessive salt that will need to be removed by several water washes, resulting in large amounts of waste, or water that needs extensive purification to be reused. Utilization of large amounts of water and production of a lot of waste is not industrially efficient.

The utilization of membrane ultrafiltration (UF) compared to dialysis, separately and in combination, were evaluated in terms of protein purity, yield, and ash content. The initial solubilization step was the same for all three purification treatments, thus the protein content and % protein residue of the pellet were not impacted (Table 2).

Ultrafiltration alone, using the benchtop membrane system, resulted in the lowest protein purity and highest ash content, demonstrating that it was not sufficient to completely remove all salt from the proteinaceous supernatant. Dialysis was significantly more effective in increasing protein purity and decreasing ash content, though the ash content was still relatively high, indicating that not all salt was removed (Table 2). The combination of UF and dialysis had significantly the highest PPI protein purity and simultaneously the lowest ash content of the three purification treatments (Table 2), thus was selected as the optimal treatment. This outcome could translate to the possible use of ultrafiltration coupled with diafiltration on a pilot or industrial scale.

Table 2. Pea protein extraction purities (%), yields (%), and ash (%) of fractions from salt extractions testing ultrafiltration and dialysis.

Purification Treatment		PPI[1]			Discarded Pellet[2]	
Ultrafiltration	Dialysis	Protein Purity (%)	Protein Yield (%)	Ash (%)	Protein Purity (%)	Protein Residue (%)
Yes	No	67.9[c]	76.1[a]	11.4[a]	7.97[a]	25.2[a]
No	Yes	86.9[b]	69.7[b]	7.19[b]	7.98[a]	25.3[a]
Yes	Yes	92.8[a^*]	72.0[ab*]	1.56[c^*]	7.70[a]	24.2[a]

[1]Pea protein isolate. [2]Pellet discarded after salt solubilization. Protein yield (%) represents the amount of protein extracted relative to the total amount of protein in the starting pea flour; Protein residue (%) represents the amount of protein left in the discarded pellet relative to the total amount of protein in the starting pea flour. Means (n = 3) in each column with different lowercase letters indicate significant differences according to the Tukey-Kramer multiple means comparison test ($P < 0.05$). A carrot (^) designates a significant difference among corresponding samples with and without ultrafiltration, while an asterisk (*) designates a significant difference among a corresponding dialyzed and non-dialyzed sample, as tested by the student's two-sample unpaired t-test ($P < 0.05$).

To the best of our knowledge, very limited work has been done on the use of solubilization in dilute NaCl, followed by UF, to produce PPI. Most studies using salt extraction recover the PPI by "salting out", which is not industry feasible. Other studies report micellar precipitation (also called "hydrophobic out"), in which the proteinaceous supernatant from "salting in" gets diluted with cold water, leading to reduced protein solubility and increased hydrophobic interactions between proteins, resulting in protein precipitation (Murray *et al.*, 1981; Owusu-Ansah and McCurdy, 1991). Ultrafiltration has been used following an alkaline or acidic solubilization step, or as further purification after isoelectric precipitation, but is not well characterized as a protein concentration step following salt solubilization. Currently, there is one known report of pea protein salt extraction coupled with ultrafiltration, though they did not test different conditions for purification and used a higher MWCO membrane (50 kDa) for UF (Tian *et al.*, 1999), which could potentially lead to loss in protein components of low molecular weight.

Furthermore, there are no known reports of salt extraction with UF and dialysis, making the optimized salt extraction a novel method.

Salt extraction is uncommon due to being not feasible for large scale production. However, our work showed that utilizing membrane filtration can make the salt extraction feasible for industry. By using membrane filtration to purify and concentrate proteins in place of "salting out" proteins, there is less waste due to reduced use of salt and water. Additionally, membrane filtration is already utilized on a large scale in the dairy industry to produce whey protein concentrates and isolates. Nevertheless, potential differences between benchtop extractions and industrial extractions must be investigated in order to promote the optimized benchtop extractions for widespread use.

3.3 Scaled-Up Production of PPI Following Optimized Extraction/Purification Conditions

Scaled-up production of PPI was performed according to the optimized benchtop extraction/purification conditions as closely as possible, with some unavoidable differences explained previously (Section 2.5). Specifically, the SU production involved different centrifuges, had overnight solubilization periods, used diafiltration in place of dialysis, included pasteurization and homogenization, and utilized spray drying instead of lyophilization. Thermal pasteurization and spray drying are suspected to be the most impactful differences, as they may have caused some protein denaturation that would not have occurred on the benchtop scale. However, the SU production achieved similar PPI purity to the benchtop counterparts. SU-pH PPI had a protein purity of 88.7%, while SU-salt PPI had a protein purity of 92.4%. Ash content of the SU-salt PPI (1.66%) was comparable to the benchtop salt-PPI (1.56%), while ash content of the SU-pH PPI (2.94%) was significantly ($P < 0.05$) lower than the benchtop pH-PPI (4.96%).

Yields were not determined for the SU PPIs, for reasons mentioned in Section 2.5. First, throughout the scaled-up production, samples (about 3 lbs, or 0.4 gallons, per sample) were pulled at several steps for a separate research

project assessing flavor development throughout the extraction process. Together, these samples pulled 10-12 lbs (1.2-1.4 gallons) of solution from each extraction that otherwise would have contributed to higher protein yield. Specifically, samples pulled from steps after membrane filtration would have removed highly concentrated protein solution, decreasing the amount of protein recovered in the final PPI. In normal production, these samples would not be pulled.

Additionally, as described in Section 2.5.1, after membrane filtration, only the high solids retentate was used for the remainder of the extraction, while the low solids retentate produced from flushing the membrane with DI water was discarded. This choice was made in order to avoid the thermal concentration step that would have been needed to increase % total solids prior to spray drying. Otherwise, the spray dried powder could be very fine and fluffy. The process for concentrating the protein solution would be to pass it through an evaporator, which would subject the protein solution to thermal treatment of 60-70°C for 30-60 minutes. Such thermal treatment would cause protein denaturation (Damodaran, 2017). Avoiding such treatment will allow better comparison between benchtop PPIs and SU PPIs. However, by choosing to continue only with the high solids retentate, protein was lost in the low solids retentate, decreasing overall yield. Future work should investigate how the structural and functional characteristics of the low solids retentate proteins that undergo thermal concentration compare to those of the high solids retentate. If differences are minimal, there is potential to combine both streams to increase process yields without majorly impacting final PPI quality.

In a similar study researching pilot plant production of PPI using membrane filtration to concentrate proteins following salt extraction, Tian et al. (1999) also had concerns over diluting protein solution to below 8-10% solids, as lower %TS would not be suitable for spray drying, leading them to use fewer washes during diafiltration. The authors reported lower final PPI protein purity (81.1%) but higher protein yield (40%) than were obtained in the present study (92.4% purity and 27.6% yield) for salt extraction with UF. It is unknown how/if they recovered the

low solids retentate from the membrane. Additional washes may have increased their protein purity by removing additional salts and small sugars but could also have decreased yields. The authors, however, did not investigate protein denaturation or functionality.

One group performing a scaled-up PPI production by alkaline solubilization with isoelectric precipitation saw final protein yields of 60% with protein purity of 90% (Gueguen, 1983). The research group solubilized pea flour at pH 9 (1:10 w/v) for 20 minutes. Soluble proteins were separated from insolubles using a decanter centrifuge followed by a clarifying centrifuge. The supernatant was then adjusted to pH 4.5 and passed through the clarifying centrifuge to precipitate proteins, followed by a wash and additional centrifugation. The protein isolate was then neutralized and spray dried. They did not perform ultrafiltration/diafiltration as a final step, so did not have separation of the high solids and low solids retentates that decreased the yield in the present study. Still, a similar protein purity was achieved compared to our SU-pH PPI utilizing similar equipment and processing. Based on this reported observation, it is possible that process yields of our SU-pH PPI could have been close to our benchtop yields, with complete recovery of protein from the UF unit.

Sumner et al. (1981) also performed pilot plant production of PPI by alkaline solubilization with isoelectric precipitation, using a double solubilization method as was done for the present study. Pea flour was solubilized at pH 9 (1:5 w/v) for 50 minutes and centrifuged. The pellet was resolubilized in 100 lbs of tap water at pH 9 for 50 minutes and centrifuged. The two supernatants were combined, clarified, adjusted to pH 4.5, and centrifuged. The precipitate was resuspended at pH 9 (time not specified) and reprecipitated by adjusting to pH 4.5 and centrifuging again. The precipitate was solubilized in 50 lbs of tap water and adjusted to pH 7 before spray drying. The protein purity of the final product was 83%, with 54% protein yield for the extraction process, slightly lower but similar to the values they obtained on a lab scale (91% protein purity and 59% protein yield). In the present study, similar protein purity was achieved for both benchtop and SU pH-extractions (87.6% and

88.7%, respectively). The work by Sumner et al. suggests that with complete protein recovery following UF, the yield of the optimized SU-pH PPI production should be comparable to or slightly lower than that of the benchtop pH-extraction (~65%). Accordingly, our SU-pH PPI yield would have been equivalent to that of the benchtop production, if there were no samples pulled for other analyses and if all streams were collected. If this speculation proves true, the SU-pH extraction would have comparable, if not better, PPI protein purity and yield compared to other reports of pilot plant pea protein extractions following alkaline extraction with isoelectric precipitation (Sumner *et al.*, 1981; Gueguen, 1983).

Currently, the only process known to be used in industrial production of pea protein is alkaline extraction with isoelectric precipitation. This may be because of concerns about scalability of ultrafiltration/diafiltration processes, namely that it is believed to be more costly to operate. However, in an economic evaluation comparing alkaline extraction with isoelectric precipitation to membrane isolation for soy protein isolates, there were no significant differences between the two processes in terms of costs (Hensley and Lawhon, 1979). Additionally, a research group performed alkaline extraction of faba beans followed by either isoelectric precipitation or UF and found no differences in operating costs and less denaturation of UF protein compared to isoelectric precipitated protein (Olsen, 1978). Therefore, our optimized salt-extraction method should be viewed as feasible for industry, especially if it produces a unique product that has structural and functional advantages over the traditional pH-extraction method.

3.4 Characterization of Structural Properties

3.4.1 Differences in Protein Profile Based on Extraction Method and Scale

SDS-PAGE reveals the presence of disulfide linkages, protein polymerization, and the relative distributions of the protein subunits. The SDS breaks all noncovalent bonds and gives the proteins a negative charge so proteins migrate down the gel based on size, while the reducing agent breaks disulfide linkages, revealing such linkages among subunits and polymers. The protein

profile of benchtop pH- and salt- PPIs, SU-pH and SU-salt PPIs, and commercially available plant protein isolates (cPPI and cSPI) were evaluated by SDS-PAGE under reducing and nonreducing conditions (Figure 3, A and B).

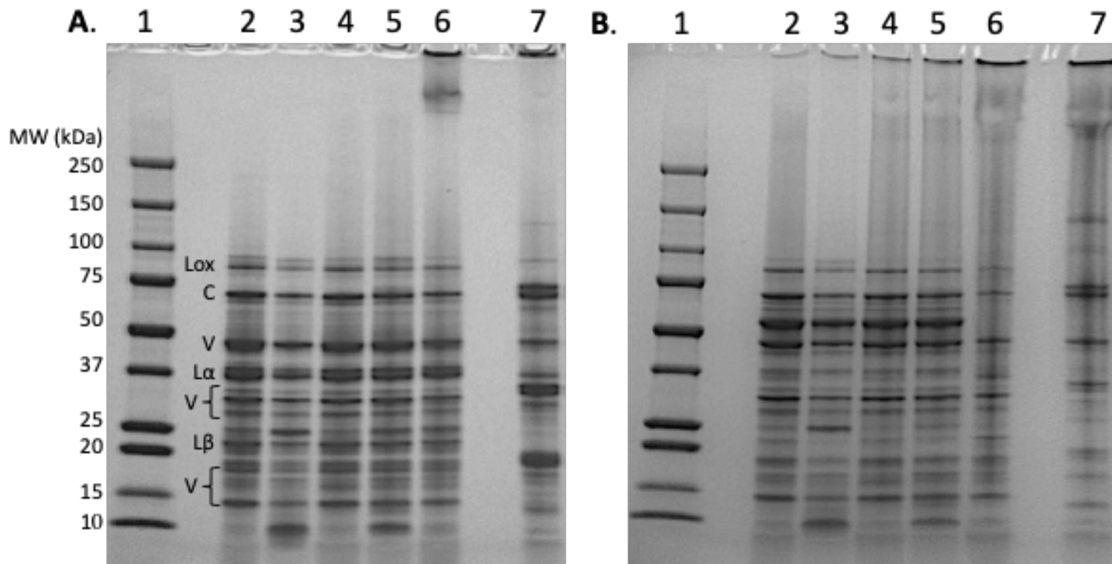

Figure 3. SDS-PAGE gel visualization of the protein profiles of protein isolate samples under reducing (A) and nonreducing (B) conditions. Lane 1: molecular weight (MW) marker; Lane 2: pH-PPI; Lane 3: salt-PPI; Lane 4: SU-pH PPI; Lane 5: SU-salt PPI; Lane 6: cPPI; Lane 7: cSPI. Lox: lipoxygenase; C: convicilin; V: vicilin; Lα: acidic subunit of legumin; Lβ: basic subunit of legumin.

The protein profiles of benchtop and SU PPI extractions were very similar. The most notable difference was between the benchtop salt-PPI and SU-salt PPI under nonreducing conditions (Figure 3B, lanes 3 and 5). The top of the lane for SU-salt PPI appears slightly smeared compared to that of the benchtop salt-PPI, indicating the presence of larger protein aggregates. The SU-pH PPI and pH-PPI both had smearing under nonreducing conditions (Figure 3B), indicating the presence of some protein aggregates, however, the smearing was more visible for the SU-pH PPI compared to the benchtop pH-PPI. Both SU PPIs showed relatively more smearing and protein bands >250 kDa that did not migrate down the gel under nonreducing conditions (Figure 3B), representing polymerization. The polymerized protein bands were not apparent with addition of reducing agent (Figure 3A), indicating that they were formed primarily through disulfide linkages.

