Study of the Molecular Structures and Functional Properties of Proteins from Seven Pea (*Pisum sativum* L.) Genotypes with Different Levels of Protein Content

by

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Abstract

The food industry has seen shifting trends towards the use of plant proteins due to a variety of factors including consumer´s perception of health, ethical and religious purposes, as well as environmental considerations. Pulses are good sources of proteins and other nutrients such as resistant starch, dietary fiber, vitamins, minerals, and polyphenols, and therefore have gained particular interest. Among the pulse family, field pea (*Pisum sativum*) is a widely produced legume that has high nutritional value and low allergenicity.

Though a lot of research has been focused on the applications of pea protein, the effect of genotype on the protein structure and functional properties has not been clearly investigated. This research intended to fill the gap by extracting and characterizing pea proteins from seven pea genotypes, consisting of high and regular protein genotypes. After extracting the pea protein, the protein molecular structures were systematically studied such as protein compositions, amino acid profiles, secondary structure, molecular weight by an array of advanced analytical tools including SDS–PAGE, Fourier-transform infrared (FTIR) spectroscopy, HPLC-Size exclusive chromatography (SE) and Differential Scanning Calorimetry (DSC). The protein solubility and functional properties including water and oil holding capacity, foaming and emulsifying properties and gelling capacity were compared between high and regular protein genotype.

Pea protein concentrates were successfully extracted from pea grains of all 7 genotype with the protein content ranging from 76.5 % to 86.2 %. Globulins are major storage proteins in pea grains with legumin (11S) and vicilin (7S) as the major globulin fractions. It is interesting to notice that the protein extracts from different pea genotype possess a wide range of 11S/7S ratio from 1.5 to 8.7. While the high protein genotype P0540-41 and P1142-6195 show relatively low 11S/7S ratio (1.5-1.9), the other high protein line P1141-5085 demonstrated an extremely high 11S/7S ratio of 8.6 5±

0.65, which is also high when compared to regular protein containing line. In this study, it was observed that the 11S/7S ratio significantly impact the pea protein denaturation temperature. For example, protein extracts from P0540-41 and P1142-6195 with lower 11S/7S ratios had relatively lower denaturation temperatures.

When analyzing nutritive value, protein concentrates from Earlystar, Greenwater and P0540-41 show higher sulfur-containing amino acids than other pea genotype, which is a known limiting essential amino acid in pea protein. The protein concentrates from Lacombe and P0540-41 have higher digestibility values of 86.3 % and 88.45 %, respectively compared to those from other pea genotype.

In general, the pea protein extracts show good foaming and emulsifying properties, and their foaming stability values are especially high. Among the protein extract samples, those from Cooper and P1142-6195 had relatively low foaming capacity at all tested pHs and the lowest 11S/7S ratio as compared to other samples. It is interesting to notice that the pea protein gelling properties are influenced by the genotypesgenotypes. The protein samples from Earlystar and P0540-41 possess better gelling capacity and their gels showed significantly increased mechanical strength. Among all the pea genotype tested, the high protein line P0540-41presents a better source to generate pea protein concentrate with good overall functional properties to support food applications.

This research is the first of its kind to compare not only the protein content of different pea genotype, but also the protein structure and functional properties between high protein genotype and regular protein genotype. Although, it is an initial study with few samples, the results demonstrate that high protein genotype are comparable, and in some instances, superiors to regular protein genotype in terms of protein functionality and nutritive value. This opens the possibility of breeding genotypesgenotypes in order to achieve not only high protein content, but also to target specific protein physical-chemical and functional properties for desirable industry processing and food applications.

Preface

This thesis is an original work by Rani Lopes Lorenzetti under the supervision of Dr. Lingyun Chen. No part of this thesis has been previously published.

Dedication

This thesis is dedicated to my grandfather, who left this earth too earlier to see it completed. May he rest in peace.

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Chapter 1 – Literature Review

1. Peas and the growing interest in plant proteins

The food industry has been shifting trends on the use of animal proteins. This can be accounted for a variety of factors, such as consumer´s perception of health, ethical and religious purposes, as well as environmental factors (Barac, et al., 2010). Therefore, there has been growing interest in substituting animal proteins by plant-based proteins.

Pulses are defined by the Food and Agriculture Organization of the United Nations (FAO) as legumes harvested solely for their seed which is consumed directly. The FAO list includes eleven primary pulses, such as peas, and excludes the oilseed legumes and those consumed in immature form as vegetables (FAO, 1994). Pulse crops belong to the family of cool season, annually grown leguminous crops (Maiti, 2001). These crops are produced on many continents worldwide. North America is the most accountable for the bulk of pulse crop production and exportation. They are inexpensive source of proteins and other components such as - starch, dietary fiber, vitamins, minerals, and polyphenols (Boye, Zare, & Petch, 2010) (Dave Oomah, Patras, Rawson, Singh, & Compos-Vega, 2011).

Field pea (*Pisum sativum*) is a widely produced legume grown around the globe for both food and feed applications. It has low allergenicity, high nutritional value, availability, and low cost (Shevkani, Singh, Kaur, & Rana, 2015) (Stone, Karalash, Tyler, Warkentin, & Nickerson, 2015). Even though pea does contain anti-nutritional factors, such as phytic acid and phenolic compounds, that can inhibit digestion and may have other possible deleterious effects, pea is still considered a highly nutritious food and is associated with health benefits beyond basic nutrition (Roy, Boye, & Simpson, 2010). Though pea has high potential to be used in food industry, there are some challenges in utilizing pea protein as a food ingredient due to limitations in functionality, and flavor and color issues (Can, Karaca, Tyler, & Nickerson, 2018). Even with these limitations, pea protein is still an excellent candidate for substituting animal protein in food products.

Peas, more specifically the yellow or green cotyledon genotypesgenotypes, are known as dry, smooth, or field peas, are the naturally dried seeds of *Pisum sativum* L. Field pea is one of the most important leguminous crops grown in 84 different countries over the world, which constitute the largest percentage (36%) of total pulse production (Dahl, 2012). The world production of peas in 2009 was more than ten million tons, the major producers being Canada, the Russian Federation, China, the USA and India (FAOSTAT, 2011).

In Canada, even though there is strong production of wheat and canola, pulse area has increased significantly since the 1980s (Bekkering, 2011). Canada has become a leading producer and exporter of pulses worldwide. According to Statistic Canada, in 2017, Canada is the leading producer of field pea. With exports expanding rapidly along with production over the last two decades, Canada now accounts for approximately 35% of global pulse trade each year (Bekkering, 2011).

Several factors played an important role in this success story, among them, the prairie soil, climate conditions and research for developing new genotypesgenotypes. The development and expansion of the pulse industry was closely tied to its profitability, research into new genotypesgenotypes that resist lodging and disease or have a shorter growing season, and the growth of processing facilities (Bekkering, 2011).

1.1. Pea protein components, structure and nutritive value

Pea is rich in protein and carbohydrate, low in fat, and contains several important vitamins and minerals (Boye, Zare, & Petch, 2010) (Swanson, 1990). The protein contains high levels of lysine (15%) but tend to be limiting in methionine and tryptophan (Mertens, et. al., 2012).

Pea composition can be separated into starch, fiber and protein fractions. The carbohydrate content consists mainly of starch, which is composed by 35–40% amylopectin; 24.0–49.0% amylose and dietary fiber (10–15% insoluble and 2–9% soluble) ranging from 60 to 65%, which also includes non-starch carbohydrates such as sucrose, oligosaccharides, and cellulose (Tiwari, Gowen, & McKenna, 2011) (Hoover, Hughes, Chung, & Liu, 2010,). Depending on the variety, maturity at harvest, and growing conditions, field pea can contain 23.1–30.9% protein, 1.5–2.0% fat, and other minor constituents such as vitamins, minerals,

phytic acid, polyphenols, saponins, and oxalates (Boye, Zare, & Petch, 2010) (Tiwari, Gowen, & McKenna, 2011) (Hoover, Hughes, Chung, & Liu, 2010).

According to Osborn, protein can be classified into 4 categories based on their solubility: albumins (water-soluble), globulin (salt solution-soluble), prolamin (alcohol-soluble) and glutelin (alkaline solution-soluble). The main protein fractions in pea are albumins and globulins which account for 10–20% and 70–80%, respectively, of the total protein within the seed (Can, Karaca, Tyler, & Nickerson, 2018) (Duranti & Scarafoni, 1999), with prolamins and glutelin present in minor amounts (Guleria, Dua, & Chongtham, 2009) (Owusu-Ansah & McCurdy, 1991) (Tsoukala, Papalamprou, Makri, Doxastakis, & Braudo, 2006). Pea globulins tend to be high in arginine, phenylalanine, leucine, and isoleucine, whereas the albumin fraction is higher in tryptophan, lysine, and threonine (Swanson, 1990). The major pea storage proteins referred as legumin (11S), vicilin (7S) and convicilin are globulins. Pea legumin is hexamer with a molecular weight (Mw) ~320 to 380 kDa. Vicilin is a trimeric protein of ~170 kDa that lacks cysteine residues and hence cannot form disulfide bonds. A third major storage protein, named convicilin, has a subunit of ~71,000 and a molecular weight in its native form of 290 kDa.

1.2. Effect of Environmental factors on pea protein content

There are a few priorities when breeding peas, which are high yield, early maturation, and resistance to lodging and disease (Vera, Warkentin, & Vandenberg, 2000).

Nikolopoulou et. al. (2007) found that between two locations with a rainfall difference of 209 mm, pea seed grown in the drier location was on average 7% higher in protein. These leads to the idea that higher temperatures and lower rainfall can be associated with higher protein content. However, McLean et al. (1974) observed only a 1.5% increase in protein content between plants grown under extreme moisture conditions of periodic wilting and maximum water capacity. Authors conducting independent studies, using different pea genotypes grown in different locations, have found a negative correlation between protein content and seed yield (Wang, Hatcher, Warkentin, & Toews, 2010) (Sosulski, McLean, & Austenson, 1974) (Al-Karaki, 1999).

1.3. Effect of genotype on pea protein content and functionalities

Plant breeding deals with the inheritance of qualitative and quantitative traits and includes the genetic improvement in existing genotypes for specific trait(s), and the creation of altogether new genotype with new gene combination called recombinant (Singh, Singh, & Singh, 2021). Through plant breeding, or plant/crop improvement, plants have been improved for their productivity, quality traits (physical and chemical), and for various other desirable traits, such as resistance to environmental stresses and harmful parasite and for their suitability to mechanical harvesting (Singh, Singh, & Singh, 2021).