All extracted PPIs contained bands identified as lipoxygenase (94 kDa) and convicilin (72 kDa), as has been seen previously in pea protein (Crévieu *et al.*, 1996; Barac *et al.*, 2006; Tzitzikas *et al.*, 2006; Barac *et al.*, 2012; Mession *et al.*, 2013). Additionally, there was a prominent band close to 50 kDa representing the unmodified vicilin (Boulter, 1983; Gueguen and Cerletti, 1994). Vicilin undergoes extensive post-translational modification, resulting in a range of lower MW vicilin proteins. As has been previously reported, bands between 13-19 kDa and 30-35 kDa were identified as modified vicilin (Gatehouse *et al.*, 1982; Gueguen and Cerletti, 1994; Crévieu *et al.*, 1996; Tzitzikas *et al.*, 2006; Barac *et al.*, 2012; Mession *et al.*, 2013). Bands close to 50 kDa and 33 kDa were most prominent, in agreement with other reports (Gatehouse *et al.*, 1981). Convicilin and vicilin do not contain disulfide bonds, so their banding was identical under reducing and nonreducing conditions (Figure 3, A and B).

However, by comparing banding under reducing and nonreducing conditions, it is apparent that the band around 60 kDa in the PPIs under the nonreducing conditions was not present under reducing conditions (Figure 3, A and B). This band represents the legumin monomer, comprised of an acidic and basic subunit linked by a disulfide bond. Under reducing conditions, the covalent disulfide bond is cleaved, resulting instead in the protein bands observed around 40 kDa and 20 kDa (Figure 3A), representing the acidic and basic legumin subunits, respectively (Crévieu *et al.*, 1996; Tzitzikas *et al.*, 2006; Barac *et al.*, 2012). As expected, this observation was similar for benchtop PPIs as well as SU PPIs.

The 60 kDa band appears to be largely absent from cPPI under nonreducing conditions (Figure 3B). However, under reducing conditions, bands around 40 and 20 kDa were present, similar to the other PPIs. This observation indicates that the legumin in cPPI had undergone polymerization via disulfide linkages, as confirmed by the presence of the dark bands at the top of the gel, as well as prominent smearing under nonreducing conditions (Figure 3B). Mession et al. (2013) reported a similar observation when comparing native PPI with PPI

subjected to thermal treatment (heating from 40°C to 90°C and incubating at 90°C for 1 hour), which they called aggregated pea protein (APP). Under nonreducing conditions, their native PPI samples had no aggregation and showed the legumin monomer band at 56 kDa, whereas APP had large MW (>200 kDa) bands of aggregated protein, which were not present under reducing conditions, similarly concluding that the aggregation involved disulfide linkages (Mession *et al.*, 2013). This phenomenon was also seen for isolated legumin subjected to thermal treatment. Legumin subjected to several thermal treatments around its onset denaturation temperature (70-85°C) showed decreasing band intensity at 60 kDa with apparent polymers of >200 kDa formed by both covalent and noncovalent bonding (Mession *et al.*, 2015). These findings suggest that cPPI was subjected to extensive thermal treatment that denatured legumin and caused polymerization involving disulfide bonding.

Under reducing conditions, cPPI still had polymerized proteins, though the legumin subunits were visible on the gel. While the reducing agent was able to cleave disulfide bonds and free the legumin subunits, it appears that some polymerization involved other covalent linkages, such as those induced by the Maillard reaction. Similarly, Marcone et al. (1998) saw polymerization of legume globulins that was determined to be partially due to disulfide bonding along with other covalent bonding. In the presence of reducing sugars in PPI, extensive thermal treatment can result in covalent linkages by the Maillard reaction, resulting in large polymers (Phillips and Williams, 2011; Damodaran, 2017). Therefore, it was concluded that cPPI was most likely subjected to excessive heat treatment during processing that caused polymerization involving the cysteine residues of legumin as well as other irreversible covalent bonding.

Similar to cPPI, cSPI had no apparent band at 60 kDa for the legumin (glycinin) monomer under nonreducing conditions. Instead, there was extensive polymerization, which involved the glycinin monomers. Under reducing conditions, it was apparent that glycinin subunits are present at relatively higher intensity than legumin counterparts in PPI. This observation indicates that the legumin:vicilin

ratio is higher in SPI than in PPI. Additionally, the molecular weights of the glycinin subunits of SPI are different than those of the legumin subunits in PPI. This observations agrees with reports from literature of SPI's glycinin acidic subunits being anywhere from 31 to 51 kDa, while the basic subunit is typically reported to be 17 to 24 kDa (Gueguen and Cerletti, 1994; L'Hocine and Boye, 2007; Mession *et al.*, 2015). As with cPPI, even under reducing conditions, cSPI had protein polymerization, indicating that polymerization was not only due to disulfide linkages.

In addition to the bands for vicilin, convicilin, and legumin, the salt-extracted PPIs both had additional bands around 9 and 25 kDa that were absent or less intense in the pH-extracted PPIs under both reducing and nonreducing conditions. This observation indicated that the 9 and 25 kDa proteins have an isoelectric point further from pH 4.5, allowing them to remain soluble during the isoelectric precipitation step of the pH extraction. These proteins are likely albumins. Albumins in pea protein have been reported to have an isoelectric point of around pH 5.5-6, so they would not be precipitated with the globulin proteins in the pH-extraction (Croy *et al.*, 1984; Swanson, 1990; Makri *et al.*, 2005). There are many different albumins in pea protein, but by far the two most common are PA_1 and PA_2, with molecular weights reported to be 6-8 kDa and 22-26 kDa, respectively (Schroeder, 1982; Gueguen and Cerletti, 1994; Crévieu *et al.*, 1996). Therefore, these two bands in the salt-PPI and SU-salt PPI were concluded to be the PA_1 and PA_2 albumins. Albumins have different structural properties than globulins, so their presence in the salt-extracted PPIs may cause some differences in functionality.

3.4.2 Protein Denaturation as Impacted by Extraction Method and Scale

Conditions during protein extraction and processing can cause partial or complete denaturation of proteins. Denaturation of the protein often results in unfolding and exposing reactive groups (i.e. hydrophobic groups, sulfhydryl groups) that lead to polymerization during further processing and storage. Denaturation state, as measured by DSC, was assessed to determine the impact

of different extraction protocols (pH- vs. salt- extraction) and scale. The benchtop and scaled-up PPIs were also compared against reference proteins, cPPI, cSPI, and nSPI. During DSC measurement, proteins are heated gradually to temperatures above their denaturation points in order to provide enough energy to cause denaturation and unraveling of protein subunits, leading to a release of heat that is seen as an endothermic peak. Absence of endothermic peaks indicates that the protein is already denatured, as no energy gets released because the protein structure is already unraveled.

Pea protein contains three main protein groups: vicilin, convicilin, and legumin. Only two endothermic peaks were observed, attributed to vicilin and legumin (Table 3). It is likely that the endothermic peak for convicilin overlapped with that of vicilin, as they are structurally similar, and therefore may denature at similar temperatures. In fact, isolated vicilin and convicilin fractions were reported to have the same denaturation temperatures (O'Kane *et al.*, 2004b). Moreover, convicilin represents a much smaller portion of total pea proteins (4-8%) than vicilin (up to 52%), thus the majority of the enthalpy of denaturation for the first peak is attributed to vicilin (Tzitzikas *et al.*, 2006). Furthermore, studies commonly report a single endothermic peak around 80-86°C for pea protein isolate due to poor separation of the vicilin and legumin peaks (Cserhalmi *et al.*, 1998; Shand *et al.*, 2007; Sun and Arntfield, 2010).

Comparing benchtop isolates, the salt-PPI had slightly higher denaturation temperatures and enthalpies of denaturation for both the vicilin and legumin compared to the pH-PPI (Table 3). This observation indicates that the salt-extraction was less denaturing than the pH-extraction. At specific concentrations, salt is thought to have a protective effect on proteins against denaturation by adding surface charges, thus increasing interaction with water and resisting unfolding, therefore leading to the higher shift in denaturation temperature and enthalpy (Kinsella, 1979; Hermansson, 1986; Shand *et al.*, 2007; Mession *et al.*, 2013). Indeed, Sun and Arntfield (2010) saw significantly higher denaturation

temperatures and enthalpies of denaturation for PPI dispersed in 0.3 M NaCl for DSC testing compared to a control PPI dispersed in water.

Table 3. Denaturation temperatures and enthalpy values for protein isolate samples.

Plant Protein Isolate	**Denaturation Temperature (°C)**	**Enthalpy of Denaturation (J/g protein)**	**Denaturation Temperature (°C)**	**Enthalpy of Denaturation (J/g protein)**
	vicilin		*legumin*	
cPPI	*	*	*	*
pH-PPI	83.3^{b}	6.21^{a}	91.6^{ab}	0.81^{b}
salt-PPI	88.5^{a}	6.82^{a}	93.5^{a}	1.54^{a}
SU-pH PPI	81.9^{c}	3.69^{b}	90.0^{b}	0.52^{b}
SU-salt PPI	82.4^{c}	4.15^{b}	90.2^{b}	0.47^{b}
	β-*conglycinin*		*glycinin*	
nSPI	73.4	1.51	90.4	6.41
cSPI	*	*	*	*

*No peak of denaturation observed. Means (n = 3) in each column with different lowercase letters indicate significant differences among pea protein samples, according to the Tukey-Kramer multiple means comparison test ($P < 0.05$).

Both vicilin and legumin in benchtop extracted PPIs had higher denaturation temperatures and enthalpies than the scaled-up PPIs (Table 3). This observation indicates that the scaling-up process resulted in partial denaturation of the proteins. Unlike benchtop extractions, in both the scaled-up pH- and salt-extractions, the protein extract solutions were pasteurized and spray dried. Both processes introduce heat that will cause some level of denaturation. A study comparing a native, lyophilized SPI with SPI subjected to pasteurization and spray drying reported that the pasteurized, spray dried SPI was partially denatured (Elmore *et al.*, 2007).

While the scaled-up PPIs may have been partially denatured compared to the benchtop PPIs, both SU PPIs were less denatured than the commercially available plant proteins (cPPI and cSPI). Neither cPPI nor cSPI showed any endothermic peaks, indicating the vicilin and legumin proteins were already completely denatured. This finding supports SDS-PAGE results, which showed that both commercial proteins had irreversible polymerization, due to aggregation of denatured proteins (Figure 3), as was also reported by other researchers (Lee *et al.*, 2003; Shand *et al.*, 2007). While the exact extraction and processing conditions of the commercial proteins are unknown, commercially available plant proteins would also go through pasteurization and spray drying steps. Both the SU PPIs and commercial plant protein isolates had these steps in common, which suggests that other steps or differences in certain steps of the commercial protein extractions were most likely responsible for the protein denaturation. These differences may include higher solubilization pH, overnight storage at the isoelectric point, thermal concentration step, and/or different spray drying conditions.

Because the cSPI was completely denatured, native SPI (nSPI) was also analyzed in order to compare the PPIs against SPI. Two endothermic peaks were seen for nSPI, identified by denaturation temperatures to be β-conglycinin (73.4°C) and glycinin (90.4°C) (Table 3). These denaturation temperatures were in line with other reported values for native SPI, 70-76°C for β-conglycinin and 86-92°C for glycinin (Hermansson, 1979; Wagner *et al.*, 1996; Rickert *et al.*, 2004; Shand *et al.*, 2007). Complementary to the SDS-PAGE observation (Figure 3), the PPIs contained a higher proportion of vicilin proteins than nSPI, indicated by the higher peak enthalpy of vicilin in PPI compared to β-conglycinin in nSPI. On the flip side, PPIs contained less legumin than glycinin in nSPI. This observation (Table 3) along with SDS-PAGE observation (Figure 3) support previous findings that the dominant protein type in PPI is vicilin, while the dominant protein in SPI is legumin (glycinin) (Tzitzikas *et al.*, 2006; Taherian *et al.*, 2011). The differences in protein

compositions as well degree of denaturation will help explain other differences in structure and consequently functionality.

3.4.3 Impact of Extraction Method and Scale on the Protein's Surface Charge

Zeta potential is a measure of surface charge, which influences solubility and reactivity of proteins. All tested proteins carried a net negative charge, as they were analyzed at pH 7, which is above their isoelectric point. The benchtop pH-PPI had the highest surface charge among all PPI samples, while the benchtop salt-PPI had the lowest surface charge (Figure 4). This is in agreement with previous reports of legume proteins produced following alkaline solubilization coupled with isoelectric precipitation having higher (more negative) surface charge than those extracted by salt (Karaca *et al.*, 2011). The observed differences in surface charges may be explained by the protein composition of the pH-PPI compared to that of the salt-PPI. The pH-PPI was produced by isoelectric precipitation, so the overall pI of pH-PPI is pH 4.5, where it carries no net charge. The salt-PPI, on the other hand, contains albumin proteins, as seen by SDS-PAGE (Figure 3). Albumins have been reported to have an isoelectric point around pH 5.5-6, higher than the isoelectric point of globulins around pH 4.5 (Croy *et al.*, 1984; Swanson, 1990; Makri *et al.*, 2005). Therefore, the overall pI of salt-PPI is likely higher than pH 4.5. Proteins carry more charge as they are farther from the isoelectric point, so it makes sense for the pH-PPI to be more negatively charged than the salt-PPI due to their different protein profiles and their respective isoelectric points.

The surface charge values for SU-pH PPI and SU-salt PPI were in between those of the benchtop pH- and salt-PPI. The differences could be in part attributed to the partial denaturation incurred during scale-up. Denaturation disrupts electrostatic interactions, hydrogen bonding, and hydrophobic interactions that stabilize the native globular protein structure, causing a change in exposed amino acids, which may affect overall surface charge (Foegeding and Davis, 2011). In the case of SU-pH PPI, the dominant effect appeared to be from increased

exposure of uncharged hydrophobic groups, causing a reduction in overall zeta potential compared to the benchtop pH-PPI. On the other hand, although it was also partially denatured, the SU-salt PPI had slightly higher surface charge than the benchtop salt-PPI. Although statistically significant, this difference could not be explained by the partial unfolding of the protein, but rather the difference in the protein subunits that get involved in polymerization. Polymerization, as noted by SDS-PAGE, involved partially the albumin subunit at 25 kDa (see lane 3 vs. 5 in Figure 3B). Albumins, as mentioned before, would have less surface charge at pH 7, thus polymerization involving albumin proteins may lead to the perceived higher net surface charge of SU-salt PPI compared to benchtop salt-PPI.

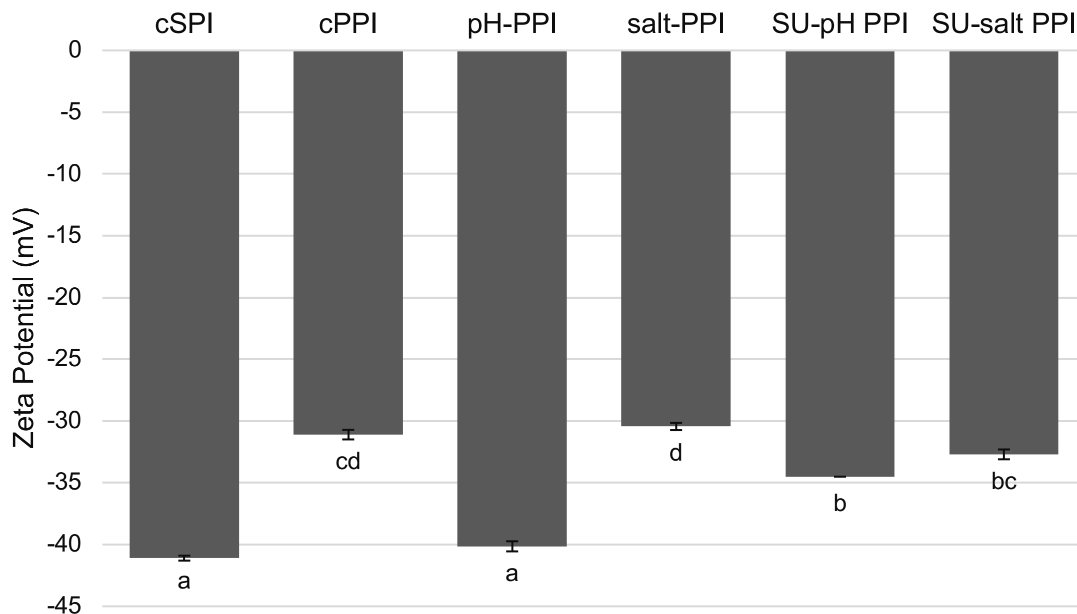

Figure 4. Zeta potential of protein isolate samples measured at pH 7. Error bars represent standard error (n = 3). Different lowercase letters below the bars indicate significant differences among the samples according to the Tukey-Kramer multiple means comparison test ($P < 0.05$).

cPPI had the lowest surface charge of most proteins measured, apart from salt-PPI. cPPI was completely denatured, as seen by DSC data (Table 3). The unraveling of the protein structure resulted in exposure of hydrophobic groups. Additionally, cPPI had irreversible protein aggregation and polymerization, as seen by SDS-PAGE (Figure 3). Although cSPI was similarly denatured and polymerized,

cSPI had higher (more negative) surface charge then most other proteins. SPI is known to have a relatively high amount of polar and charged amino acid residues on its surface, which seemed to be maintained even with denaturation (Lampart-Szczapa, 2001). There was no significant difference in net charge between the completely denatured cSPI and nSPI (nSPI = -39.2 mV, full data in Appendix K, Figure 11). This observation suggests that denaturation and subsequent polymerization may have varying impact on surface charge of soy protein compared to pea protein, probably attributed to differences in protein profile and content of legumin vs. vicilin-type proteins.

3.4.4 Impact of Extraction Method and Scale on the Protein's Surface Hydrophobicity

Proteins naturally contain patches of both hydrophilic and hydrophobic amino acids on their surfaces, although the relative amount varies between proteins and can be affected by processing. Typically, surface hydrophobicity tends to be lower for the native protein compared to the denatured counterpart, because most of the hydrophobic amino acids tend to orient toward the interior moiety of globular proteins, hiding from the aqueous environment. Therefore, high surface hydrophobicity indicates protein unfolding due to the denaturation of the native protein structure and exposure of previously hidden hydrophobic groups (Kato and Nakai, 1980; Alizadeh-Pasdar and Li-Chan, 2000).

Extraction method did not have a significant impact on surface hydrophobicity, while the extraction scale did (Figure 5). Although not statistically significant, the slightly lower surface hydrophobicity of salt-extracted PPI than that of pH-extracted PPI could be attributed to the presence of albumins. Albumins are known to be hydrophilic proteins with lower surface hydrophobicity than globulins (Papalamprou *et al.*, 2009; Karaca *et al.*, 2011). Therefore, their presence in the salt-extracted PPIs may have resulted in the slightly lower surface hydrophobicity. This observation is complementary to the DSC results (Table 3), where the impact

of extraction method was minimal on denaturation enthalpy, confirming that there was not a major difference in unfolding.

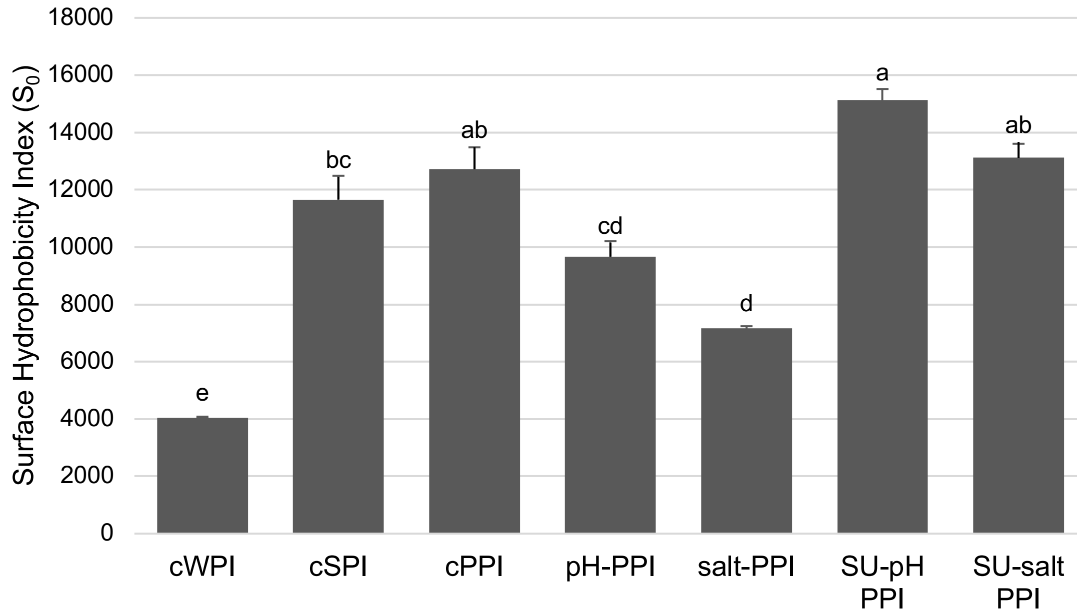

Figure 5. Surface hydrophobicity index of protein isolate samples measured at pH 7. Error bars represent standard error (n = 3). Different lowercase letters above the bars indicate significant differences among the samples according to the Tukey-Kramer multiple means comparison test (*P < 0.05).*

On the other hand, extraction scale significantly impacted surface hydrophobicity. The SU PPI samples had significantly higher surface hydrophobicity than their benchtop counterparts. DSC results revealed that the scaling-up process caused partial denaturation compared to benchtop production (Table 3). The benchtop PPIs maintained a more native globular protein structure that had significantly lower surface hydrophobicity.

There were no significant differences in surface hydrophobicity among cPPI and SU PPIs. Based on DSC results (Table 3), cPPI was completely denatured, while the SU PPIs were only partially denatured. Accordingly, it may have been anticipated that cPPI would have higher surface hydrophobicity than the SU PPIs. However, legumin protein subunits formed polymers, as noted by SDS-PAGE (Figure 3). Upon complete denaturation, surface hydrophobicity will reach a

maximum, however, once polymers are formed, it will be reduced (Wang and Ismail, 2012). Denatured proteins are attracted to each other via hydrophobic forces; once in close proximity, hydrophobic interactions occur and facilitate disulfide polymerization (Rickert *et al.*, 2004). While both the SU PPIs and cPPI had protein aggregates, protein aggregation was more extensive in cPPI (Figure 3). Higher degree of polymerization will have a detrimental impact on some functional properties, such as solubility, as will be discussed in later sections.

cSPI was also completely denatured (Table 3), had irreversible protein polymerization (Figure 3), and had similar surface hydrophobicity to cPPI. SPI, however, is unique in that it is known to have relatively high surface hydrophobicity along with fairly high surface charge (Lampart-Szczapa, 2001). The balance of surface charge and surface hydrophobicity affects how the protein interacts with its surrounding, thus, it impacts functional behavior such as solubility, gelation, and emulsification, as will be discussed in later sections.

3.5 Characterization of Functional Properties

3.5.1 Protein Solubility of the Different Isolates

Protein solubility is considered the most important functional property, as it is a prerequisite for most other functional properties (Singh *et al.*, 2008; Taherian *et al.*, 2012). Additionally, there is a large market for high protein performance beverages, valued at $1.8 billion in the U.S. in 2019 (Franz, 2020). Traditionally, WPI has been considered the “gold standard” for use in high protein beverages, as it is highly soluble (at 5-10% protein) even post-thermal treatment, at both neutral and acidic pH (Table 4). WPI maintains high solubility even near its isoelectric point when not thermally treated because its globular proteins have very low surface hydrophobicity, as shown in Figure 5 and supported by Wang and Ismail (2012). Plant proteins have a relatively high surface hydrophobicity (Figure 5), thus tend to have poor solubility compared to WPI, especially as the pH gets closer to the isoelectric point. Along with testing protein solubility at pH 7, solubility

was measured at pH 3.4 to indicate potential use of pea protein in acidic high protein beverages as a replacement for WPI.

Table 4. Percent protein solubility of isolates tested at 5% protein concentration, at pH 3.4 and 7, under not-heated and heated conditions (80°C for 30 minutes).

Protein Isolate	% Protein Solubility			
	pH 7		pH 3.4	
	Not-heated	Heated at 80°C	Not-heated	Heated at 80°C
cWPI	99.7^{a}	99.7^{a}	99.4^{a}	100.0^{a}
cSPI	79.7^{d}	86.9^{b*}	23.7^{f}	25.9^{d}
cPPI	22.2^{f}	39.0^{d*}	8.9^{g}	20.4^{d*}
pH-PPI	87.4^{b}	85.7^{b}	43.6^{e}	65.4^{c*}
salt-PPI	84.1^{c}	72.0^{c*}	88.7^{b}	90.1^{b}
SU-pH PPI	88.4^{b}	86.8^{b}	68.4^{c}	61.6^{c}
SU-salt PPI	70.4^{e}	71.5^{c}	64.0^{d}	68.6^{c}

Means (n = 3) in each column with different lowercase letters indicate significant differences according to the Tukey-Kramer multiple means comparison test ($P < 0.05$). An asterisk (*) indicates a significant difference among a not-heated and heated sample ($P < 0.05$).

At pH 7, the pH-extracted PPIs had significantly higher protein solubility than the salt-extracted PPIs at both the benchtop and pilot plant scales (Table 4). The observed difference, however, was not large, for the most part, and could be attributed to the selective solubilization of proteins at pH 7.5 when following the pH-extraction method. Following salt-extraction, on the other hand, the proteins were solubilized in 0.5 M NaCl, so the solubilized proteins would not have selective solubility at a specific pH. Additionally, compared to the salt-PPI and SU-salt PPI, the pH-PPI and SU-pH PPI had higher surface charge at pH 7 (Figure 4), which helped improve solubility by promoting higher levels of interaction with water.

Similarly, Karaca et al. (2011) reported that pH-extracted PPI had higher surface charge than salt-extracted PPI at pH 7, resulting in better solubility.

At neutral pH, the SU PPIs had comparable solubilities to the benchtop counterparts. While relatively not far behind, the SU-salt PPI had significantly lower solubility than the benchtop salt-PPI when not heated, yet comparable solubility when heated. This observation can be attributed to the additional thermal treatment applied during scaling-up (pasteurization and spray drying). The thermal treatment caused partial denaturation (Table 3) of the major proteins in SU-salt PPI (Table 3), and significantly increased the surface hydrophobicity compared to that of the benchtop salt-PPI (Figure 5). However, heating the protein solutions at 80°C for 30 minutes most likely resulted in partial denaturation of the proteins in the benchtop salt-PPI, but did not cause further impacts to the proteins in SU-salt PPI. In addition to partial denaturation of the major proteins (Table 3), denaturation of thermally labile albumin proteins in the salt-extracted PPIs could further explain the observed difference in solubility, which was not noted between the benchtop pH-PPI and SU-pH PPI. Albumins are known to be sensitive to heat and have been reported to have a lower denaturation temperature than globulins (Deak *et al.*, 2008). Cserhlami et al. (1998) performed DSC measurement on five varieties of peas separated into albumin and globulin fractions and found the albumins denatured between 53-61°C, while the globulins denatured at ≥80°C. Similarly, in a study of chickpea protein, the albumin fraction had a lower denaturation temperature (57°C) than the whole chickpea protein isolate (70-80°C) (Papalamprou *et al.*, 2009).