The breeding of pea cultivar has long been driven by the yield, disease resistance, protein content, and mineral content without considering the functionality of protein. In fact, the impact of yellow pea cultivar on the functionality of pea protein has been previously examined and pea protein prepared from different genotype performed differently in their functionalities (Barac, et al., 2010) (Stone, Avarmenko, Warkentin, & Nickerson, 2015). This is not unexpected because each cultivar differs inherently on protein contents, protein composition and conformation. For instance, the ratio of legumin and vicilin, two major globulin proteins in pea accounting for more than 70% of total protein, varies from 0.4 to 2.0 depending on the genotype (Lam, Can Karaca, Tyler, & Nickerson, 2018). Regarding the role of pea composition on the protein functionality, mixed results have been documented so far. Some studies have shown that vicilin has better emulsifying and foaming properties including capacity and stability than legumin (Dagorn-Scaviner, Gueguen, & Lefebvre, 1987), while others found legumin has the higher emulsifying and foaming stability compared to vicilin (Liang & Tang, 2013).

1.4. Allergenicity and bioactivity of pea protein and peptides

Compared to soybean protein, pea protein is generally not recognized as a major food allergen with relatively high nutritional value and without genetic modification, offering a clean label for food products (Day, 2013) (Krefting, 2017). Although proteins in these pulse crops have been found to be allergenic, allergic reactions have been limited and have been confined mostly to Europe, Asia and the Mediterranean (San Ireneo, et al., 2000), probably

due to higher consumption of pulses in these populations. Severe anaphylaxis to pulse crops is infrequent (San Ireneo et al., 2000) which may partially explain why pea, chickpea, lentil and bean are not included in the priority allergen list. Multiple proteins in pulses can provoke allergic reactions and these proteins are often thermo-stable. Furthermore, pea, lentil, bean and chickpea allergens have been reported to be cross-reactive (San Ireneo et al., 2000). (Szymkiewicz & Jedrychowski, 1998).

Many studies suggested that pea protein (in many cases, pea protein hydrolysates and specific peptide fractions) has antioxidant (Ndiaye et al., 2012) (Sun & Xiong, 2015), antihypertensive (Aluko et al., 2015) (Liao et al., 2019), anti-inflammatory (Ndiaye et al., 2012), lowering cholesterol (Sirtori et al., 2012) and modulating intestinal bacteria activities (Swiatecka et al., 2011).

Enzymatic protein hydrolysates of yellow pea seed have been shown to possess high antioxidant (Pownall, Udenigwe, & Aluko, 2010) and anti-bacterial activities (Niehues, Euler, Georgi, & al, 2010). Such hydrolysates may be used as food components in order to beneficially influence human health, by restoration of improperly working antioxidant machinery, which consequently, demonstrates anti-inflammatory and/or antioxidant properties.

2. Protein Extraction Methods

The extraction of protein from pea seeds for use in other food applications has become more common due to their nutritional, functional and economic benefits (Dijkink & Langelaan, 2002) (Makri, Papalamprou, & Doxastakis, 2005) (Singh, Kaur, Rana, & Sharma, 2010). Several methods have been applied to extract proteins from pulse flours. The extraction methods may influence the protein composition and functionalities of the protein isolates.

2.1. Dry Processing

There are two extensively studied dry separation processes, which are air classification and electrostatic separation. Dry fractionation has been proposed as a more sustainable approach

to prepare plant protein-enriched ingredients with retained native functional properties (Schutyser, Pelgrom, van der Goot, & Boom, 2015). A disadvantage of dry fractionation is the low purity obtained, compared to wet fractionation (Wang, Zhao, Wit, Boom, & Schutyser, 2016).

2.1.1. Air Classification

One of the methods that can be used to separate protein from other components of the pea is air classification. This technique separates the flour into fractions of different particle sizes and nutritional compositions (Andersson, Andersson, & Åman, 2000). This technique feeds air currents into a classifier chamber. The air induces centrifugal and gravitational forces inside the chamber that separate the feed flour into fine and coarse particles differing in size and density (Lundgren, 2011). Schutyser et al. (2015) explored the impact of milling settings to optimize the separation of starch granules from cell wall fibers and protein bodies for starch rich legumes (pea, lentil, chickpea, and bean). Feed particles must be sufficiently small and disaggregated for air classification to fractionate cell components (Andersson, Andersson, & Åman, 2000) (King & Dietz, 1987). Because of this, size reduction of the feed material usually precedes air classification to improve separation efficiency. Wu & Nichols (2005) investigated fine grinding and air classification and concluded that the yield of the protein fraction increased with the intensity of grinding before air classification. The starch content of the starch fraction increased with the intensity of grinding and subsequent air classification. After fine milling, the larger starch granules are physically detached from the smaller protein-rich particles, which allows separation (Tyler & Panchuk, 1982). Too coarse milling, however, leads to the presence of aggregates of protein bodies, starch granules, and other cell components, which does not allow subsequent separation. Too fine milling leads to extensive starch damage and affects separation negatively, as the starch granule fragments, and protein bodies have similar sizes. The protein content of the fine fraction depends on the initial protein content of the flour and the dispersibility of the flour (Dijkink, Speranza, Paltsidis, & Vereijken, 2007) (Reichert, 1982). Complete separation of protein from starch is hindered because some proteins are still adhered to the starch granules after milling (Vose, Separating grain components by air classification, 1978). A second milling step can be applied to increase the protein yield. However, a side effect is that more damaged starch will

be present in the second protein fraction, decreasing purity (Tyler, Youngs, & Sosulski, 1981).

2.1.2. Electrostatic separation

Alternatively to air classification, which relies on particle size and/or density, electrostatic separation relies on triboelectric charging as a driving force for separation (Hemery, et al., 2011). Tribo-electrostatic separation has been long applied in the mining (Cangialosi, Notarnicola, Liberti, & Stencel, 2008) (Dwari & Hanumantha Rao, 2009) and plastics (Park, Jeon, Yu, Han, & Park, 2007) (Wu, Li, & Xu, 2013) industries, and is currently attracting attention by the food industry (Hemery, et al., 2011) (Hemery, et al., 2009) (Wang, Smits, Boom, & Schutyser, 2015). Due to their different tribo-electrostatic properties, different materials such as protein and fiber, charge either positively or negatively when sliding along a surface made of a different material (Xing, Wit, Kyriakopoulou, Boom, & Schutyser, 2018,).

The advantage of this approach is that mixed particles of similar size but of different composition may be separated. For example, Pelgrom et al. (2015) applied electrostatic separation as a post-treatment to further increase the protein content of the fractions obtained by air classification. It was observed that electrostatic separation could deliver lupine protein enriched fractions (protein concentration of ~ 59 g/100 g dry solids) not only from the fine fraction, but also directly from the coarse fraction and whole flour.

There is still limited information on the functional properties of electrostatically enriched protein fractions compared to the wet-fractionated plant protein products.

2.2. Wet extraction

2.2.1. Alkaline Extraction

Alkaline extraction can be followed by isoelectric precipitation or ultrafiltration to collect proteins.

Alkaline solution extraction followed by isoelectric precipitation is a conventional extraction method and is largely used throughout industrial and research processes. As in dry

processing, previously to the extraction, the sample must be made into flour form. Then, the proteins are defatted, suspended in water and a suspension of protein and fiber is obtained. The solubilized proteins are separated from insoluble components, such as fibers and starch at pH 9. Then, protein can be collected by isoelectric precipitation or ultrafiltration. In the isoelectric precipitation methods, proteins are separated from other soluble components, such as soluble fibers, by precipitation at their iso-electric point (pH 4.5–4.8 for pea globulins). Subsequently, the pH is readjusted to 7 and a dry protein isolate is obtained after a final drying step (Berghout, Pelgrom, Schutyser, Boom, & van der Goot, 2015) (Boye, Zare, & Petch, 2010).

The advantages of alkaline extraction followed by isoelectric precipitation method include high purity of the protein extracts to obtain protein concentrates or isolates, low cost, convenient and easy to scale-up. There are some disadvantages to this method. For example, the use of an excessive amount of water and subsequent drying make the wet extraction procedure a water and energy intensive process (Trivelato, Mayer, Barakat, Fulcrand, & Aouf, 2016). The dilution steps result in a consumption of 50 kg water/kg recovered protein (Berghout, Pelgrom, Schutyser, Boom, & van der Goot, 2015). Moreover, the harsh extraction conditions, especially the high temperatures and extreme pH, affect the native functional protein properties (Matsumiya & Murray, 2016) (Föste, Elgeti, Brunner, Jekle, & Becker, 2015).

2.2.2. Salt Extraction

Pea protein is mainly composed of globulins, as previously stated, and globulins are salt soluble. In this sense, salt extraction is a suitable method to extract pea globulins.

Salt extraction (SE) takes advantage of the salting-in and salting-out phenomena of proteins, followed by a desalting process to lower the ionic strength of the protein environment (Boye, Zare, & Petch, 2010) (Canadian Intellectual Property Office: Gatineau, QC Patente Nº 1,028,552, 1978.). Briefly, flour is stirred for 10–60 minutes in a salt solution of specified ionic strength at a 1:10 (w/v) ratio, followed by the removal of insoluble matter by settling, decanting, screening, filtering, or centrifuging. The supernatant is then desalted and dried (Boye, Zare, & Petch, 2010) (Canadian Intellectual Property Office: Gatineau, QC Patente

Nº 1,028,552, 1978.) (Gueguen & Barbot, 1988). Desalting can be achieved by dialysis, ultrafiltration or micellar precipitation. The concentration and choice of salt or mixture of salts are selected according to the salting-in characteristics of the protein to be isolated as well as the salting-out characteristics of any unwanted proteins, since proteins precipitate at an array of ionic strengths (Berg, Tymoczko, & Stryer, 2002) (Patent and Trademark Office: Washington, DC Patente Nº U.S. Patent No. 4,321,192, 1892.). Other factors to consider include adverse interactions between the salt and sample components and ensuring the use of food-grade salts (Canadian Intellectual Property Office: Gatineau, QC Patente Nº 1,028,552, 1978.) (Ahmed, 2005). Generally, salting-in of proteins occurs at low ionic strength, between 0.1 and 1 M. Some advantages of SE are that extreme alkaline or acidic pH, or elevated temperature, is not required. Extraction occurs at the natural pH level of the protein/water/salt mixture of 5.5–6.5, although Crévieu et al. (1996) suggested the use of a slightly alkaline pH to maximize protein solubility (Canadian Intellectual Property Office: Gatineau, QC Patente Nº 1,028,552, 1978.). The addition of acid or base might be needed to maintain the pH within this range, or a salt solution with a buffering capacity may be used.