At pH 3.4, the pH-PPI had significantly lower solubility than the salt-PPI. This observation is again mostly attributed to the extraction process used to prepare each PPI. The pH-extraction selected for proteins with an isoelectric point of 4.5, so they would be expected to have lower surface charge near their pI and hence lower solubility. The salt-extraction, on the other hand, was not selective of proteins based on their solubility at a certain pH. Therefore, the salt-solubilized proteins could have a higher isoelectric point on average. This assumption is

supported by the lower surface charge of salt-PPI at pH 7 compared to pH-PPI (Figure 4). Tian et al. (1999) reported that proteins prepared by alkaline extraction with isoelectric precipitation had lowest solubility at pH 4-5, and solubility between 20-30 percent at pH between 3 and 4. Meanwhile, they saw that the lowest solubility for salt-extracted PPI was at pH 5-6, while solubility between pH 3 and 4 was over 50 percent (Tian *et al.*, 1999). The higher isoelectric point of salt-PPI indicates that it carries higher net positive charge at pH 3.4 compared to pH-PPI, thus, explaining the significantly higher solubility at pH 3.4, both with and without thermal treatment.

Both SU PPIs retained good solubility at pH 3.4, around 60-70 percent (Table 4). The SU-pH PPI had significantly higher solubility than the benchtop pH-PPI at pH 3.4 when not heated. This observation can be explained by their different ash contents. At low levels, salt can help increase protein solubilization by "salting in" the proteins. However, after a certain point, salt content may be high enough to shield surface charge on the protein, thereby decreasing solubility (Zhou, 2005; Duong-Ly and Gabelli, 2014). The benchtop pH-PPI had significantly higher ash content than the SU-pH PPI (4.96% ash for benchtop pH-PPI, 2.94% ash for SU-pH PPI; $p < 0.05$). The exact content of salt that causes "salting out" is dependent on each protein and the type of salt, but it appears that the higher salt content of the pH-PPI was sufficient to shield some surface charge and result in lower solubility compared to SU-pH PPI. This effect was not seen at pH 7, likely because the proteins carried higher charge further from their isoelectric point, so the salt content might not have had a major impact. However, at a pH closer to pH 4.5, the proteins were less charged, so the effects of shielding by salt ions were pronounced.

While the SU-pH PPI had higher solubility at pH 3.4 than the benchtop counterpart, the opposite was true for the salt-extracted PPIs. Unlike the SU-pH PPI and pH-PPI, the ash contents of the SU-salt PPI and salt-PPI were not statistically different (1.66% ash for SU-salt PPI, 1.56% ash for benchtop salt-PPI). The lower solubility of the SU-salt PPI compared to its benchtop counterpart may

be explained by it being more denatured and aggregated (Table 3 and Figure 3). At acidic pH, the net surface charge would be lower than the net surface charge at pH 7, resulting in potentially less repulsion among the partially denatured proteins, allowing for hydrophobic interactions, resulting in aggregation and subsequent precipitation, which would explain the lower solubility of the SU-salt PPI.

Thermal treatment at pH 3.4 did not affect solubility of the salt-PPI, SU-pH PPI, or SU-salt PPI, but the solubility of pH-PPI at pH 3.4 was significantly higher upon heating. Heating pH-PPI at pH 3.4 near its isoelectric point, where surface charge would be low, would make the protein more prone to denaturation and unfolding. At higher pH (pH 7), further from its isoelectric point and with higher net surface charge, proteins are more resistant to denaturation. While denaturing proteins can often lead to a decrease in protein solubility due to new exposure of hydrophobic groups, in the case of pH-PPI, solubility was increased with heating. It is possible that heating disrupted hydrogen and electrostatic bonding, leading to an increase in freed functional groups that would interact with water instead, thus the higher solubility of pH-PPI at pH 3.4 (Damodaran, 2017).

In addition to comparing protein solubility across extraction protocol and scale, all extracted PPIs were compared against reference plant proteins, cSPI and cPPI. Specifically, comparing the commercial isolates to the SU PPIs was of particular interest, since they were processed using similar equipment and could potentially be direct competitors in the plant protein ingredient market. The scaled-up PPIs were relatively comparable to the cSPI in solubility (ranging from 70-88%) at pH 7 under heated and not-heated conditions, and significantly superior to cPPI. At pH 3.4, both SU PPIs had significantly higher protein solubility than cSPI and cPPI. As shown by SDS-PAGE and DSC results (Figure 3 and Table 3), the cSPI and cPPI were completely denatured and had high molecular weight polymers, which explains their low solubility. In agreement, Shand et al. (2007) observed lower solubility of commercial PPI and SPI compared to native PPI and SPI. The authors attributed this observation to the denaturation and polymerization upon spray drying (Shand *et al.*, 2007). However, the SU PPIs in the current study were

also spray dried, indicating that the poor solubility of cPPI and cSPI were caused by harsh extraction/processing conditions prior to spray drying. These findings confirm that scaling-up production of PPI under controlled and optimized conditions will contribute to relatively high and acceptable solubility compared to current industrial processes.

3.5.2 Gelation of the Different Protein Isolates

While the native conformation of globular proteins is desirable for solubility, partial denaturation is needed in order to form a gel. Heating the protein solution above its denaturation temperature disrupts hydrogen bonding and electrostatic interactions that take part in stabilizing the secondary and tertiary protein structure, allowing the protein molecule to unfold and expose hydrophobic groups and buried reactive sulfhydryl groups (Shand *et al.*, 2007). The primary interactions responsible for thermally induced gelation are hydrophobic interactions and disulfide bonding, as these interactions are enhanced by heating (Fukushima, 2011). These protein-protein interactions contribute to the formation of a 3D protein network in which water is entrapped (O'Kane *et al.*, 2004a). Disulfide interactions, specifically, contribute to gel strength and springiness. Hydrogen bonding and electrostatic interactions, which are destabilized with heating, reform during cooling and contribute further to the consistency of the gel network (Kinsella and Melachouris, 1976). Larger proteins tend to be better for gelation because they are able to form a more extensive gel network and interact at more points than smaller proteins would be able to (Kinsella, 1979).

Of all the protein isolates analyzed, cSPI formed the strongest gel (Figure 6). This was not surprising, as SPI is known for its gelling abilities due to its high legumin (glycinin) to vicilin (β-conglycinin) ratio (Shand *et al.*, 2007; Sun and Arntfield, 2011a; Tulbek *et al.*, 2016). Legumin contains cysteine residues that can form disulfide linkages inter- or intra-molecularly, providing structure and strength to the gel (Wolf, 1970). PPI has a lower legumin proportion compared to SPI, and is higher in vicilin proteins, which cannot form disulfide bonds. Because of its

higher content of legumin, cSPI formed a strong gel at 15% protein, while at 15% protein, none of the PPI gels kept their shape when they were removed from the tubes to measure their strength. PPI gels were prepared at 20% protein instead in order to form a gel that kept its shape and could be measured by the Texture Analyzer.

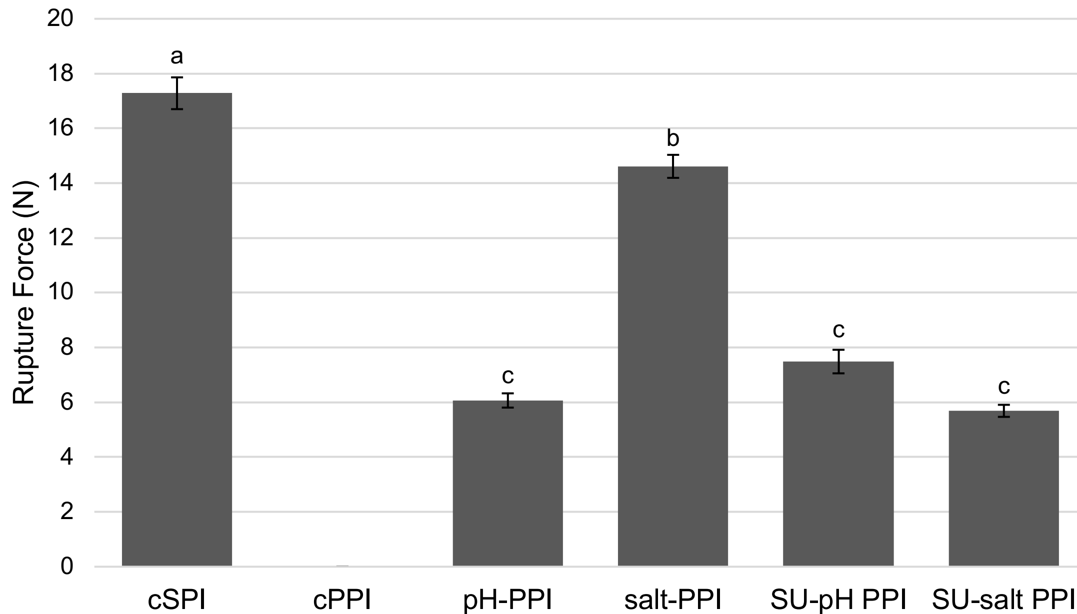

Figure 6. Gel strength of protein isolate samples. SPI gels were prepared at 15% protein (w/v) and heated for 10 minutes at 95°C. PPI gels were prepared at 20% protein (w/v) and heated for 20 minutes at 95°C. cPPI did not form a measurable gel. Error bars represent standard error (n = 4). Different lowercase letters above the bars indicate significant differences among the samples according to the Tukey-Kramer multiple means comparison test ($P < 0.05$).

Along with being a better gelling agent than PPI, SPI gels tend to be more elastic. Under the same gelling conditions, SPI gels had higher elasticity and hardness than PPI gels, which was attributed to a higher extent of cross-linking (Shand *et al.*, 2007; Taherian *et al.*, 2011). Though elasticity was not measured explicitly in this study, SPI gels appeared springier. When pressed with the Texture Analyzer, the SPI gels bounced back to their starting cylindrical shape, while the PPI gels were completely flattened, most likely due to the deficiency in stabilizing disulfide linkages.

While legumin is a primary force behind the strength of SPI gels, it is believed that PPI gels are formed by a different mechanism (O'Kane *et al.*, 2004a). Disulfide bonding still occurs between legumin proteins in PPI, but the legumin content of PPI is insufficient for a good distribution of disulfide bonds throughout the gel network, thus, disulfide bonds contribute minimally to the gel strength of PPI (O'Kane *et al.*, 2005). Instead, PPI gels are believed to be primarily formed by hydrophobic interactions among vicilin proteins, and are therefore less elastic than SPI gels (Shand *et al.*, 2007; Sun and Arntfield, 2011a).

The salt-PPI had significantly the highest gel strength among the PPI samples, while the gel strengths of the pH-PPI, SU-pH PPI, and SU-salt PPI were not significantly different (Figure 6). Salt-PPI had significantly lower surface charge than the other PPIs (Figure 4). A relatively lower surface charge will contribute to reduced repulsion among proteins, thus facilitating protein-protein interactions, and consequently the formation of a better protein network and higher gel strength. High surface charge of proteins, on the other hand, promotes solubility by enhancing interactions with water, but retards gel formation by causing repulsion among the proteins (Panyam and Kilara, 1996). Protein-protein interactions are required for gelation to occur, but the presence of preformed large polymers/aggregates may have a negative effect on gel strength (Sun and Arntfield, 2010).

When proteins are already partially denatured and aggregated, upon further heating to induce gelation, simultaneous denaturation and random aggregation will occur, resulting in a coagulum rather than a gel. On the other hand, proteins that are not already denatured, upon heating they will first denature and then proceed to aggregate, leading to the formation of a more ordered protein network and a better gel (Tombs, 1974; Hermansson, 1979). As indicated by DSC and SDS-PAGE (Table 3 and Figure 3), the SU-pH and SU-salt PPI samples were partially denatured and aggregated by processing, and the pH-PPI also showed slight aggregation. While SU-pH PPI, SU-salt PPI, and pH-PPI still formed a gel, the gels were formed by highly random associations and so had low structural strength.

The salt-PPI, however, was least denatured and did not have any signs of aggregation (Figure 3 and Table 3). Together, the low denaturation state and low surface charge of salt-PPI, enabled it to form a more ordered structure, resulting in significantly higher gel strength than all other PPIs.

Even at 20% protein, cPPI did not form a gel. cPPI had low surface charge, similar to salt-PPI, but was completely denatured, extensively aggregated, and irreversibly polymerized. Accordingly, heating cPPI caused further, yet random, aggregation of the proteins. Along with having a higher degree of random aggregation than the SU-salt PPI, SU-pH PPI, and pH-PPI, cPPI was significantly lower in solubility. SU-salt PPI, SU-pH PPI, and pH-PPI had high solubility, thus were still able to interact with and entrap water, forming a gel. cPPI, however, formed a coagulum of proteins that was unable to entrap water. Taherian et al. (2011) similarly reported that commercial PPI did not form a gel at 20% protein, an observation they attributed to poor solubility due to denaturation and polymerization that occurred during protein extraction (Taherian *et al.*, 2011). Both the benchtop and SU PPIs were superior to cPPI in their ability to form a gel, as they were less denatured during extraction and processing, and were accordingly able to participate in both protein-protein interactions as well as protein-water interactions.

While cSPI was also denatured and aggregated by processing, it was superior in gel strength over all PPIs (Figure 6). Similar to cPPI, cSPI also had random aggregation of previously denatured proteins, which resulted in it having half the gel strength of nSPI (nSPI rupture force = 35 N, full data in Appendix L, Figure 12). However, the gel strength of cSPI was significantly higher than that of all PPIs, even though it was measured at 15% protein compared to 20% protein used to form PPI gels. This phenomenon can be attributed to the much higher legumin content of SPI compared to that of PPI, and the associated ability to form more disulfide linkages. Disulfide linkages contribute order and strength to the structure of the gel, which seems to have compensated for the random aggregation in cSPI to allow it to still achieve a relatively high gel strength (Tombs, 1974). The

benchtop salt-PPI approached cSPI in terms of gel strength, though scaling up the salt-extraction reduced gelling ability. Without further modification, PPI is inferior to SPI in terms of gel formation and strength, mostly due to the relatively lower legumin content.