Using a 0.25 M NaCl solution at pH 6.5 and a micellization standing time of 6 hours, Mwasaru et al. found that pigeon pea yielded a product with a protein extractability of 40.2% total seed protein, whereas cowpea yielded a product with 36.7% protein extractability. These values are comparable to those of alkaline-extracted samples at pH 10.5 and 8.5, respectively, where yields increased with alkalinity. Meanwhile, Gueguen & Barbot (1988) reported that up to 95% yield might be attainable using the micellization method. Another widely used method for desalting is dialysis. Dialysis is a membrane separation process driven by a chemical potential gradient to diffuse water and low molecular weight solutes, such as salt, across a semipermeable membrane. For pea proteins, Gueguen & Barbot (1988) and Crévieu et al. (1996) used membranes with cutoffs of 8,000 Da and 12,000–14,000 Da, respectively. Diffusion requires time for both sides to equilibrate and is complete when the chemical gradient becomes negligible (Patent and Trademark Office: Washington, DC Patente Nº U.S. Patent No. 4,321,192, 1892.). Multiple changes of fresh, precooled liquid against which the sample is dialyzed ensures that very low concentrations of solutes remain in the sample. For example, Gueguen and Barbot cited a 130-hour process that required five changes of water of 20 times the extract volume. Crévieu et al. (1996) dialyzed globulin solutions against two

changes of 10 times the extract volume of ammonium carbonate, which required 70 hours and resulted in a 66.8% yield. Dialysis is also useful for separating albumin and globulin fractions. According to the Osborne protein classification, centrifugation of the dialyzed sample results in dissolved albumin fractions in the supernatant and precipitated globulin fractions in the pellet (Gueguen & Barbot, 1988). The presence of phenolic compounds within the pea can also be reduced through additional steps during processing, such as the use of charcoal filters and alcohol washes. Although phenolic compounds are known to have antioxidant activity, they can also promote cross-linking of proteins, which negatively affects protein digestibility, enzymatic activity, and functionality, and can lead to undesirable flavor and color compounds within the food product.

2.3. New technologies

There are many new technologies being studied for pulse extractions. Although studies specifically on pea protein extraction are not always available, the following techniques can be considered for future novelty studies.

2.3.1. Enzyme-assisted extraction

Enzyme-assisted extraction using protease was found to improve both the extraction of oil and protein from soy flour (Rosenthal, Pyle, Niranjan, Gilmour, & Trinca, 2001) (Sari, Mulder, Sanders, & Bruins, 2015). The effects of protease were also studied with positive effects in combination with membrane recovery (De Moura, Campbell, De Almeida, Glatz, & Johnson (2011) and enhanced protein solubility from full fat extruded flakes (De Almeida, De Moura Bell, & Johnson, 2014). Protease may improve the solubility of soy proteins, especially when they are denatured and/or aggregated. In general, the smaller the proteins, the better their solubility. In addition, protease may cleave proteins from cell wall materials. Cellulases have also been used to degrade cell walls, although the results were limited (Kasai, Murata, Inui, Sakamoto, & Kahn, 2004) (Kasai, Imashiro, & Morita, 2003) (Rosenthal, Pyle, Niranjan, Gilmour, & Trinca, 2001). The main issues with cellulases are that most of them are not food-grade and that the food-grade enzymes are most active at pH 5; at this pH soy proteins tend to aggregate since their pI is 4.5. One enzyme that has shown promise in previous research (Rosset, Acquaro, & Beléia, 2014) is Viscozyme L, a multi-component

carbohydrolase containing arabanase, cellulose, hemicellulose and xylanase. Viscozyme L resulted in a protein yield improvement of 23% at pH 9 from defatted soy flakes versus a control sample upon a 30 min incubation at a 1:20 solid-to-liquid ratio at 60 °C (Rosset, Acquaro, & Beléia, 2014). A few disadvantages of this method are enzyme treatment times are usually long (30 min–h), and expenses of both enzyme and processing are relatively high.

3. Protein composition in extracts especially Legumin/Vicilin Ratio as impacted by variety and processing

3.1. Legumin/Vicilin in peas

The globulins can be classified based on their sedimentation coefficients into two main types, legumin (11S) and vicilin (7S) (Mertens, Dehon, Bourgeois, Verhaeghe-Cartrysse, & Blecker, 2012). These two components exhibit unique functional attributes due to their different amino acid profile, size, and structure (Can, Karaca, Tyler, & Nickerson, 2018). The expression of these two components is determined by several factors, such as, the processes employed in production and the cultivar growing environment of the raw material, which implicate directly their functionality, and therefore, their use.

Legumin is a hexametric molecule (molecular weight: ~ 380 kDa) where each monomer (molecular weight: ~ 60kDa) contains an acidic (40 kDa) and basic (20 kDa) subunit linked by a disulphide bond (Can, Karaca, Tyler, & Nickerson, 2018). This monomer is located within a non-covalently linked, quaternary structure (Mertens, Dehon, Bourgeois, Verhaeghe-Cartrysse, & Blecker, 2012) (Sikorski, 2001). Legumin oligomer heterogeneity depends on both molecular weight and isoelectric point of its subunits (O'Kane, Happ, Vereijken, Gruppen, & van Boekel, 2004).

Vicilin is a trimeric molecule (molecular weight: ~ 150k Da) where each monomer can contain gaps in its polypeptide chain, giving rise to a variety of smaller subunits. Similar to legumin, vicilin is held together by hydrophobic interactions and has no covalent disulfide bonds. Vicilin is glycosylated and has a more hydrophilic surface than legumin, which makes

it more water soluble. Vicilin contains low levels of sulfur-containing amino acids (methionine, cysteine) and tryptophan, and higher levels of basic (arginine, lysine) and acidic (aspartic acid, glutamic acid) amino acids (Jackson, Boulter, & Thurman, 1969). N-terminal amino groups typically are represented by serine, glutamic acid, and aspartic acid (Sikorski, 2001).

The ratio of legumin-vicilin (Lg/Vc) of field peas at maturity can range from 0.4 to 20 (Schroeder, 1982). There are some differences in the vicilin and legumin formation throughout seed development, as they have different synthesis rates. Vicilin synthesis is dominant from early development until 17 days after flowering, whereas legumin is rapidly synthesized in the later stages of development, from 20 days after flowering and onward (Chandler, Spencer, Randall, & Higgins, 1984). This is directly connected to the seed nutrient necessity during its development, as these two components are storage proteins.

The Lg/Vc ratio has been extensively studied, by comparing the impact of environmental factors, phases of seed maturity, as well as genetic variation. Danielsson (1952) measured a change in Lg/Vc ratio from 0.37 to 0.67 in field pea seed sampled 20 days apart, which demonstrates how the Lg/Vc increases throughout the seed development. This agrees with Chandler et. al. (1984) studied the Lg/Vc along the stages of development. Tzitzikas et al. (2006) analyzed the genetic variation in pea seed globulin protein composition of 59 different genotypes. These authors found that vicilin proteins mostly dominate, having Lg/Vc ratios ranging from 0.12 to 0.77. Boye et. al. reported Lg/Vc ratios ranged from 0.23 to 0.50 for wrinkled pea genotype, and from 0.31 to 1.67 for smooth pea genotype. This contradicted Cousin et. al., who verified that even though wrinkled genotypes had lower protein content, they had much higher Lg/Vc ratio than smooth pea genotypes (Cousin, Maltese, & Burghoffer, 1992).

Legumin and vicilin are environmentally sensitive in pea and are highly susceptible to extrinsic factors such as agronomic practice, environmental conditions, and even the method used to determine protein composition (Mertens, Dehon, Bourgeois, Verhaeghe-Cartrysse, & Blecker, 2012). An example of environmental impact on vicilin synthesis was studied by Chandler et al. (1984) When pea is grown under sulfur-deficient conditions, vicilin synthesis

is maintained throughout development, whereas the synthesis of the relatively sulfur-rich legumin is greatly compromised or undetectable.

Another key influential factor on the properties of pea protein is the extraction condition, i.e., extraction and drying methods, which is the most basic knowledge needed for producing satisfactory products. The extraction process can affect protein functionalities because it involves exposing protein to an external environment (e.g., pH, heat, salts) that can alter protein structure, composition, conformation, and/or surface charge which altogether lead to different functionalities (Cui, et al., 2020). Several studies have successfully elucidated the impacts of different extraction methods (e.g., alkaline extraction-isoelectric precipitation, salt extraction-dialysis, ultrafiltration, diafiltration and micellar precipitation) on the functional properties of pea protein (Boye, Zare, & Petch, 2010) (Fuhrmeister & Meuser, 2003) (Can Karaca, Low, & Nickerson, 2011) (Stone, Karalash, Tyler, Warkentin, & Nickerson, 2015).

Alkaline extraction - isoelectric precipitation is based on dissolving the protein in alkaline medium. Another method is to dissolve the protein at acidic conditions far away from the isoelectric point (Mession, Assifaoui, Cayot, & Saurel, 2012). Then, the proteins are precipitated at their isoelectric point at pH 4.5. Since the isoelectric point of globulins and albumins is different, mainly globulins are extracted with this method. In contrast, a mixture of globulins and albumins is extracted by salt extraction (Stone, Karalash, Tyler, Warkentin, & Nickerson, 2015). In micellar precipitation, the protein is extracted in a neutral salt solution. The soluble proteins are precipitated by reducing the salt content in the solution leading to micelle-type form (Tanger, Engel, & Kulozik, 2020). Hydrophobic interactions may play a major role in stabilization of the micelles. Only globulins are extracted with this procedure. They may be less denatured compared to proteins extracted by alkaline extraction - isoelectric precipitation (Arntfield, Ismond, & Murray, 1985) (Paredes-López & Ordorica-Falomir, 1986).

3.2. Methods of determination of Legumin/Vicilin

Various methods have been applied to the quantification of the legumin and vicilin. Most methods utilize the molecular masses and chemical properties of the two fractions for their separation.

Ultracentrifugation is one of these methods. Proteins are subjected to a strong centrifugal field which separates the two fractions based on their different on their size and density, and the concentration distribution of the rate of settling is measured by light absorption and refraction (Svedberg, 1979). Another commonly used method is size exclusion HPLC, which separates molecules based on their molecular weight by filtration through a gel column.

Differential scanning calorimetry (DSC) is another method in which protein fractions are differentiated by their temperatures and enthalpies during denaturation (Chambers, Bacon, & Lambert, 1992). The two major components in pea protein are legumin and vicilin. Each of them has a different size, and therefore a different denaturation temperature. Denaturation temperature is the temperature in which the tertiary structure of the protein is lost. DSC is a thermo-analytical technique in which the difference in the amount of heat required to increase the temperature of a sample and reference is measured as a function of temperature (Karoui, 2001).

Immunological techniques can also be applied to legumin and vicilin determination through the formation of complexes with antibodies and proteins, which are subsequently characterized (Casey, 1979). In these techniques, each protein has a specific antibody, and therefore, the complexes are only formed when both are available. An example of characterization is Laurell's rocket immunoelectrophoresis, where proteins pass through an agarose gel containing antibodies and are analyzed against a standard (Laurell, 1966).

4. Protein Structure Characterizations

4.1. Molecular weight – SDS page and HPLC

The most used technology to obtain high resolution analytical separation of mixtures of proteins is sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Walker, 2002) (Gallagher & Wiley, 2008). The procedure involves initial denaturation of component proteins with an anionic detergent that also binds to them, imparting to all proteins a negative charge proportional to their molecular mass. This step is followed by electrophoresis through a porous acrylamide gel matrix that separates proteins with excellent resolution based on

molecular mass. Largely unchanged since its introduction the early 1970s, this method works well in applications that do not require retention of native features of protein structure or function (Walker, 2002) (Laemmli, 1970). Thus, assessment of purity of protein samples, evaluation of protein expression, and immunochemical identification and quantification of proteins (western blotting) are methods that utilize SDS-PAGE (Walker, 2002) (Gallagher & Wiley, 2008) (Simpson, 2003).

An obvious limitation of SDS-PAGE resides in its deliberate denaturation of proteins prior to electrophoresis. Enzymatic activity, protein binding interactions, detection of protein cofactors, etc. generally cannot be determined on proteins isolated by SDS-PAGE. Instead, other methods must be employed to separate native proteins for investigations of structure-function relationships (Walker, 2002) (Wittig & Schägger, 2005). One such alternative is the blue-native PAGE technique (Schägger & von Jagow, 1991). This method has been used in the determination of protein-protein interactions, in which proteins in the sample are separated as oligomers in first dimension BN-PAGE, followed by a denaturing second dimension SDS-PAGE to identify the monomers within the oligomers (Camacho-Carvajal, Wollscheid, Aebersold, Steimle, & Schamel, 2004) (Swamy, Siegers, Minguet, Wollscheid, & Schamel, 2006) (Wittig, Braun, & Schagger, 2006) (Schamel & Reth, 2000) (Schägger, et al., 2004). However, as a one-dimensional separation method, it faces the opposite problems of SDS-PAGE. While BN-PAGE retains the native state of proteins, it falls short of the high resolution of proteomic mixtures that is attained with SDS-PAGE and can add ambiguities to successful molecular weight determinations (Raab, Pioselli, Munro, Thomas-Oates, & Feldmann, 2009) (Sussulini & Becker, 2011) (Lasserre, et al., 2006).

Another method that is widely used for characterizing molecular mass is High Performance Liquid Chromatography (HPLC). In theory, HPLC provides the capability of determining the “absolute” molecular weights of proteins without the need to dissociate them into subunits. HPLC SEC is nondestructive, and the samples may be recovered for use in subsequent studies. Compared with techniques such as analytical centrifugation, HPLC SEC is rapid, and samples may be analyzed easily at various pH values, ionic strengths, and temperatures and in the presence or absence of ligands.

4.2. Protein conformation - Fourier-transform infrared spectroscopy (FTIR)

Protein conformation may be defined as the arrangement in space of its constituent atoms which determine the overall shape of the molecule. The conformation of the protein arises from the bonding arrangements within its structure, and therefore is dependent on the pea composition. The conformational and composition differences of pea protein have not been sufficiently investigated. However, it is known that in most functional properties the unfolding of the protein affects the functionality. Therefore, it is important to understand how the protein's 3D structure differs among different pea genotypes.

FTIR is an established method to analyze the secondary structure of proteins because it is a non-destructive method that requires little sample preparation, and it can be applied to a wide variety of conditions (Kong & Yu, 2007). Infrared spectroscopy is based on molecular vibrations. Chemical bonds undergo various forms of vibrations such as stretching, twisting and rotating. The energy of most molecular vibrations corresponds to that of the infrared region of electromagnetic spectrum. Many of the vibrations can be localized to specific bonds or groupings, such as the C═O and O–H groups (Haris & Severcan, 1999). Typical group frequencies of interest to biochemists include CO, –COOH, COO−, O–H and S–H. With developments in FTIR instrumentation it is now possible to obtain high quality spectra from dilute protein solutions in H2O (Haris & Chapman, 1992) (Surewicz, Mantsch, & Chapman, 1993). The overlapping H2O absorption can be digitally subtracted from the spectrum of the protein solution. In addition, the broad infrared bands in the spectra of proteins can be analyzed in detail using second derivative and deconvolution procedures. These procedures can be utilized to reveal the overlapping components within the broad amide bands. Protein secondary structure can be determined through analysis of the amide I band (stretching vibrations of C=O in the peptide bond) (Jackson and Mantsch 1995). The sensitivity of amide I to conformational changes makes it possible to study not only protein folding and unfolding, but also aggregation processes.

The most important advantage of FTIR spectroscopy for biological studies is that spectra of almost any biological material can be obtained in a wide variety of environments. Spectra of a protein can be obtained in single crystals, in aqueous solution, organic solvents, detergents

micelles, lipid membranes, etc. The chemical environment in which a peptide or protein exists influences its structure and stability. This has important implications for the formulation, storage and delivery mechanisms for protein therapeutics. There is increasing evidence indicating that the environment can be important in determining the secondary structure formed by an amino acid sequence. Other advantages of the technique include the following: the amount of protein required is relatively small (10 μg); the size of the protein is not important; there is no light scattering or fluorescent effects; kinetic and time-resolved studies are possible; and inexpensive compared to the cost of X-ray diffraction, NMR, ESR and CD spectroscopic equipment.

4.3. Protein Denaturation Properties- Differential scanning calorimetry (DSC)

The stability of the native state of a protein determines under which conditions the protein is folded and thus active. Accurate measurements of protein stability are important if we wish to understand the underlying interactions that stabilizes a protein structure and manipulate proteins to be more (or less) stable. Protein stabilities are typically determined by gradually changing temperature or the concentration of a chemical denaturant and measuring the unfolding by a spectroscopic technique or calorimetry.

Functions of proteins are developed by folding of linear peptide chains into programmed ternary conformations. Since the folded conformation is constructed not only by covalent bonds such as disulfide bonds but also by non-covalent interactions including hydrogen bonding and hydrophobic interactions, protein higher-order structures are perturbed by a change of temperature, pressure and pH and contamination of a denaturation agent (Muraoka, et al., 2014). Once the folded structure is collapsed, the hydrophobic amino acid residues are exposed to the aqueous environment and readily interact with other hydrophobic parts similarly appeared by unfolding. This is a crucial step in the gelation process in proteins, as it involves the denaturation of the proteins followed by their aggregation, and then association of the aggregates to form a three-dimensional gel network structure. On the other hand, formation of large aggregates will lead to insoluble precipitates. Denaturation of protein is also important when considering industrial processing, as most processes involve heating steps.

Protein denaturation can be measured by differential scanning calorimetry (DSC), where the excess heat capacity of unfolding is quantified, and gives a direct measure of the enthalpy for folding and the melting temperature. DSC thus provides a full thermodynamic description of the folding process if it is reversible, though fitting DSC experiments and determining the thermodynamic stability at ambient temperatures is not always trivial.

5. Functional properties of pea protein in relation to structure as impacted by extraction method

The functional properties of pea protein discussed here refer to the techno functionality, including solubility, water holding capacity (WHC) and oil holding capacity (OHC), emulsifying properties, foaming properties, and gelling properties. These properties will determine the behavior and performance of pea protein in food systems during preparation, processing, storage, and consumption, thereby affecting food texture, stability, and organoleptic characteristics.

5.1. Solubility

Protein solubility can be defined as the equilibrium between protein–protein (hydrophobic) and protein–solvent (hydrophilic) interactions, expressed as Protein-Solvent ↔ Protein-Protein + Solvent-Solvent (Hall, 1996). At pH values above and below the isoelectric point (pI), solubility is increased due to electrostatic repulsion brought on by positive and negative net charges on the protein surface (Hall, 1996). A protein exhibits the lowest solubility at its isoelectric pH since it carries a zero net charge, thereby minimizing electrostatic repulsive forces. Under these conditions, hydrophobic interactions between neighboring proteins can lead to aggregation, and once the aggregates are sufficient in size and number, precipitation occurs (Zayas, 1997). In general, pea globulins have an pI of 4-5; thus, pea protein isolates exhibit the lowest solubility between pH 4 and 6 irrespective of the extraction method or pea cultivar (Boye, Zare, & Petch, 2010) (Taherian, et al., 2011) (Withana-Gamage, Wanasundara, Pietrasik, & Shand, 2011).

Extraction and processing conditions may influence pea protein solubility. Using a pea protein isolate obtained from a commercial manufacturer, Taherian et al. (2011) suggested that hydrophobic segments of legumin might have become exposed during processing, resulting in increased surface hydrophobicity of the product. Shevkani et al. (2015) tested isolates from five pea genotypes produced by AE/IEP under similar conditions of pH 2–9, and also found a U-shaped solubility profile. The authors reported solubility values of 66–77% at pH 2 and 70–95% at pH 9, but only 2–4% at pH 5. Karaca et al. (2011) observed a negative correlation between solubility and surface hydrophobicity, as well as a positive correlation between solubility and surface charge. These authors reported that at pH 7, pea protein isolates prepared from the CDC Leroy cultivar (forage market class) had solubilities of 61% and 38% when extracted by alkalyne extraction/isoelectric point (AE/IEP) and SE, respectively. Pea showed lowered solubility relative to Kabuli chickpea, faba bean, lentil, and soy isolates, in addition to high surface hydrophobicity and low surface charge. Boye et al. (2010) reported that a pea protein isolate sourced from the CDC Golden cultivar (yellow market class) displayed higher solubility at pH values 1 and 7 compared to isolates from red and green lentil and desi and Kabuli chickpea.

Differences were noted for pea protein isolates extracted by AE/IEP and UF, where AE/IEP resulted in a solubility of 90% at pH 1, but 29% at pH 3, whereas UF isolates exhibited solubilities of 60% and 56%, respectively. Vose (1980) also reported such difference for protein isolates prepared from Trapper (forage market class) pea, where AE/IEP isolates exhibited 66% solubility at pH 3 and 7. At these pH levels, an isolate prepared using UF displayed lower solubility but exhibited 15% higher solubility overall between pH 2 and 10. Stone et al. (2015) tested protein isolates prepared from green (CDC Striker), yellow (CDC Meadow), and dun (CDC Dakota) market classes of pea using three extraction methods, and reported the highest solubility from isolates produced using SE (86–91%), followed by AE/IEP (63–64%), and then by micellization (43–49%). The authors hypothesized that protein–protein hydrophobic interactions in the AE/IEP and micellization samples might be responsible for the reduced solubility.