3.5.3 Emulsifying Properties of the Different Protein Isolates

Emulsifying abilities of a protein can be tested in a number of ways, with the most common three being emulsification capacity (EC), emulsion stability (ES), and emulsion activity index (EAI). EC determines the amount of oil that can be emulsified per unit of protein and requires proteins to have a relatively flexible structure and a good hydrophilic/lipophilic balance (HLB) so that they are able to interact with both the water and oil phases. ES measures how long the protein can stabilize the emulsion before the oil and water phases begin to separate. Emulsions are thermodynamically unstable due to the positive free energy at the water:oil interface (Kinsella, 1979). Proteins can provide long-term stability by creating a thick, continuous film around oil droplets to decrease tension at the interface and act as a physical barrier between oil droplets to prevent coalescence. Along with physical separation, proteins mostly carry a net negative charge at neutral pH that causes repulsion between proteins adsorbed on oil droplets and thus delays phase separation (Kinsella, 1979; Dagorn-Scaviner *et al.*, 1986). To obtain high ES, ideally proteins should form a thick protein layer at the interface and have a relatively high charge on the surface to cause repulsion among emulsified oil droplets to prevent coalescence. EAI indicates how easily proteins can migrate to the water:oil interface and unfold to orient their hydrophobic residues to the oil phase and hydrophilic residues to the water. Proteins that have flexible structures and good solubility have high EAI (Nakai, 1983; Barac *et al.*, 2010; Feyzi *et al.*, 2018).

cSPI had more than double the emulsification capacity (EC) compared to all PPIs tested (Figure 7). Protein denaturation can increase emulsification capacity, to a certain extent, by increasing surface hydrophobicity (Osen *et al.*,

2014). cSPI was completely denatured (Table 3) yet maintained good solubility at pH 7 (Table 4). On the other hand, while cPPI was also denatured, it had poor solubility compared to cSPI at pH 7. As mentioned earlier, EC requires a good HLB in order for the protein to interact effectively with both the oil and the water phases. Scaled-up PPI samples were partially denatured and had similar solubility to cSPI at pH 7 (Table 4) but had significantly higher surface hydrophobicity (Figure 5), while benchtop PPIs were not denatured and had significantly lower surface hydrophobicity than cSPI. It is therefore suspected that the cSPI had the best HLB due to its moderate surface hydrophobicity and good solubility, contributing to the relatively high EC. The SU PPIs likely were more dominated by high surface hydrophobicity, whereas the benchtop PPIs were dominated by high solubility. The cPPI had relatively high surface hydrophobicity coupled with very low solubility, resulting in the lowest EC of all protein isolates. The poor solubility and high extent of aggregation inhibited cPPI from effectively interacting with either phase, resulting in low EC values.

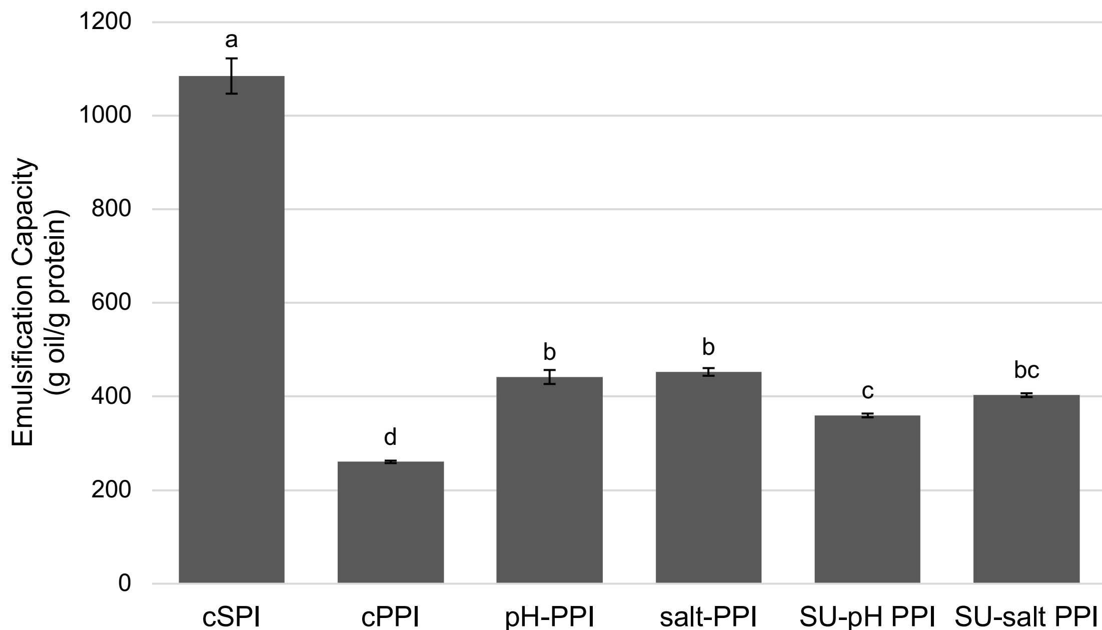

Figure 7. Emulsification capacity of protein isolate samples. Error bars represent standard error (n = 3). Different lowercase letters above the bars indicate significant differences among the samples according to the Tukey-Kramer multiple means comparison test (*P* < *0.05).*

The benchtop and SU PPIs all had comparable EC values (Figure 7). As seen by SDS-PAGE (Figure 3), the benchtop PPIs and SU PPIs had very similar protein profiles, with some differences in degree of polymerization and denaturation, which could have contributed to the slight differences in EC.

Both the benchtop and SU pH-PPIs had higher ES than their salt counterparts (Figure 8A). This observation could be attributed to the presence of low molecular weight albumins in the salt-extracted PPIs. Globulin proteins, generally, are good for stabilizing emulsions because they can form a thick film at the water:oil interface that prevents coalescence and creaming. The pH-extracted PPIs contained mostly globulin proteins and were not diluted by albumins as in the case of the salt-extracted PPIs. Similarly, Makri et al. (2005) reported that PPI prepared by alkaline extraction with isoelectric precipitation had superior emulsion stability compared to PPI prepared by alkaline solubilization with UF, which they attributed to the higher stabilizing effects of globulins compared to albumins. Additionally, the pH-extracted PPIs had higher surface charge than the salt-extracted PPIs (Figure 4), which would cause greater repulsion among proteins adsorbed at the interface of the oil droplets, thus delaying coalescence.

Comparing the ES of benchtop PPIs and corresponding SU PPIs, there were no significant differences (Figure 8A). Differences in denaturation, surface hydrophobicity, and surface charge between benchtop and SU PPIs may have had counter as well as balanced effects that culminated to no significant change in ES. For instance, low extent of polymerization might contribute to a thicker protective layer around the oil droplets, while higher surface charge would aid in preventing coalescence.

Of all protein isolates tested, cPPI had the highest emulsion stability (Figure 8A). However, cPPI also had the lowest EAI (Figure 8B). As cPPI was highly denatured and aggregated, it makes sense for EAI to be low, because high emulsion activity requires proteins to be flexible and quick to migrate to the water:oil interface. The poor solubility and high amount of denatured and aggregated proteins in cPPI would cause them to interact with each other rather

than efficiently migrating to the water:oil interface. Indeed, Taherian et al. (2011) reported that commercial PPI had the lowest emulsion activity compared to PPIs prepared from 6 cultivars, which the authors attributed to its corresponding lowest solubility. However, once the proteins got to the interface, the large aggregates of protein would provide a thick layer around oil droplets, preventing coalescence and resulting in relatively high ES. It has been proposed that the thickness of the interfacial layer is the primary factor determining ES (Ivey *et al.*, 1970; Dickinson, 2010).

Salt-PPI, on the other hand, had the lowest ES values, but the highest EAI. Unlike cPPI, salt-PPI was mostly not denatured, as indicated by the high enthalpy values (Table 3), and had high protein solubility (Table 4). Additionally, salt-PPI contained more low MW proteins than pH-PPI (Figure 3). These characteristics enable the proteins to migrate easily to the interface and reduce surface tension, resulting in a high EAI. However, the low MW proteins in salt-PPI would be less capable of forming thick, protective films around the oil droplets, resulting in low ES.

Another factor to consider when assessing ES is the viscosity of the aqueous phase of the emulsions. Making the viscosity of the aqueous phase more similar to that of the oil phase causes reduced rate of oil separation (Zayas, 1997). Functionality testing was corrected for protein contents, therefore, protein isolates with lower protein purity would have a higher amount of nonprotein constituents, like carbohydrates and lipids, in the aqueous phase. These nonprotein constituents could contribute to the viscosity of the solution and help make the aqueous phase more similar to the oil phase, therefore decreasing the force driving phase separation (Zayas, 1997). A more viscous aqueous phase decreases the interfacial tension, thus may help improve emulsion stability. Overall, protein purity negatively correlated with emulsion stability for all protein isolates ($r = -0.83$). Salt-PPI, which had the highest protein purity (92.8% protein) and contained the lowest amount of carbohydrates and lipids, while having highly soluble protein, resulted in an aqueous phase with low viscosity, potentially similar to that of water. cPPI,

on the other end, which had the lowest protein purity (81.2% protein) and therefore contained a higher amount of nonprotein constituents, resulted in a more viscous aqueous phase that was likely more similar to the oil phase.

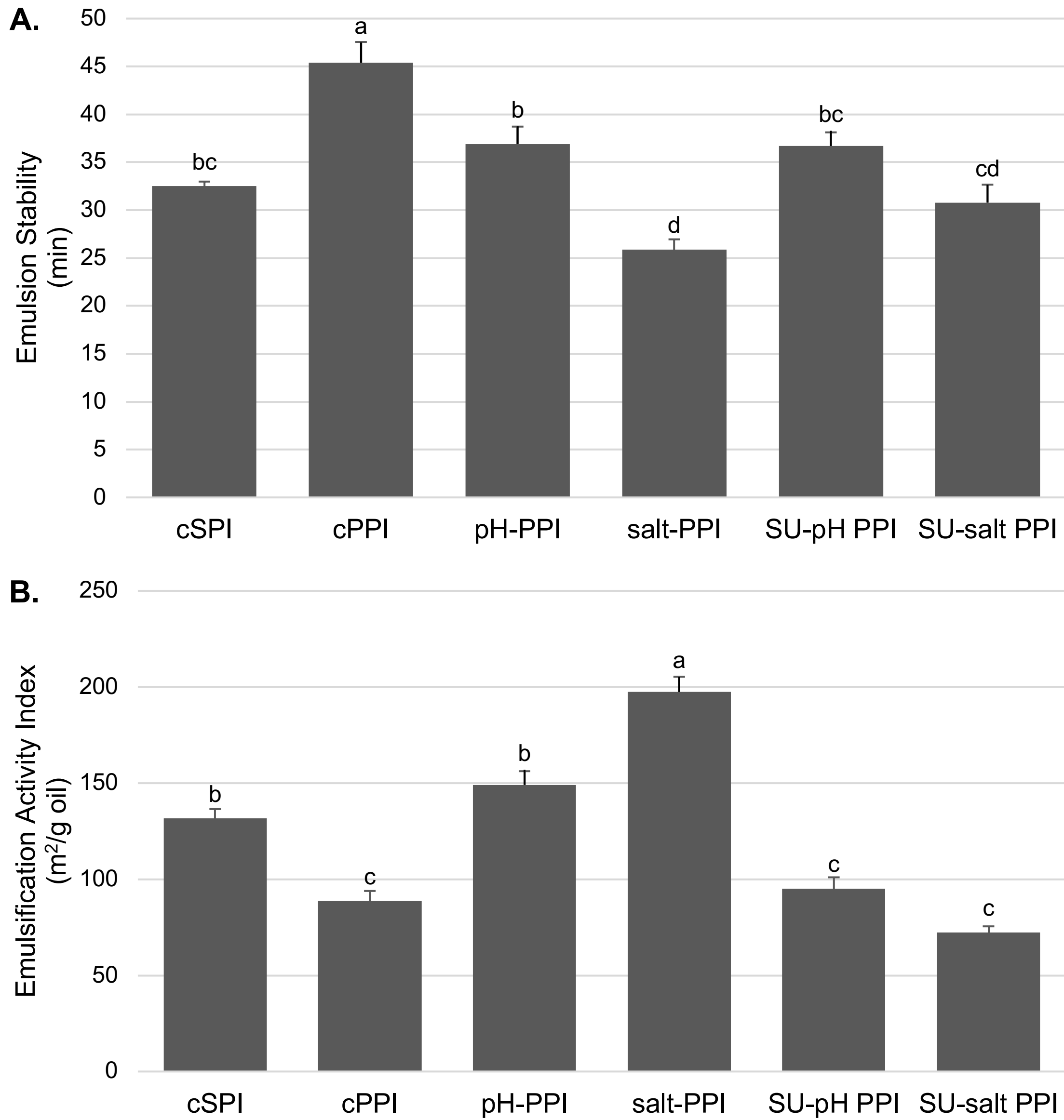

Figure 8. Emulsion stability (**A**) and emulsification activity index (**B**) of protein isolate samples. Error bars represent standard error (n = 3). Different lowercase letters above the bars indicate significant differences among the samples according to the Tukey-Kramer multiple means comparison test (*P < 0.05).*

Benchtop PPIs had significantly higher EAI than the SU PPIs (Figure 6B). While the polymerized proteins of the SU PPIs helped provide stability to the emulsion, it hindered initial emulsion formation. The non-polymerized proteins of the benchtop PPIs were able to migrate relatively quicker to the interface. The benchtop salt-PPI had significantly higher EAI than the benchtop pH-PPI, probably because the albumins in salt-PPI aided in quickly forming the emulsion. However, this effect was not seen for the SU-salt PPI compared to the SU-pH PPI, because the albumins in SU-salt are suspected to be partially denatured based on solubility data (Table 4).

It was expected that PPIs would have better emulsifying properties than SPI, as PPI contains a higher proportion of vicilin proteins, which are known to be good emulsifiers (Dagorn-Scaviner *et al.*, 1986; Arntfield and Maskus, 2011; Liang and Tang, 2013a). The vicilin (β-conglycinin) fraction typically has good emulsion forming capability due to its relatively low MW and flexible conformation, compared to the legumin (glycinin) fraction, which is better for stabilizing emulsions because it is a larger protein that can form a thicker film (Fukushima, 2011). However, changes to the protein structure due to processing, as is the case for cSPI, may cause deviation from the expected emulsification properties of a native soy protein. cSPI had moderate surface hydrophobicity along with good protein solubility that contributed to its relatively high EC and fairly high EAI. Furthermore, the predenatured state of glycinin in cSPI could have resulted in improved flexibility, leading to enhanced emulsifying abilities.