Depending on the type and concentration of the salt present, proteins may undergo “salting-in” or “salting-out”. In the case of the former, thiocyanate, perchlorate, barium, and calcium

salts promote protein–water interactions and ordering of hydration layers surrounding the protein to increase solubility (Hall, 1996) (Damodaran, Parkin, & Fennema, 2008) (Walstra, 2003). In contrast, sulfate, hydrogen phosphate, ammonium, and potassium salts promote ion–water interactions, which act to disrupt the hydration layers surrounding the proteins to cause the exposure of hydrophobic moieties (Hall, 1996) (Damodaran, Parkin, & Fennema, 2008) (Walstra, 2003). Consequently, aggregation and precipitation ensue, depending on the ionic strength and level of hydrophobicity.

Generally, protein solubilization increases as the temperature is raised from 0–50 °C up to a temperature where non-covalent bonds (e.g., hydrogen bonds) become destabilized and secondary and tertiary structures are lost (Hall, 1996). Protein denaturation induces interaction between hydrophobic groups, which leads to precipitation and decreased solubility. However, Walstra (2003) stated that hydrophobic interactions increase greatly between 0 °C and 60 °C. In contrast, organic solvents such as acetone lower the dielectric constant of the solvent medium, where the dielectric constant is defined as an index of resistance to an electric current passing through a sample. This unfolds protein molecules through increased repulsive, intramolecular electrostatic forces, and promotes intermolecular electrostatic forces between oppositely charged groups, resulting in precipitation (Nielsen, 2010) (Damodaran, Parkin, & Fennema, 2008).

5.2. Water Holding Capacity

Water-holding capacity (WHC) is defined as the amount of water that can be absorbed per gram of protein material (Boye, Zare, & Petch, 2010) or the ability of proteins needed to retain water against gravity (Shevkani, Singh, Kaur, & Rana, 2015). It is often used interchangeably with the term's hydration capacity, water binding capacity, and water absorption capacity in the literature. Because conventional food products can comprise more than 50% water, poor WHC can further trigger liquid loss during processing and unfavorably alter the texture of products (Hall, 1996). Water binding occurs through a combination of ion–dipole, dipole–dipole, dipole-induced dipole, and hydrophobic interactions (Damodaran, Parkin, & Fennema, 2008). The association between water and protein is affected by the protein matrix structure, especially pore size (Hall, 1996).

The amino acid composition of a protein is one determinant of WHC. Water molecules bind to the charged groups, backbone peptide groups, amide groups, hydroxyl groups, and nonpolar residues of amino acids, where each group varies in its capacity to bind water molecules (Damodaran, Parkin, & Fennema, 2008). Highly charged proteins exhibit greater electrostatic attraction toward water (Stone, Karalash, Tyler, Warkentin, & Nickerson, 2015).

Moreover, WHC is the lowest at the isoelectric pH of a protein because protein–protein interactions are at their greatest. WHC also increases at low salt concentrations due to salt ions binding water to proteins (Damodaran, Parkin, & Fennema, 2008).

The extraction and processing conditions influence protein structure, consequently the protein WHC. Fuhrmeister and Meuser (2003) reported WHC values of ~4.0 g/g for a pea protein isolate (Pisane HD, Cosucra, Warcoing, Belgium) and ~4.6 g/g for a soy protein isolate (Soyamin 90, Lucas Meyer Ltd., Hamburg, Germany), both of which were commercially produced. They found that isolates from wrinkled pea extracted using acid, heat, and heat-acid precipitation had WHC values of 1.5–2.7 g/g. Also, greater WHC was found in more denatured isolate products. The increase in WHC can be attributed to the exposure of hydrophilic groups (Damodaran, Parkin, & Fennema, 2008). Swanson (1990) also reported that the WHC of pea protein concentrates increased with the severity of heat treatment. Boye et al. (2010) found that protein concentrates extracted using AE/IEP held more water than those recovered by UF, although not significantly more. They reported that protein concentrates from yellow pea (2.7g) showed higher WHC than green lentil, red lentil, desi chickpea, and kabuli chickpea. Like these results, Withana-Gamage et al. (2011) found that pea protein isolates prepared by AE/IEP had a WHC value of ~2.6 g/g, which was lower than those of isolates prepared from five genotypes of desi and kabuli chickpea. Swanson (1990) reported that at pH 7, a pea protein isolate was able to retain 2.7–2.8 times its initial weight of water, which was less than soy protein isolate at four to five times its initial weight. Using isolates produced from three genotype of pea (yellow, green, and dun market classes) and by three extraction methods, Stone et al. (2015) found WHC values for AE/IEP isolates similar to those in the above-mentioned studies (2.4–2.6 g/g). The authors reported higher values for protein isolates extracted by micellization, citing that the exposure of side chains and polar groups permitted greater hydrogen bonding. In other studies where protein isolates

were prepared using the AE/IEP method, Shevkani et al. (2015) obtained higher WHC from five pea genotypes (3.9–4.8 g/g), whereas Stone et al. (2015) reported slightly lower values for seven pea genotype (1.9–2.4 g/g), with no differences amongst genotype.

5.3. Oil Holding Capacity

Oil-holding capacity (OHC), or oil absorption capacity, is defined as the amount of oil that can be absorbed per gram of protein (Lin & Zayas, 1987). Lipids and proteins interact through the binding of the aliphatic chains of lipid to the nonpolar side chains of amino acids; therefore, proteins with higher hydrophobicity tend to have a greater propensity to hold oils (Withana-Gamage, Wanasundara, Pietrasik, & Shand, 2011) (Sanjeewa, 2008). OHC values can be influenced by the matrix structure of a protein, the type of lipid present, and the distribution and stability of lipids. The latter is affected by both droplet size and distribution and the presence of emulsifying agents (Hall, 1996). Understanding the OHC is important as it relates to the emulsifying capacity of a protein and is an important characteristic when pea protein ingredients are used in meat binder applications. The reported OHC values for pulse isolates are quite variable, and relate to, amongst other factors, the type and variety of pulse and the processing conditions used to prepare the isolate (Boye, Zare, & Petch, 2010). Using isolates of Miranda yellow pea precipitated by acid, magnesium, or calcium via 13 pH and temperature combinations, Soetrisno & Holmes (1992) found OHC to be consistently lower as the extraction temperature was decreased. They also found that the interaction of high pH and temperature decreased OHC for both magnesium- and calcium-precipitated isolates, whereas the interaction of low pH and temperature affected only magnesium precipitation. This suggests that the choice of precipitating agent can affect the OHC of salt-extracted protein isolates; however, no possible mechanism was proposed. The highest OHC values were 5.22 g/g and 5.10 g/g for magnesium and calcium-precipitated pea protein isolates, respectively. An AE/IEP pea protein isolate (cultivar undisclosed) was reported by Withana-Gamage et al. (2011) to have an OHC value of 2.70 g/g, which was much lower than those of isolates from kabuli and desi chickpea (3.06–5.74 g/g). Meanwhile, Boye et al. (2010) reported an OHC of 1.20 g/g for a pea protein isolate prepared by AE/IEP, which was between those of isolates from fababean (1.60 g/g) and soy (1.10 g/g) in terms of magnitude. Comparable results were found by Stone et al. (2015), who observed OHC values of 1.1–1.3

g/g for seven pea genotype produced by AE/IEP. The OHC values of commercially available isolates from smooth pea (Pisane HD, Cosucra) and soybean (Soyamin 90, Lucas Meyer Ltd.) were reported to be 1.59 g/g and 1.23 g/g, respectively. Boye et al. (2010) conveyed that the OHC values of isolates from yellow pea (CDC Golden), red and green lentil, and kabuli and desi chickpea were similar when the isolates were prepared by AE/IEP. However, red lentil and yellow pea had the greatest OHC at 2.26 g/g and 1.77 g/g, respectively, when prepared by UF. Fuhrmeister and Meuser (2003) found that a wrinkled pea isolate prepared by UF had an OHC of 1.32 g/g, whereas the OHC of isolates precipitated by acid, heat, or acid-heat treatments did not exceed 0.87 g/g. Meanwhile, the OHC of an acid-precipitated Miranda yellow pea isolate was 5.34 g/g, which Soetrisno and Holmes (1992) attributed to the exposure of hydrophobic groups during denaturation in the extraction process. Significant OHC differences attributed to the isolate production method were also observed by Stone et al. (2015). Using three genotype of pea from the yellow, green, and dun market classes, the authors reported OHC values of ~3.7 g/g, ~5.3 g/g, and ~3.6 g/g when the isolates were produced by AE/IEP, SE, and micellization, respectively.

5.4. Emulsification

An emulsion is the dispersion or suspension of two immiscible liquids created by mechanical agitation, resulting in a dispersed phase of submicron droplets suspended within a continuous phase (Hall, 1996). In foods, emulsions are of either oil-in-water (O/W) type, such as milk and mayonnaise, or water-in-oil (W/O) type, such as butter and margarine (Alzagtat & Alli, 2002). The emulsifying capacity of a protein is the ability to emulsify oil. Emulsifying properties are useful functional characteristics which play an important role in the development of new sources of plant protein products for uses as foods.

Emulsifying ingredients from dairy and egg proteins are used for milk, creams, salad dressings, mayonnaise, soups, margarine, and butter (Lam & Nickerson, 2013). With the consideration of sustainability, emulsifying ingredients from plant proteins are favorable in food formulations. Proteins adsorb to the interface to minimize the interfacial tension between the two phases (A. C. Y. Lam, Can Karaca, Tyler, & Nickerson, 2018). They align at the interface according to their amphiphilic nature and conform to train, loop, and tail

configurations to form a viscoelastic interfacial film. Trains lie along the interface, whereas loops and tails protrude into the continuous phase to facilitate repulsion (Alzagtat & Alli, 2002; Walstra, 2003).

Emulsions are thermodynamically unstable because such an arrangement increases the interfacial area, thereby increasing the interfacial free energy of the system. Over time, O/W emulsions are prone to the phenomena of creaming, flocculation, and coalescence as the system attempts to minimize its free energy (Walstra, 2003). Creaming is the reversible rise of dispersed droplets to the surface against gravity due to density differences (0.05 g/cm3 for most food-grade oils) between the two phases (Damodaran, 2005). Flocculation is the reversible or irreversible aggregation of dispersed droplets due to an imbalance of attractive (van der Waals) and repulsive (electrostatic and steric) forces. Coalescence occurs when the continuous phase film separating the dispersed phase is ruptured, resulting in the irreversible merging of individual dispersed droplets into larger droplets (Damodaran, 2005) (Dickinson, 2010). Proteins adsorb to the interface to minimize the interfacial tension between the two phases (Alzagtat & Alli, 2002). They align at the interface according to their amphiphilic nature and conform to train, loop, and tail configurations to form a viscoelastic interfacial film. Trains lie along the interface, whereas loops and tails protrude into the continuous phase to facilitate repulsion (Damodaran, 2005) (Lam & Nickerson, 2014). Proteins differ in the minimum amount required for monolayer coverage of droplets and in the rate of adsorption to the oil–water interface. These factors, along with homogenizer energy output, determine droplet size, of which smaller radii confer a more stable emulsion (McClements, 2004). The net charge of a protein and its ability to rapidly reorient to the interface determine its molecular flexibility, which is cited as the most important characteristic of a good emulsifier (Damodaran, 2005). Globular proteins are less flexible and require more time to align to the interface (McClements, 2004).

Emulsions are more stable away from the pI of a protein and at low ionic strength when the electrostatic repulsive forces are greater. At pH away from the pI, because the dispersed droplets are farther apart, the interactions between proteins adsorbed to different droplets are weakened. This in turn might promote stronger interactions between proteins adsorbed to the same droplet to form a robust interfacial film and deter coalescence (McClements, 2004).

Likewise, emulsions are the least stable close to the pI of a protein and at high ionic strength, because the dispersed phase is in close proximity and the electrostatic repulsion is weak relative to the attractive forces between droplets (Lam & Nickerson, 2014). Instability is also promoted by low temperature since crystallized water molecules force dispersed droplets closer together, as well as by uneven emulsifier distribution on the droplet surface (McClements, 2004). Meanwhile, partially denatured proteins and the use of more polar oils can improve stability, since hydrophobic groups are exposed and less unraveling of proteins is necessary (Damodaran, 2005) (McClements, 2004).

Emulsion properties have been measured using many methods. A common method is by the determination of the emulsion activity index (EAI), which estimates the interfacial area that can be stabilized per unit weight of protein, or by the determination of the emulsion stability index (ESI), which measures the ability of an emulsion to resist changes over time (Can Karaca, Low, & Nickerson, 2011) (Boye, Zare, & Petch, 2010). Similar to ESI, emulsion stability (ES) is the percentage of an emulsion that has not succumbed to separation (as a serum layer) after a specified length of time (Liu, Elmer, Low, & Nickerson, 2010). Emulsion capacity (EC) is a measure of the maximum amount of oil that can be emulsified per unit weight of protein before the O/W emulsion reaches its inversion point and becomes a W/O emulsion, as signified by a large drop in conductivity (Can, Karaca, Tyler, & Nickerson, 2018) (Hall, 1996).

Measurement techniques varied amongst authors and the values were reported using various units, making comparison difficult. Using protein isolates produced from three genotype of pea, Stone et al. (2015) found a significant interaction between cultivar and the method of extraction for EC. The authors reported comparable results between isolates prepared by AE/IEP or SE for CDC Striker (188–194 g/g) and CDC Meadow (~194 g/g), but a higher value for SE (244 g/g) than for AE/IEP (188 g/g) for CDC Dakota. In contrast, using AE/IEP and SE isolates from several legumes, Can Karaca et al. (2011) found that EC was significantly affected by the extraction method, but not by the legume source when tested using isolates from chickpea, faba bean, lentil, pea, and soy. At pH 7.0, a yellow pea (CDC Leroy) isolate prepared by AE/IEP had an EC of 477.78 g oil/g protein, whereas an SE isolate yielded a value of 484.45 g oil/g protein. The authors reported that the EC values for legume

isolates increased when surface charge and solubility increased, and surface hydrophobicity decreased. They also found that EAI, ESI, creaming stability, and mean droplet size were all significantly affected by the extraction method, legume source, and the interaction of these two factors, where AE/IEP isolates yielded higher values overall with smaller droplet size. However, Boye et al. (2010) proposed that EAI and ESI were affected only by the legume source for isolates prepared from yellow pea, desi and kabuli chickpea, and green and red lentil. It is unknown whether isolates from different pea genotypes would also display significant differences in their emulsification characteristics. Boye et al. (2010) reported that both AE/IEP and UF isolates from a yellow pea cultivar (CDC Golden) had nearly identical EAIs of 4.6 m^2/g, whereas Withana-Gamage et al. (2011) reported 0.7 m^2/g for an isolate from an unknown variety of pea protein isolate. Acid-precipitated wrinkled pea isolates yielded EAIs of 10.1 m^2/g and 14.0 m^2/g at pH 3.4 and 4, respectively (Boye, Zare, & Petch, 2010). Can Karaca et al. (2011) stated that EAI was positively correlated to surface charge and solubility and reported values of 42.9 m^2/g and 42.7 m^2/g for AE/IEP and SE pea protein isolates, respectively, at pH 7.0.

Adebiyi and Aluko (2011) found droplet size to be reduced at neutral and alkaline pH values. They proposed that decreased solubility and a more folded protein structure at acidic pH led to lower molecular flexibility. In a previous study, Aluko et al. (2009) reported an oil droplet size range of 14–15 μm using a commercial yellow pea isolate (Nutri-Pea Ltd.) prepared by AE/IEP. They found that a higher protein concentration led to smaller droplet size at pH 3, but not at isoelectric or neutral pH. Dagorn-Scaviner, Gueguen, & Lefebvre (1986) investigated the impact of Vn/Lg ratio on the emulsifying properties of pea globulins, to find those with higher vicilin contents showing more surface activity at the oil–water interface, resulting in better emulsifying properties. Liang & Tang (2013) investigated the effect of partially purified legumin and vicilin isolates and a mixed isolate on the emulsifying properties of pH (3.0, 5.0, 7.0, and 9.0). The authors reported the poorest emulsifying properties near the pI of pea (close to pH 5.0), with improved emulsifying properties at pH 3.0. The authors concluded that the emulsifying properties of the pea proteins were related to their solubility, surface hydrophobicity, ability to absorb to the oil–water interface, and the strength of the interfacial films formed. The authors found pea legumin was better at forming emulsions than vicilin; however, vicilin formed more stable emulsions. Gharsallaoui, Cases,

Chambin, & Saurel (2009) reported pea proteins absorbed faster at pH 7.0 than under acidic conditions (pH 2.4); however, the interfacial films were inhomogeneous in nature and weak. In contrast, under acidic pH values, the interfacial films were thicker and showed greater elastic properties giving stabilized emulsions. Tsoukala et al. (2006) also reported pea protein's ability to create interfacial films needed to stabilize O/W emulsions, and that the partial hydrolysis of pea proteins can lead to faster absorption but ultimately a weaker film and less stable emulsion.

5.5. Foaming Properties

Foams are dispersions of gas bubbles within a liquid (usually water) or solid continuous phase and can be generated by sparging (forcing gas into the liquid phase through an aperture), whipping (beating atmospheric air into the liquid phase), shaking, or pouring (such as a glass of beer) (Hall, 1996). Due to high free energy at the gas–liquid interface, foams are thermodynamically unstable and undergo coalescence and disproportionation to reduce the interfacial area (Dickinson, 2010). Disproportionation (Oswald ripening) is the diffusion of gas from small to large bubbles due to higher pressure within the former (Wierenga & Gruppen, 2010). Solubilized proteins diffuse and adsorb to the gas–liquid interface, which reduces surface tension. They then unfold and orient hydrophobic regions to the gas phase and hydrophilic regions to the liquid phase to assume train and loop formations. A cohesive, continuous film is then formed around gas bubbles due to interactions between polypeptides (Wierenga & Gruppen, 2010) (Kinsella, 1981).

Ideally, the protein should adsorb rapidly to the gas–liquid interface and possess high molecular flexibility for quick reorientation. Newly formed bubbles tend to burst instantaneously due to the high surface tension in water. Accordingly, the foam volume (capacity) is dependent on how quickly new air cells are formed and stabilized relative to the rate of collapse (Kinsella, 1981). Foaming capacity (FC) is the amount of interfacial area that can be created by the protein (Damodaran, Parkin, & Fennema, 2008). It is positively correlated to the average hydrophobicity (difference in the free energy of amino acid side chains when exposed to a nonpolar solvent or water) of proteins and can be enhanced by partial denaturation to increase surface activity (Damodaran, 2005) (Kinsella, 1981). Because

average hydrophobicity is derived using all amino acids in a protein, as opposed to only those exposed to the surface in surface hydrophobicity, the correlation of a functional property to average hydrophobicity suggests that the proteins exist in a more unfolded state to expose amino acids buried in the core (Damodaran, 2005). Boye et al. (2010) reported the FC of isolates prepared from CDC Golden yellow pea to range from 95 to 105% when prepared by AE/IEP or UF. Shevkani et al. (2015) reported a wider range of results (87–132%) for AE/IEP isolates produced from five pea genotypes, whereas Stone et al. observed higher FC for isolates produced from three pea genotype using AE/IEP, SE, and micellization (133–263%).

Foam stability (FS) is the ability of a protein to stabilize a foam against stresses (Damodaran, 2005). Stable foams tend to be resistant to gas diffusion, drainage and thinning of lamella fluid, and mechanical shock. Accordingly, stable protein-based foams should possess interfacial films that are cohesive through hydrogen bonding and electrostatic and hydrophobic interactions. Intermolecular associations should result in a network structure of high surface elasticity to allow for some deformation (Wierenga & Gruppen, 2010) (Kinsella, 1981). Foams are the most stable at the isoelectric pH of a protein. Because of minimal electrostatic repulsion, protein–protein interactions and adsorption to the interface are maximized, which promotes viscous film formation and steric stabilization. Boye et al. (2010) reported that ~40% of the liquid remained in a foam after 5 minutes when stabilized by a protein isolate prepared from CDC Golden yellow pea, whether prepared by AE/IEP or UF. Barac et al. (2010) measured slightly lower FS (~35%) after only 3 minutes for isolates prepared from two pea genotypes by AE/IEP. In comparison, Stone et al. (2015) reported values of 68–70% after a 30-minute waiting period at pH 7 for isolates prepared from three pea genotype by AE/IEP. Following similar testing parameters, Shevkani et al. (2015) was able to achieve 94–96% FS for isolates prepared from five pea genotypes by AE/IEP. The high FS attained by Shevkani et al. (2015) may be a consequence of the high homogenizer speed (15,000 rpm) used in foam formation, which was almost twice as fast as that used by Stone et al. (2015) (7,200 rpm). Wierenga and Gruppen (2010) reported that upon diffusion to the interface, proteins are less likely to adsorb as coverage approaches a maximum. Because protein-based foams can be formed at millimolar concentrations, excess proteins continually exchange between the continuous phase and the interface, and thus

intermolecular associations gradually increase with aging to form a more cohesive film (Kinsella, 1981) (Ikeda & Nishinari, 2001).