3.5.4 Foaming Properties of the Different Protein Isolates

Proteins with high molecular flexibility are good for forming foams, as they have high surface activity and can easily migrate to the water:air interface and decrease surface tension (Kinsella, 1981; Taherian *et al.*, 2011; Toews and Wang, 2013). Additionally, as with emulsification, a balance of hydrophobicity and hydrophilicity is needed for the protein to interact with both the air and water phases (Makri *et al.*, 2005). However, the tension at the water:air interface is

greater than the tension at the water:oil interface, so foams require more extensive unfolding than emulsions (Graham and Phillips, 1979; Nakai, 1983). Therefore, foaming is more dependent on “exposable hydrophobicity”, caused by protein denaturation, rather than surface hydrophobicity (Graham and Phillips, 1979; Schwenke, 2001). While hydrophobicity plays a role in foaming, surface charge is believed to be a more important factor (Nakai, 1983). Unlike emulsification where a relatively high surface charge is desirable in order to prevent coalescence, low surface charge is better for foaming, as it decreases the energy barrier for adsorption to the interface and encourages protein-protein hydrophobic interactions, leading to formation of a thick, cohesive film around air bubbles that stabilizes the foam against rupture (Nakai, 1983; Tsoukala *et al.*, 2006). However, too much interaction between proteins at the interface causes aggregation and increases film rigidity, leading to foam breakdown (Kinsella and Melachouris, 1976; Kinsella, 1981).

The benchtop and SU salt-PPIs each had significantly higher foaming capacity and stability than the benchtop and SU pH-PPI counterparts (Figure 9). This observation can be attributed to the presence of albumins in the salt-extracted PPIs. Because albumins have a more open, flexible structure than the compact structure of globulins, they can more easily migrate to the water:air interface and interact with both the hydrophobic air phase and hydrophilic water phase. Previous research findings similarly noted that protein isolates containing albumins had better foaming properties than those that do not (Vose, 1980; Sathe and Salunkhe, 1981; Makri *et al.*, 2005; Taherian *et al.*, 2011). Based on the solubility data of salt-PPI and SU-salt PPI at pH 7 (Table 4) and their protein profiles (Figure 3B, lanes 3 and 5), it is believed that the albumin proteins in SU-salt PPI were at least partially denatured and involved in polymerization, which explains why the salt-PPI had significantly higher foaming capacity and foaming stability than the SU-salt PPI.

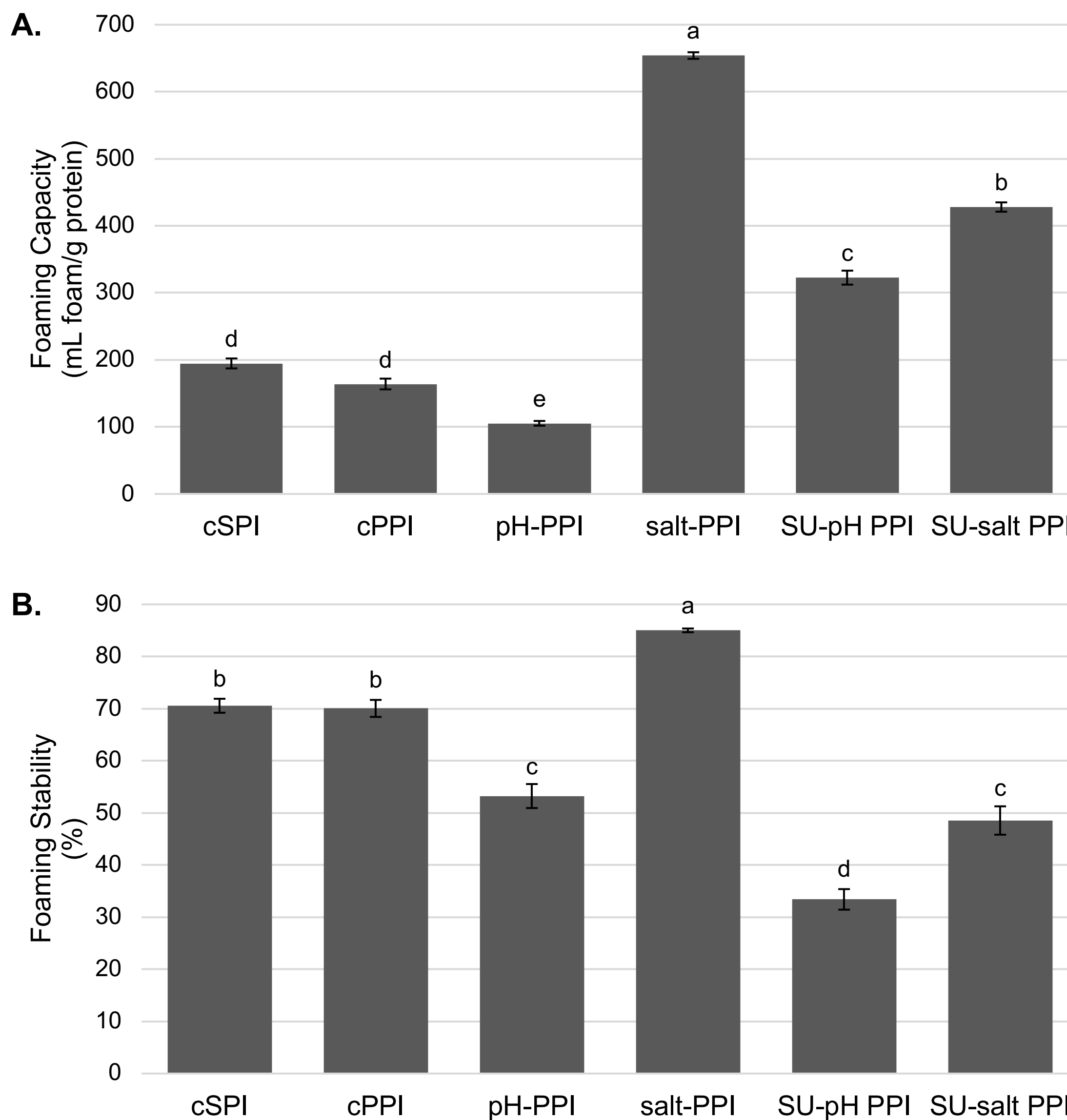

Figure 9. Foaming capacity (**A**) and stability (**B**) of protein isolate samples. Error bars represent standard error, n = 3. Different lowercase letters above the bars indicate significant differences among the samples according to the Tukey-Kramer multiple means comparison test (*P < 0.05).*

In addition to lacking albumin proteins, the benchtop and SU pH-PPIs had higher surface charge than their salt-extracted counterparts (Figure 4), contributing further to their lower foaming abilities. Lower surface charge decreases repulsions between proteins and enables proteins to more easily adsorb at the interface, enabling the formation of a cohesive film around air bubbles (Nakai, 1983). It is

reported that proteins foam better as they approach their isoelectric point due to reduced repulsion between proteins (Townsend and Nakai, 1983; Makri *et al.*, 2005; Aluko *et al.*, 2009; Dickinson, 2010; Lam *et al.*, 2018). The pH-PPI had the highest surface charge of all PPIs tested, which contributed to low foaming capacity. Similarly, others have reported that foam stability negatively correlated with surface charge (Nakai, 1983). The higher surface charge of the pH-PPI and SU-pH PPI compared to the salt-PPI and SU-salt PPI negatively impacted foam formation and foam stability due to potential repulsion between proteins. Similarly, differences in surface charge may explain differences in FC and FS of benchtop salt-PPI compared to both of the SU PPIs.

Similar to pH-PPI, cSPI and cPPI had low foaming capacity, yet fairly high foaming stability (Figure 9). This observation could be attributed to a poorly formed foam that is easy to maintain (Kinsella, 1981; Boye *et al.*, 2010b). The lower volume of foam means that there is less surface area for the protein to cover. All protein isolates were measured at the same protein concentration, so those with lower FC covered less surface area with the same amount of protein, essentially resulting in a more concentrated density of proteins at the interface. Higher protein concentration contributes to higher foam stability, as there are more proteins available to form a thicker film at the interface (Kinsella, 1981; Taherian *et al.*, 2011). Furthermore, as with emulsion stability, increasing the viscosity of the continuous phase helps increase foaming stability by reducing the rate of drainage of lamellar fluid from the air bubbles (Townsend and Nakai, 1983; Zayas, 1997). pH-PPI, cSPI, and cPPI had the lowest protein purities of the isolates, which would contribute to higher viscosity since there would be a higher amount of nonprotein constituents in the continuous phase. The relatively high FS of pH-PPI, cSPI, and cPPI, therefore, was the result of having a low volume of foam to stabilize with a fairly viscous continuous phase.

The FS of salt-PPI, SU-salt PPI, and SU-pH PPI followed a different trend. The salt-PPI had both the highest foaming capacity as well as the highest foaming stability of all protein isolates (Figure 9). Foaming stability is influenced by several

factors, including formation of a thick film at the interface. Intermolecular interactions among proteins at the interface are required in order to form a thick, cohesive film that is resistant to rupture. However, proteins should also be highly soluble so that when localized ruptures occur, protein molecules can migrate to the areas of the film that need reinforcement and prevent further collapsing of the foam (Kinsella, 1981). While protein-protein interactions are necessary to form a cohesive film, excessive protein aggregation causes lower FS because the proteins would be less capable of flowing to reinforce weak portions of the foam. The salt-PPI was not denatured nor aggregated by extraction, thus potentially formed a flexible and thick film that enabled it to have high FS along with high FC. However, the SU-pH PPI and SU-salt PPI were partially denatured and had some protein aggregation that potentially reduced molecular flexibility and caused lower FS.

Comparing the commercial plant proteins against the SU PPIs, cSPI and cPPI had significantly lower FC than both the SU-pH and SU-salt PPIs. The commercial proteins were completely denatured and irreversibly polymerized, so they did not have the structural flexibility to align at the water:air interface. Though the SU PPIs still had some denaturation and polymerization, it was less extensive than that of cSPI and cPPI, enabling the SU PPIs to have a higher FC. However, cSPI and cPPI had significantly higher FS than the SU PPIs, which could be attributed to the combined effects of easier maintenance of less foam and the increased viscosity of the continuous phase.

Chapter 4: Conclusions, Implications, and Recommendations

Conditions for pea protein extraction by alkaline solubilization with isoelectric precipitation were optimized, with a focus on selecting conditions that would be nondenaturing and feasible for industrial production, while still obtaining high protein purity and yield. Additionally, purification techniques for a relatively novel extraction method of salt solubilization coupled with membrane filtration were investigated. The optimized pH- and salt- extractions had PPI protein purity and yield competitive or superior to what has been reported previously for pea protein extractions. The optimized extractions were scaled-up to pilot plant production and achieved similar protein purity. The effect of scaling up on protein yields was not determined due to sampling throughout the extraction and the recovery of only the high solids retentate from the UF unit.

Structural characterization revealed that the salt-extraction was less denaturing than the pH-extraction, indicated by reduced aggregation and higher thermal stability of the salt-PPIs. The relatively more native state of the salt-PPI contributed to its highest gel strength among all the PPIs, as it was able to form a more ordered gel network. Another key structural difference was that the salt-extraction retained albumin proteins, whereas the pH-extraction did not. The presence of albumins in salt-PPIs contributed to lower surface charge and lower surface hydrophobicity, at pH 7, compared to the pH-PPIs, which together caused differences in functionality. While both the pH- and salt-PPIs had good solubility at pH 7, the salt-PPI has slightly lower solubility because the presence of albumins increased its isoelectric point compared to the pH-PPI. However, the lower isoelectric point of the pH-PPI caused it to have lower solubility at pH 3.4 compared to salt-PPI. Furthermore, compared to the pH-PPI, the salt-PPI had lower ES, but higher EAI, FC, and FS, largely due to its albumin content and lower surface charge. pH-PPI and salt-PPIs had similar EC due to having similar solubility and surface hydrophobicity at pH 7.

Pea protein extraction following alkaline solubilization with isoelectric precipitation is commonly used to produce PPI, but typically harsher conditions are

employed, such as higher solubilization pH, overnight storage at the isoelectric point, and higher thermal treatment, all contributing to protein denaturation and polymerization, thus limiting functionality. However, this study demonstrated that high protein purity and yield can be achieved using mild, nondenaturing conditions for pH extraction. Furthermore, this study demonstrated the success of a novel salt extraction process in producing a functional PPI. Salt solubilization coupled with membrane filtration has not been thoroughly investigated. This study, therefore, fills a gap in literature and shines light on the potential of salt-extracted PPI by demonstrating the differences in structural and functional properties compared to the traditional alkaline solubilization method. Additionally, the feasibility of scaling up optimized extractions from the benchtop was elucidated.

SU PPIs had higher levels of denaturation and aggregation than their benchtop counterparts, caused by the pasteurization and spray drying steps done in the pilot plant. This structural difference resulted in a slight decrease in functional properties of SU PPIs compared to their benchtop counterparts. However, the SU PPIs were less denatured and aggregated than cPPI, which would also have gone through pasteurization and spray drying, indicating that denaturation cPPI was produced under harsher extraction and processing conditions. Despite being completely denatured, cSPI maintained better gelation and emulsification properties, attributed to its naturally higher legumin content and desirable hydrophobicity/hydrophilicity balance compared to PPI samples. On the other hand, both the benchtop and SU PPI samples had similar solubility at pH 7 but superior solubility at pH 3.4. It is worth noting that the SU PPIs had overall better functionality than cPPI. This finding demonstrates that industrial produced PPI can have acceptable protein functionality, if extraction and processing conditions are mild and controlled.

The optimized extraction methods were proven to be scalable, though some denaturation was inevitable. Nevertheless, the SU PPIs were less denatured than commercially available PPI. Future work should investigate how evaporation of the low solids retentate from UF affects the structural and functional properties of the

protein to determine the potential to combine both the high and low solids retentates in order to increase process yields.

While the SU PPIs can be seen as an improvement to the commercially available PPI, further research is needed to make them competitive with soy protein. Protein modification techniques, such as enzymatic hydrolysis, glycation, and cold plasma treatments, should be investigated, as they have potential to increase specific functional properties by altering the protein's structure. Furthermore, flavor formation throughout the extraction process should be characterized in order to determine where off-flavors are being produced so that procedures may be adapted to minimize their production. Off-flavors remain an issue for pea protein, hindering expanded use in various applications. Lastly, in order to market the SU PPIs for wide-scale production, their nutritional quality must be assessed through the evaluation of PDCAAS. These investigations coupled with the optimized extractions from this study will all contribute to the advancement of pea protein as a viable alternative to soy protein.

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Appendix A: Sample Calculation for Determining Protein Yields Using Mass Balance of pH Extraction Optimization

Calculating Protein Yields of Each Fraction in pH Extraction Optimization

$$\%\ protein\ yield\ in\ PPI = \frac{PPI\ mass\ (g) \times PPI\ protein\ purity\ (\%)}{pea\ flour\ mass\ (g) \times pea\ flour\ protein\ purity\ (\%)}$$

$$\%\ protein\ residue\ in\ pellet = \frac{pellet\ mass\ (g) \times pellet\ protein\ purity\ (\%)}{pea\ flour\ mass\ (g) \times pea\ flour\ protein\ purity\ (\%)}$$

$$\%\ protein\ lost\ in\ supernatant = \frac{supernatant\ mass\ (g) \times supernatant\ protein\ purity\ (\%)}{pea\ flour\ mass\ (g) \times pea\ flour\ protein\ purity\ (\%)}$$

Calculating Protein Yields of Each Fraction for Optimized pH Extraction (pH 7.5, double solubilized (1 hr x 2), precipitated at pH 4.5, and dialyzed)

$$\%\ protein\ yield\ in\ PPI = \frac{(1.7966\ g) \times (85.0000\ \%)}{(10.0591\ g) \times (23.3015\ \%)} = 65.1519\ \%$$

$$\%\ protein\ residue\ in\ pellet = \frac{(6.1367\ g) \times (4.3970\ \%)}{(10.0591\ g) \times (23.3015\ \%)} = 11.5119\ \%$$

$$\%\ protein\ lost\ in\ supernatant = \frac{(1.8223\ g) \times (28.1250\ \%)}{(10.0591\ g) \times (23.3015\ \%)} = 21.8660\ \%$$

Table 5. Mass balance for pea protein pH-extraction optimization using pH 7.5 solubilization, 1 hour solubilization, double solubilization, isoelectric precipitation at pH 4.5, and dialysis (rep 1).

	Mass (g)	Protein Purity (%)	Mass of Protein (g)	Protein Yield (%)
Pea Flour	10.0591	23.3015	2.3439	
Pellet	6.1367	4.3970	0.2698	11.5119
Supernatant	1.8223	28.1250	0.5125	21.8660
PPI	1.7966	85.0000	1.5271	65.1519
Sum of Fractions	9.7556		2.3095	
Recovered Material (%)	96.9828			98.5299

Appendix B: Sample Calculation for Determining Protein Yields Using Mass Balance of Salt Extraction Optimization

Calculating Protein Yields of Each Fraction in Salt Extraction Optimization

$$\%\ protein\ yield\ in\ PPI = \frac{PPI\ mass\ (g) \times PPI\ protein\ purity\ (\%)}{pea\ flour\ mass\ (g) \times pea\ flour\ protein\ purity\ (\%)}$$

$$\%\ protein\ residue\ in\ pellet = \frac{pellet\ mass\ (g) \times pellet\ protein\ purity\ (\%)}{pea\ flour\ mass\ (g) \times pea\ flour\ protein\ purity\ (\%)}$$

Calculating Protein Yields of Each Fraction in Optimized Salt Extraction (0.5 M NaCl 1 hr, ultrafiltered and dialyzed)

$$\%\ protein\ yield\ in\ PPI = \frac{(1.6921\ g) \times (92.3500\ \%)}{(10.0472\ g) \times (21.6116\ \%)} = 71.9666\%$$

$$\%\ protein\ residue\ in\ pellet = \frac{(6.8204\ g) \times (7.4700\ \%)}{(10.0472\ g) \times (21.6116\ \%)} = 23.4638\ \%$$

Table 6. Mass balance for pea protein salt-extraction using ultrafiltration and dialysis (rep 1).

	Mass (g)	Protein Purity (%)	Mass of Protein (g)	Protein Yield (%)
Pea Flour	10.0472	21.6116	2.1714	
Pellet	6.8204	7.4700	0.5095	23.4638
PPI	1.6921	92.3500	1.5627	71.9666
Sum of Fractions	8.5125		2.0721	
Recovered Material (%)	84.7251			95.4304

Appendix C: Sample Calculation to Determine Volume of Water Needed to Resuspend Precipitate at 1:10 (w/v) in Scaled-Up pH Extraction Using Precipitate Mass and Total Solids (%)

$$mass\ of\ solids\ in\ precipitate = (wet\ precipitate\ mass) \times (precipitate\ \%\ total\ solids)$$

$$\frac{(1\ kg)}{(10\ L)} = \frac{mass\ of\ solids\ in\ precipitate}{volume\ of\ water\ needed\ for\ resuspension}$$

$$mass\ of\ solids\ in\ precipitate = (47.4\ kg) \times (49.58\%\ TS) = 23.5\ kg\ of\ solids$$

$$\frac{(1\ kg)}{(10\ L)} = \frac{23.5\ kg}{volume\ of\ water\ needed\ for\ resuspension}$$

$$(23.5\ kg) \times (10\ L) = (1\ kg) \times (volume\ of\ water\ needed\ for\ resuspension) = 235\ L$$

235 L of water was added to 47.4 kg precipitate at 49.58% TS in order to reach a 1:10 w/v suspension.

Appendix D: Sample Calculation to Determine Volume of 0.5 M NaCl Needed to Resuspend Precipitate at 1:5 (w/v) in Scaled-Up Salt Extraction Using Precipitate Mass and Total Solids (%)

$$mass\ of\ solids\ in\ precipitate = (wet\ precipitate\ mass) \times (precipitate\ \%\ total\ solids)$$

$$\frac{(1\ kg)}{(5\ L)} = \frac{mass\ of\ solids\ in\ precipitate}{volume\ of\ 0.5\ M\ NaCl\ needed\ for\ resuspension}$$

$$mass\ of\ solids\ in\ precipitate = (38.5\ kg) \times (46.20\%\ TS) = 17.8\ kg\ of\ solids$$

$$\frac{(1\ kg)}{(5\ L)} = \frac{17.8\ kg}{volume\ of\ 0.5\ M\ NaCl\ needed\ for\ resuspension}$$

$$(17.8\ kg) \times (5\ L) = (1\ kg) \times (volume\ of\ 0.5\ M\ NaCl\ needed\ for\ resuspension) = 89\ L$$

89 L of 0.5 M NaCl was added to 38.5 kg precipitate at 46.20% TS in order to reach a 1:5 w/v suspension.

Appendix E: Sample Calculation for Determining Surface Hydrophobicity Index

Net Relative Fluorescence Intensity (RFI) at a single protein concentration

$RFI_{initial}$ = $Sample_{initial}$ – $Blank_{initial}$
RFI_{final} = $Sample_{final}$ – $Blank_{final}$
Net RFI = RFI_{final} – $RFI_{initial}$

Where:
$Sample_{initial}$ = fluorescence of protein sample before ANS probe is added
$Blank_{intiail}$ = fluorescence of buffer blank before ANS probe is added
$Sample_{final}$ = fluorescence of protein sample after ANS probe is added and 15 minute incubation
$Blank_{final}$ = fluorescence of buffer blank after ANS probe is added and 15 minute incubation

Example calculation for pH-PPI at 0.05% protein

$RFI_{initial}$ = 18 – 15.5 = 2.5
RFI_{final} = 519 – 20 = 499
Net RFI = 499 – 2.5 = 496.5

Surface Hydrophobicity Index

Net RFI values for all concentrations of protein solution (0.05%, 0.025%, 0.02%, 0.015%, 0.01%, and 0.005% protein) are plotted against protein concentration, as seen in Figure 10.

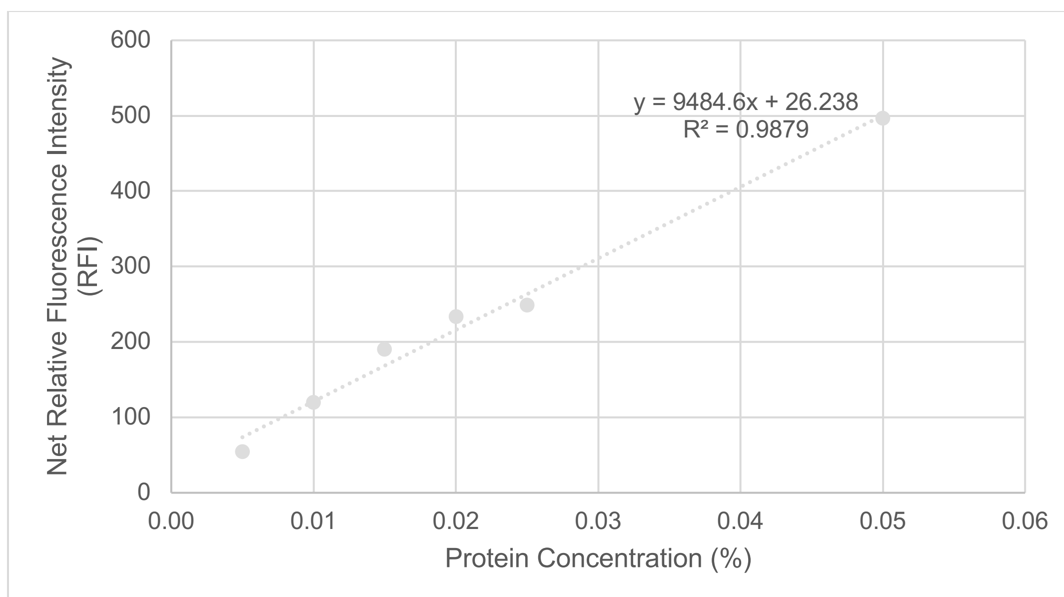

Figure 10. Net Relative Fluorescence Intensity (RFI) plotted against protein concentration (%) for pH-PPI to determine surface hydrophobicity index.

The slope of the trendline in Figure 10 is the surface hydrophobicity index (9484.6). The final value for surface hydrophobicity index is the average of three replicates.

Appendix F: Sample Calculation for Determining Protein Solubility

Protein Solubility

$$\%\,protein\ solubility = \frac{\%\,supernatant\ protein}{\%\,initial\ protein} \times 100\%$$

$$\%\,protein\ solubility = \frac{4.13}{4.72} \times 100\% = 87.42\%$$

Appendix G: Sample Calculation for Determining Emulsification Capacity

Emulsification Capacity

$$EC = \frac{volume\ of\ oil\ titrated\ (mL) \times\ density\ of\ oil\ (\frac{g}{mL})}{mass\ of\ protein\ (g)}$$

$$EC = \frac{48\ mL \times\ 0.93\ (\frac{g}{mL})}{0.1\ g} = 446.4\ \frac{g\ oil}{g\ protein}$$

Where:

0.93 g/mL = density of corn oil
0.1 g = grams of protein in 5 mL of a 2% protein solution

Appendix H: Sample Calculation for Determining Emulsion Stability and Emulsion Activity Index

Emulsion Stability (ES):

$$ES\ (\text{min}) = \frac{A_0}{A_0 - A_{10}} \times 10\ min$$

$$ES\ (\text{min}) = \frac{0.2604}{0.2604 - 0.1822} \times 10\ min = 33.30\ min$$

Where:

A_0 = absorbance at 0 min
A_{10} = absorbance at 10 min

Emulsion Activity Index (EAI):

$$EAI\left(\frac{m^2}{g}\right) = \frac{2T}{(1-\phi)C} = \frac{2(2.303 \times A_0)}{l(1-\phi)C}$$

$$EAI\left(\frac{m^2}{g}\right) = \frac{2(2.303 \times 0.2604)}{0.01\ m(1-0.25)1\ g/m^3} = 160\frac{m^2}{g}$$

Where:

C = weight of protein per volume of aqueous phase
    = 0.1% protein solution = 0.1 g protein/100 mL = 1 g/m^3
ϕ = volume fraction of oil
    = 1.67 mL oil in 5 mL of 0.1% protein solution
    = (1.67 mL)/(1.67 mL + 5 mL) = 0.25
A_0 = initial absorbance at 500 nm
    = 0.2604
l = path length of the cuvette
    = 10 mm = 0.01 m
T = turbidity of oil at 500 nm
    $Turbidity\ of\ oil\ (T) = \frac{2.303 \times A_0}{l}$

Appendix I: Sample Calculation for Determining Foaming Capacity and Foaming Stability

Foaming Capacity (FC):

$$foaming\ capacity\ \left(\frac{mL\ foam}{g\ protein}\right) = \frac{total\ solution\ volume\ (mL) - liquid\ volume\ (mL)}{mass\ of\ protein\ (g)}$$

$$FC\ \left(\frac{mL\ foam}{g\ protein}\right) = \frac{75\ mL - 49\ mL}{0.25\ g} = 104\frac{mL\ foam}{g\ protein}$$

Foaming Stability (FS):

$$foaming\ stability\ (\%) = \frac{total\ solution\ volume_{end} - liquid\ volume_{end}}{total\ solution\ volume_{initial} - liquid\ volume_{initial}} \times 100$$

$$FS\ (\%) = \frac{65\ mL - 50\ mL}{75\ mL - 49\ mL} \times 100\% = 57.69\%$$

Appendix J: ANOVA Tables

Table 7. Analysis of variance on the effect of pH-extraction protocol on PPI protein purity.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
PPIs prepared in benchtop pH-extraction optimization	Extraction protocol	6	14.089	6.545	0.00232
	Error	13	2.153		

Table 8. Analysis of variance on the effect of pH extraction protocol on PPI protein yield.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
PPIs prepared in benchtop pH-extraction optimization	Extraction protocol	6	65.09	53.68	2.25e-8
	Error	13	1.24		