Likewise, FC and FS usually improve at higher protein concentrations (Kinsella, 1981). Aluko et al. (2009), using a commercial yellow pea protein isolate prepared by AE/IEP (Nutri-Pea Ltd.), found that FC increased with protein concentration up to 50 mg/mL at pH 3, but decreased at pH 5 and 7. For all pH levels, FC decreased to between 50 and 120% when the protein concentration was increased to 100 mg/mL, whereas values above 200% were possible at other concentrations and pHs. This was possibly due to limited protein solubility. The addition of sugars also had been shown to improve FS by increasing lamella fluid viscosity to hinder drainage; however, FC was impaired (Kinsella, 1981) (Damodaran, 2005). Foaming properties were also enhanced when proteins were salted-out in a salt solution, but impaired by the presence of lipids, which adsorbed more readily to the gas–fluid interface than proteins due to their higher surface activity (Damodaran, Parkin, & Fennema, 2008). Using a protein isolate prepared from Eclipse yellow pea by UF, Taherian et al. (2011) observed enhanced FS when NaCl was added up to 0.25%, with no improvement with a further increase in salt concentration. Greater stability was attributed to improved solubility and arrangement of proteins at the interface.

5.6. Gelation

Gelation is one of the most important functional properties of the globular proteins as it is used to modify food texture (Ikeda & Nishinari, 2001). A protein gel is defined as a three-dimensional and well defined network assembled from protein molecules. Gelation mechanism of globular proteins consists of two stages, conformational change, or partial denaturation of protein molecules, followed by gradual association or aggregation into a three-dimensional matrix structure that traps water, fat, and other food ingredients (Corredig, 2006). Protein gelation can be induced by heat treatment, pH, salts, pressure or shearing, and the presence of various solvents (Culbertson, 2005).

Protein gelling properties are very important for their food applications, since several foods are marketed in the form of gel that offers convenience to the consumers. Examples include

jam, jelly, confectionery products, desserts, quick-set gels, and other gel products based on fruits and vegetables (Banerjee & Bhattacharya, 2012).

Most of the food protein gels are formed through a heat treatment. Heat-induced gelation of pea proteins has been studied by several researchers and has been reported to be affected by many factors such as cultivar, extraction procedure, heterogeneity of the protein, solvent parameters, and heating procedure (O'Kane, Happe, Vereijken, & Gruppen, 2004) (Shand, Ya, Pietrasik, & Wanasundara, 2007) (Sun & Arntfield, 2011) (Mession, Sok, Assifaoui, & Saurel, 2013) (Munialo, van der Linden, Akt, & de Jongh, 2015). Pea protein isolates have been reported to form weaker and less elastic gels compared to soy protein isolate when processed under the same conditions (O'Kane, Happ, Vereijken, Gruppen, & van Boekel, 2004) (Mession, et al., 2013).

Gelation is impacted by protein composition. The ratio between globulins/albumins and/or legumin/vicilin can vary based on species and the methods of protein extraction (Gueguen, 1983). Differences in ratio and structure can lead to functionality differences (Yerramilli, Longmore, & Ghosh, 2017). O'Kane et al. (2004) compared the gelling characteristics of two vicilin fractions named vicilin 1° and vicilin 2° from two pea genotypes (Solara and Supra), which were obtained in a salt fractionation procedure of the globular proteins extracted from pea flour under alkaline conditions. Vicilin 2° was reported to contain a substantial amount of convicilin (~70 kDa). Although these two fractions showed similar thermal denaturation at pH 7.6, their gelation behavior was different. Vicilin 1° had a minimum gelling concentration of 10% (w/v) and formed turbid gels, while vicilin 2° had a minimum gelling concentration of 14% (w/v) and formed transparent gels at pH 7.6.

Sun and Arntfield (2011) investigated the effect of pH (3.0–10.0) on the gelation characteristics of pea protein isolate in relation to denaturation at different salt concentrations (0–2.0 M). A protein isolate prepared from Canadian yellow pea by the SE method was used. Both pH and ionic strength were found to be effective on the gelation characteristics of pea protein. The stiffest gel was reported to form at pH 4.0 in 0.3 M NaCl. The denaturation temperature of the pea protein was altered by pH and hence gelation temperatures. The gelling temperature was also affected by ionic strength in such a way that higher salt concentration resulted in higher gelling temperature at pH 5.65 as salt had a stabilization

effect that inhibited pea protein denaturation. Munialo et al. (2015) studied the transitioning in mechanical responses of pea protein gels as a result of changes in the network structure. A pea protein isolate extracted from commercial green peas by the IEP method was used. The authors followed two different approaches: (i) changing the pH from 3.0 to 4.2 at a fixed protein concentration (100 mg/ mL), and (ii) changing the protein concentration from 100 to 150 mg/mL at a fixed pH of 3.0. Variation of the pH during the gel formation of pea proteins at a fixed protein concentration was reported to result in changes in the aggregate size of the proteins and structural changes in the network. The microstructure of the gels was not altered by variation of the protein concentration at a fixed pH. On the other hand, variation of the pH at a fixed protein concentration was reported to result in significant changes in the gel structure, which was correlated with the fracture properties of pea protein gels. A structural transitioning from finer to coarser pea protein networks was shown to occur around pH 3.7, where the mechanical deformation properties changed significantly.

Mession et al. (2013) investigated the effect of fractionation of globular pea proteins on their heat-induced aggregation followed by acid cold-set gelation. Mixed globular pea proteins were extracted from smooth yellow peas using a salt-extraction method at pH 8.0 followed by UF. The globulin fractions Vicilin 7S and Legumin 11S were separated by chromatography. It was reported that the denaturation temperature of pea proteins increased with increasing legumin content. The acidic (α) and basic (β) legumin subunits (56–58 kDa) denatured and aggregated at a temperature range of 75–85 °C. The gluconoδ-lactone (GDL)-induced cold-set gelation of protein thermal aggregates was triggered at pH values higher than 6, even at low protein concentrations. Mixed pea globulins and vicilin-enriched samples were reported to result in higher final moduli values of the acid gels, while legumin-enriched samples displayed low gelling properties.

Cold-set gelation involves a two-step process that involves the preheating of the protein dispersion and the induction of gelation by the addition of salts or acids at low or room temperatures (Maltais, Remondetto, & Subirade, 2008) (Vilela, Cavallieri, & Da Cunha, 2011). Cold-set gelation methods have been considered as potential alternatives to produce healthy foods, as they allow the incorporation of thermal-sensitive valuable compounds and

enable the introduction of gel structures to foods, without the necessity of deleterious heating (Alting, de Jongh, Visschers, & Simons, 2002).

6. Research rationale

A growing trend in substituting animal-based products for alternative plant-based has been occurring in the upcoming years. There are several factors that have motivated this, which include but are not limited to health, religious or environmental reasons. The food industry is also following that trend, and pea protein has shown to be a good candidate due to its low cost, high nutritional value, availability, and lack of allergenicity. Peas usually present approximately 23% of protein, depending on the cultivar and production process, but in order to increase nutritive value, yield and increase attraction, breeders have developed new genotype that contain approximately 30% protein. These genotypes were developed considering the total protein fraction, but studies were not conducted on how this increase in protein will affect the functionalities. The main goal of this study was to understand how these genotypes differ in functionality when compared to regular protein genotypes. It is also our goal to understand how the high and regular protein genotypes or genotype differ at molecular level including protein compositions and structures, and then how such structural difference impact the protein and physical-chemical properties and functionalities.

6.1. Objectives

Overall, the present investigation is undertaken to: (1) study and compare physicochemical, structural, rheological, and functional properties of protein isolates from different pea genotypes, including high protein and (2) to establish possible relationships between these properties, to predict their use in industry practices.

6.1.1. Specific Objectives

1. To perform the protein extraction from pea grains of different genotype, including high protein genotype and normal genotypes, to obtain pea protein concentrates.

2. To study the functional properties of the protein concentrates from different pea genotypes, including solubility, water/oil holding, emulsifying, gelling, and foaming capacity.

3. To study the structural differences of proteins among the seven genotypes to understand how the increase in protein content influences the protein functionality.

7. Significance of this work

The outcome of this research will lead to fill the knowledge gaps by understanding the differences in compositions and structures of pea protein in high protein genotype and how such changes impact the protein functional properties that directly determine their utilizations in industrial food processes. This research will also demonstrate how breeding can be used to add value to plant-based food ingredients. The knowledge generated may guide breeders to develop more high protein genotypes not based only on their content but taking into consideration of their functionality.

Chapter 2 – Effects of genotypes on the functional properties of pea proteins

1. Introduction

Pulses are defined by the FAO as legumes harvested solely for their seed which is consumed directly. The FAO list includes eleven primary pulses, including peas, and excludes the oilseed legumes and those consumed in immature form as vegetables (FAO, 1994). Pulse crops belong to the family of cool season, annually grown leguminous crops (Maiti, 2001). These crops are produced on many continents worldwide. North America is accountable for the bulk of pulse crop production and exportation. The legumes are considered as the second most important source of human food after cereals. They are inexpensive source of proteins and other nutrients such as resistant starch, dietary fiber, vitamins, minerals and polyphenols.

Field pea is one of the most important leguminous crops grown in 84 different countries over the world, which constitute the largest percentage (36%) of total pulse production (Dave Oomah, Patras, Rawson, Singh, & Compos-Vega, 2011). The world production of peas in 2009 was more than ten million tons, the major producers being Canada, the Russian Federation, China, the USA and India (Dahl, 2012).

Peas, more specifically the yellow or green cotyledon genotypes known as dry, smooth, or field peas, are the naturally dried seeds of *Pisum sativum* L. and are grown around the world for human and animal consumption. Pea composition can be separated into starch-, fiber- and protein-enriched products for use in the development of novel foods. Field pea contains 20–40% protein depending on the variety and environmental factors (FAOSTAT, 2011). The main protein classes in pea are albumins and globulins which account for 18–25% and 55–80% of the total protein, respectively, with convicilin, prolamins, and glutelins present in minor amounts (Koyoro & Powers, 1987) (Guleria, Dua, & Chongtham, 2009; Owusu-Ansah & McCurdy, 1991). Pea globulins tend to be high in arginine, phenylalanine, leucine, and isoleucine, whereas the albumin fraction is higher in tryptophan, lysine, and threonine (Schroeder, 1982). The globulins can be further classified based on their sedimentation coefficients into two main types, legumin (11S) and vicilin (7S) (Tsoukala, Papalamprou, Makri, Doxastakis, & Braudo, 2006). Legumin is a hexameric molecule (molecular weight: ~ 380,000 Da) where each subunit (molecular weight: ~ 60,000 Da) contains one "acidic" and one "basic" peptide linked by a disulfide bond. Vicilin is a trimeric molecule (molecular weight: ~ 150,000 Da) where each monomer can contain breaks in its polypeptide chain, giving rise to a variety of smaller subunits. The protein solubility of albumin and globulin fractions differ as the former is considered water-soluble and the latter salt-soluble (Swanson, 1990).