Table 9. Analysis of variance on the effect of pH-extraction protocol on pellet protein purity.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
Pellets prepared in benchtop pH-extraction optimization	Extraction protocol	6	49.23	17.19	1.72e-5
	Error	13	6.21		

Table 10. Analysis of variance on the effect of pH-extraction protocol on pellet protein residue.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
Pellets prepared in benchtop pH-extraction optimization	Extraction protocol	6	68.63	17.62	1.49e-5
	Error	13	3.89		

Table 11. Analysis of variance on the effect of pH-extraction protocol on supernatant protein purity.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
Supernatants prepared in benchtop pH-extraction optimization	Extraction protocol	6	27.708	15.51	8.66e-5
	Error	13	3.274		

Table 12. Analysis of variance on the effect of pH-extraction protocol on protein lost to supernatant.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
Supernatants prepared in benchtop pH-extraction optimization	Extraction protocol	6	40.84	55.49	1.38e-7
	Error	13	1.35		

Table 13. Analysis of variance on the effect of pH-extraction protocol on PPI ash content.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
PPIs prepared in benchtop pH-extraction optimization	Extraction protocol	6	0.30844	16.96	1.09e-5
	Error	13	0.01819		

Table 14. Analysis of variance on the effect of solubilization pH on PPI protein purity in the pH-extraction.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
PPIs prepared in benchtop pH-extraction optimization	Solubilization pH	2	13.252	3.3349	0.105
	Error	6	3.957		

Table 15. Analysis of variance on the effect of solubilization pH on PPI protein yield in the pH-extraction.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
PPIs prepared in benchtop pH-extraction optimization	Solubilization pH	2	115.88	99.03	2.54e-5
	Error	6	1.17		

Table 16. Analysis of variance on the effect of solubilization pH on pellet protein purity in the pH-extraction.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
Pellets prepared in benchtop pH-extraction optimization	Solubilization pH	2	9.181	9.349	0.0143
	Error	6	0.982		

Table 17. Analysis of variance on the effect of solubilization pH on pellet protein residue in the pH-extraction.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
Pellets prepared in benchtop pH-extraction optimization	Solubilization pH	2	88.04	10.96	0.00993
	Error	6	8.03		

Table 18. Analysis of variance on the effect of solubilization pH on supernatant protein purity in the pH-extraction.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
Supernatants prepared in benchtop pH-extraction optimization	Solubilization pH	2	1.3294	3.252	0.11
	Error	6	0.4088		

Table 19. Analysis of variance on the effect of solubilization pH on protein lost to supernatant in the pH-extraction.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
Supernatants prepared in benchtop pH-extraction optimization	Solubilization pH	2	4.992	40.17	0.000336
	Error	6	0.124		

Table 20. Analysis of variance on the effect of solubilization pH on PPI ash content in the pH-extraction.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
PPIs prepared in benchtop pH-extraction optimization	Solubilization pH	2	0.007723	0.539	0.609
	Error	6	0.014322		

Table 21. Analysis of variance on the effect of salt-extraction protocol on PPI protein purity.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
PPIs prepared in benchtop salt-extraction optimization	Extraction protocol	2	506.4	734.4	6.49e-8
	Error	6	0.7		

Table 22. Analysis of variance on the effect of salt-extraction protocol on PPI protein yield.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
PPIs prepared in benchtop salt-extraction optimization	Extraction protocol	2	31.72	9.96	0.0124
	Error	6	3.19		

Table 23. Analysis of variance on the effect of salt-extraction protocol on pellet protein purity.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
Pellets prepared in benchtop salt-extraction optimization	Extraction protocol	2	0.07937	1.496	0.297
	Error	6	0.05306		

Table 24. Analysis of variance on the effect of salt-extraction protocol on pellet protein residue.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
Pellets prepared in benchtop salt-extraction optimization	Extraction protocol	2	0.9714	1.315	0.336
	Error	6	0.7389		

Table 25. Analysis of variance on the effect of salt-extraction protocol on PPI ash content.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
PPIs prepared in benchtop salt-extraction optimization	Extraction protocol	2	73.11	234	2.03e-6
	Error	6	0.31		

Table 26. Analysis of variance on the effect of pea protein isolate type on thermal denaturation temperature for first peak on DSC.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
pH-PPI, salt-PPI, SU-pH, and SU-salt*	Pea Protein Isolate Type	3	27.615	597.1	9.6e-10
	Error	8	0.046		

*cPPI was completely denatured before analysis.

Table 27. Analysis of variance on the effect of pea protein isolate type on enthalpy of denaturation for first peak on DSC.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
pH-PPI, salt-PPI, SU-pH, and SU-salt*	Pea Protein Isolate Type	3	7.026	15.39	0.0011
	Error	8	0.456		

*cPPI was completely denatured before analysis.

Table 28. Analysis of variance on the effect of pea protein isolate type on thermal denaturation temperature for second peak on DSC.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
pH-PPI, salt-PPI, SU-pH, and SU-salt*	Pea Protein Isolate Type	3	7.648	13.31	0.00178
	Error	8	0.574		

*cPPI was completely denatured before analysis.

Table 29. Analysis of variance on the effect of pea protein isolate type on enthalpy of denaturation for second peak on DSC.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
pH-PPI, salt-PPI, SU-pH, and SU-salt*	Pea Protein Isolate Type	3	0.7258	18.13	0.00063
	Error	8	0.0400		

*cPPI was completely denatured before analysis.

Table 30. Analysis of variance on the effect of plant protein isolate type on zeta potential.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
pH-PPI, salt-PPI, SU-pH, SU-salt, cPPI, and cSPI	Pea Protein Isolate Type	5	63.23	148	2.37e-10
	Error	12	0.43		

Table 31. Analysis of variance on the effect of protein isolate type on surface hydrophobicity.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
pH-PPI, salt-PPI, SU-pH, SU-salt, cPPI, cSPI, and cWPI	Protein Isolate Type	6	43993549	52.47	8.46e-10
	Error	14	838452		

Table 32. Analysis of variance on the effect of protein isolate type on protein solubility at pH 7 for not-heated samples.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
pH-PPI, salt-PPI, SU-pH, SU-salt, cPPI, cSPI, and cWPI	Protein Isolate Type	6	1926.6	1391	<2e-16
	Error	14	1.4		

Table 33. Analysis of variance on the effect of protein isolate type on protein solubility at pH 7 for heated (80°C) samples.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
pH-PPI, salt-PPI, SU-pH, SU-salt, cPPI, cSPI, and cWPI	Protein Isolate Type	6	1141.9	738.5	<2e-16
	Error	14	1.5		

Table 34. Analysis of variance on the effect of protein isolate type on protein solubility at pH 3.4 for not-heated samples.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
pH-PPI, salt-PPI, SU-pH, SU-salt, cPPI, cSPI, and cWPI	Protein Isolate Type	6	3082.6	2000	<2e-16
	Error	14	1.5		

Table 35. Analysis of variance on the effect of protein isolate type on protein solubility at pH 3.4 for heated (80°C) samples.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
pH-PPI, salt-PPI, SU-pH, SU-salt, cPPI, cSPI, and cWPI	Protein Isolate Type	6	2460	273	4.09e-12
	Error	14	9		

Table 36. Analysis of variance on the effect of plant protein isolate type on gelation.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
pH-PPI, salt-PPI, SU-pH, SU-salt, and cSPI*	Plant Protein Isolate Type	4	114.4	164.3	3.44e-12
	Error	15	0.7		

*cPPI did not form a gel

Table 37. Analysis of variance on the effect of plant protein isolate type on emulsification capacity.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
pH-PPI, salt-PPI, SU-pH, SU-salt, cPPI, and cSPI	Plant Protein Isolate Type	5	260616	297.3	3.83e-12
	Error	12	877		

Table 38. Analysis of variance on the effect of plant protein isolate type on emulsion stability.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
pH-PPI, salt-PPI, SU-pH, SU-salt, cPPI, and cSPI	Plant Protein Isolate Type	5	190.62	24.83	1.02e-7
	Error	12	7.68		

Table 39. Analysis of variance on the effect of plant protein isolate type on emulsion activity index.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
pH-PPI, salt-PPI, SU-pH, SU-salt, cPPI, and cSPI	Plant Protein Isolate Type	5	9829	87.72	1.87e-12
	Error	12	112		

Table 40. Analysis of variance on the effect of plant protein isolate type on foaming capacity.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
pH-PPI, salt-PPI, SU-pH, SU-salt, cPPI, and cSPI	Plant Protein Isolate Type	5	1267426	764.2	1.43e-15
	Error	14	166		

Table 41. Analysis of variance on the effect of plant protein isolate type on foaming stability.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
pH-PPI, salt-PPI, SU-pH, SU-salt, cPPI, and cSPI	Plant Protein Isolate Type	5	1065.4	102.8	5.56e-10
	Error	14	10.4		

Appendix K: Zeta Potential of Protein Isolates Including nSPI

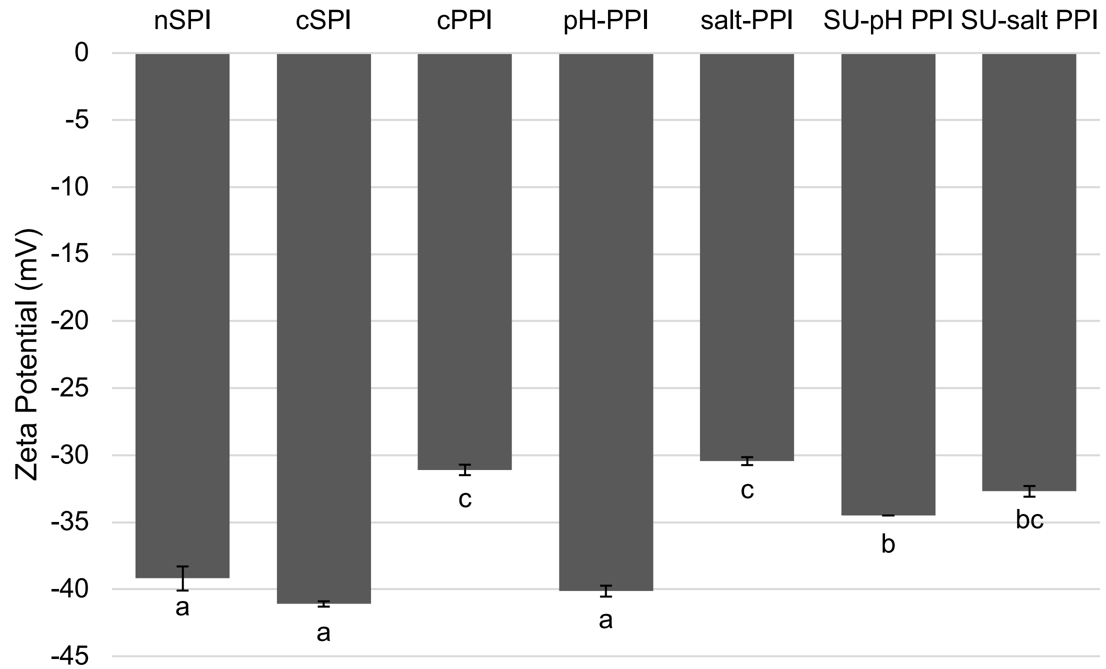

Figure 11. Zeta potential of protein isolate samples including nSPI measured at pH 7. Error bars represent standard error (n = 3). Different lowercase letters below the bars indicate significant differences among the samples according to the Tukey-Kramer multiple means comparison test (*P < 0.05).*

Appendix L: Gel Strength of Protein Isolates Including nSPI

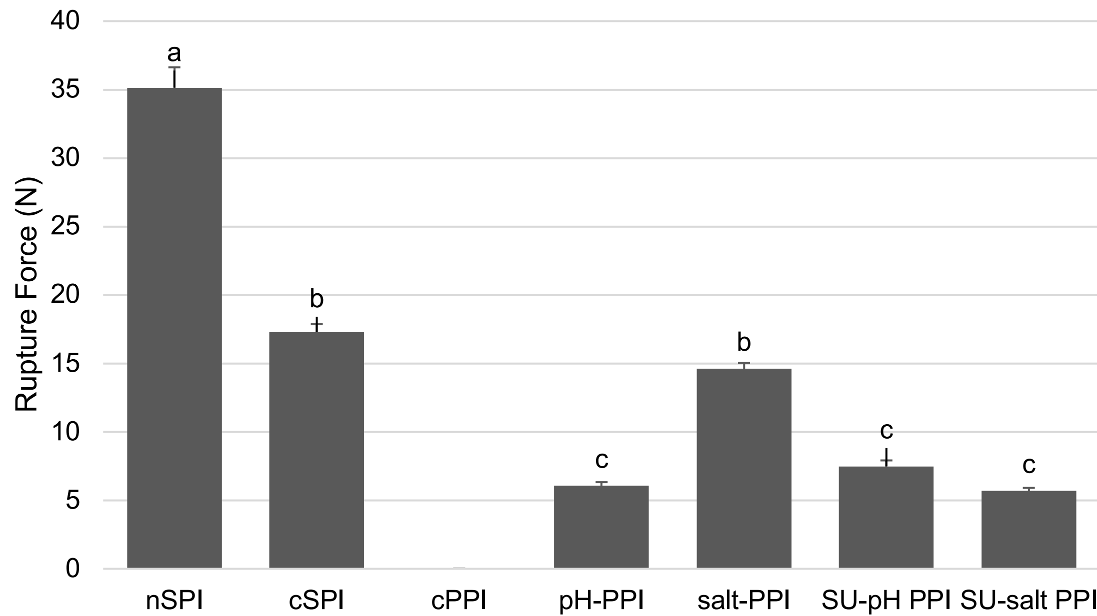

Figure 12. Gel strength of protein isolate samples including nSPI. SPI gels were prepared at 15% protein (w/v) and heated for 10 minutes at 95°C. PPI gels were prepared at 20% protein (w/v) and heated for 20 minutes at 95°C. cPPI did not form a measurable gel. Error bars represent standard error (n = 4). Different lowercase letters above the bars indicate significant differences among the samples according to the Tukey-Kramer multiple means comparison test ($P < 0.05$).