Plant breeding deals with the inheritance of qualitative and quantitative traits and includes the genetic improvement in existing genotypes for specific trait(s), and the creation of altogether new genotype with new gene combination called recombinant (Singh, Singh, & Singh, 2021). Through plant breeding, or plant/crop improvement, plants have been improved for their productivity, quality traits (physical and chemical), and also for various

other desirable traits, such as resistance to environmental stresses and harmful parasite and for their suitability to mechanical harvesting (Singh, Singh, & Singh, 2021).

The breeding of pea cultivar has long been driven by the yield, disease resistance, protein content, and mineral content without considering the functionality of protein. In fact, the impact of yellow pea cultivar on the functionality of pea protein has been previously examined and pea protein prepared from different genotype performed differently in their functionalities (Barac, et al., 2010) (Stone, Avarmenko, Warkentin, & Nickerson, 2015). This is not unexpected because each cultivar differs inherently on protein contents, protein composition and conformation. For instance, the ratio of legumin and vicilin, two major globulin proteins in pea accounting for more than 70% of total protein, varies from 0.4 to 2.0 depending on the genotype (Lam, Can Karaca, Tyler, & Nickerson, 2018). Regarding the role of pea composition on the protein functionality, mixed results have been documented so far. Some studies have shown that vicilin has better emulsifying and foaming properties including capacity and stability than legumin (Dagorn-Scaviner, Gueguen, & Lefebvre, 1987), while others found legumin has the higher emulsifying and foaming stability compared to vicilin (Liang & Tang, 2013).

Pea seed protein composition is complex genetically, with multigene families encoding different proteins, which are then subject to considerable post-translational processing (Bourgeois, et al., 2011). These authors showed that pea seed protein composition is predominantly under genetic control, with 60% of the protein 'spots' from a two-dimensional electrophoresis analysis varying in abundance between genotypes (Bourgeois, et al., 2011). However, environmental factors have also been shown to influence the pea seed proteome; heat and drought stress can interrupt seed maturation and may result in lower accumulation of those seed proteins that typically accumulate later in development (Bourgeois, et al., 2009).

The predominant storage proteins in pea are the globulins legumin and vicilin, thought to be synthesized by at least 40 genes (Casey, et al., 2001). Loci related to the synthesis of legumin (Lycett, Croy, Shirsat, & Boulter, 1984), vicilin (Lycett, et al., 1983), and convicilin (Newbigin, et al., 1990), have been identified, and may be considered as suitable targets for mutagenesis for altering seed protein composition (Domoney, Ellis, & Davies, 1986) (Ellis,

Domoney, Castleton, Cleary, & Davies, 1986). However, pea seed protein content is a quantitatively variable trait, with globulins encoded by multigene families (Bourgeois, et al., 2009), and so mutations affecting single genes have little influence on total protein concentration (Chinoy, Welham, Turner, Moreau, & Domoney, 2011) (Rayner, Moreau, Isaac, & Domoney, 2018), unless the mutation is at a locus which controls a large proportion of phenotypic variation.

Current knowledge on the genetics of nutritional traits in pea will greatly assist with crop improvement for specific end uses, and further identification of genes involved will help advance our knowledge of the control of the synthesis of seed compounds (Robinson & Domoney, 2021).

The extraction of protein from pea seeds for use in other food applications has become more common due to their nutritional, functional and economic benefits (Adebiyi & Aluko, 2011; Mertens, Dehon, Bourgeois, Verhaeghe-Cartrysse, & Blecker, 2012) (Dijkink & Langelaan, 2002).

Isolation of plant proteins can be carried out using either wet or dry process. In dry method, the plant parts (e.g., roots, stems or seeds) are ground into fine powders from where starch and protein are separated on the basis of their densities (Makri, Papalamprou, & Doxastakis, 2005). On the other hand, wet method involves solubilization of protein in an alkaline solution, separation of solubilized protein from the insoluble materials, and then precipitation of the dissolved protein to recover it from the solution. Extraction of pulse proteins may be relatively easy using wet processes, as they are highly soluble under alkaline and acidic conditions. The precipitation is usually carried out at isoelectric point (pI) of the protein of pH 4.5 at which its solubility is the lowest (Singh, Kaur, Rana, & Sharma, 2010). After the protein extraction is complete, the samples are then spray dried.

In spite of high protein content and low cost of the legume proteins, their application in food formulation depends upon functional properties which have been defined as the physical and chemical properties of proteins that influence their behavior in food systems during processing, storage, cooking and consumption (Rezaei, 2019). The functional properties of proteins have been classified according to the mechanism of action on three main groups: (i) properties related with hydration (water and oil absorption, solubility, thickening &

wettability) (ii) properties related with the protein structure and rheological characteristics (viscosity, elasticity, adhesiveness, aggregation & gelification), and (iii) properties related with the protein surface characteristics (emulsifying and foaming, formation of protein-lipid films, whippability) (Wang & Timilsena, 2017). The most important functional properties of protein in food include its solubility, water- and fat-binding capacities, gel forming, and rheological behaviors, emulsifying capabilities, foaming and whipping abilities are closely related to the protein composition and molecular structures in the extracts such as molecular weight, charge distribution, surface hydrophobicity and conformation, as well as environmental factors such as pH, temperature, ionic strength and presence of other food components (Kinsella & Melachouris, 1976; Rezaei, 2019).

Pea proteins show peculiar functional benefits including solubility, emulsifying, and foaming capacity and emulsion and foam stability as well as gel and film forming capacity. These functionalities are closely affected by their composition.

Legumins result with more rigid conformation due to the compact quaternary structure and disulfide bridges as well as hydrophobic interactions; while vicilins are characterized by a more flexible structure (Barac et al., 2015). Nutritionally, vicilins have higher amounts in arginine, isoleucine, leucine, phenylalanine, and lysine compared to legumins, while this later is richer in sulfur-containing amino acids. Compared to vicilins, convicilins present cysteine in their amino acid sequences (Barac et al., 2015; Djoullah et al., 2018; Lan et al., 2018). From a functional point of view, no data was found reporting the functionality of convicilins. These structural and compositional differences result in different functionalities, where vicilins present better gelling and emulsifying properties than legumins due to structural flexibility. The authors also highlighted those stronger elastic gels are formed through more crosslinking of vicilin polypeptides (Djoullah et al., 2018). Due to the increasing interest in pea protein applications for the (re)formulation of food and beverages products, a better understanding of their functional properties is still required.

Current applications for pea protein include vegan style yogurts and non-dairy based sports products, as well as, partial dairy protein replacers for therapeutic beverages and powders (Makri, Papalamprou, & Doxastakis, 2005). Boukid, Rosell, & Castellari (2021) investigated the impact of pea protein on nutritional and technological properties of foodstuffs, as well as

their applications. The authors described the successful use of pea protein in products such as bread (Erben & Osella, 2017) (Millar, Barry-Ryan, Burke, McCarthy, & Gallagher, 2019), pasta (Wee, Loud, Tan, & Forde, 2019), baked goods (Assad-Bustillos, et al., 2020), snacks (Arribas, et al., 2017), meat products (Baugreet, et al., 2018), and fermented (Akin & Ozcan, 2017) and non-fermented bevarages (Trikusuma, Paravisini, & Peterson, 2020).

The value of pea grain has been drastically improved by manufacturing it to protein ingredients, e.g., pea protein concentrates and isolate. The utilization of pea protein ingredients as functional ingredients in food products depends on their functional attributes such as solubility, emulsifying and foaming properties, etc. Therefore, achieving desirable functional attributes is of great importance for successfully developing pea protein fortified products. Though a lot of research has been focused on the applications of pea protein, the effect, however, of genotype on the functional properties of pea proteins has not been clearly investigated. This research extracted and characterized pea proteins from seven pea genotypes, including high protein genotype and compared their functional properties (solubility, gelling capacity, water and oil holding capacity, foaming capacity and stability, emulsifying capacity) and correlated these properties to their technical properties.

2. Materials and Methods

2.1. Materials

Severn pea genotype or breeding genotype, including four regular protein genotypes of yellow and green peas and three high protein genotype obtained from the Agriculture and Agri-Food Canada (AAFC) field pea breeding program were analyzed in this study. The detailed information of materials is shown in Table 1.

Protein standards (Sigma-Aldrich Co., Oakvillem Ontario, Canada) including thyrogloulin (660 kDa), γ-globulin (150 kDa), albumin (43 kDa), ribonuclease A (14 kDa) and p-aminobenzoic acid (0.14 kDa) were used for Mw calculation. D2O, DOH, NaOH, HCl, hexadecane, pepsin (3460 units/mg solid or protein) and pancreatin (activity at least equivalent to 1 USP specification) were purchased from Sigma-Aldrich Co. (Oakville,

Ontario, Canada). All chemicals were reagent grade and used without further purification. Water used in this study was purified by Milli-Q Advantage A10 system (EMD Millipore Corporation, MA, USA).

2.2. Methods

2.2.1. Pea protein isolation

Pea seeds were dehulled by a Forsberg Model 2 Huller (Forsbergs, Inc., Thief River Falls, MN, USA) and ground into flour by a laboratory mill (Retsch ZM 200, Retsch GmbH, Germany) equipped with 500 μm sieves. Pea flour was defatted by a double hexane extraction with flour to hexane ratio of 1:5 for 2h each. The pea flour was separated from hexane by centrifugation at 8,000g for 15 min. Then the pea flour was air-dried in a fume hood at 22°C overnight.

2.2.2. Alkaline Extraction followed by isoelectric precipitation

Defatted pea flour was dispersed in distilled water in flour to water ratio of 1:10. Then the pH of the mixture was adjusted to 9 by 1 mol/l NaOH and stirred for 2h at room temperature (22°C). The suspension was centrifuged at 8,000g for 15 min at 4°C. The supernatant was adjusted to pH 4.5 using 1 mol/l HCl to precipitate the proteins which were then collected by centrifugation at 8,000 g for 15 min at 4°C. The pea protein samples were neutralized by 1 mol/l NaOH before freeze-dried (Day, 2013).

2.2.3. Protein Content – Leco Analysis

After freeze-drying, pea protein samples were stored in plastic containers at 4 °C. The protein content was determined by nitrogen analyzer (FP-428, Leco Corporation, St. Joseph, MI, USA) using a protein conversion factor of 6.25 (Tang & Sun, 2011; J. Yang, Liu, Zeng, & Chen, 2018).

2.2.4. SDS – PAGE

Samples were diluted with 2 × Laemmli sample buffer (Bio-Rad, CA, USA). In order to achieve reducing conditions, 2-mercaptoethanol was added to the sample buffer.

Subsequently, the samples were heated at 90 °C for 4 min, cooled and loaded into 12% Mini-Protean® TGX™ precast gels (Bio-Rad, CA, USA), and subjected to electrophoresis at a constant voltage of 150 V. Precision Plus Protein™ Prestained Standards (Bio-Rad) was used as a molecular weight marker. After electrophoresis, the gels were stained in an aqueous mixture of 40% (v/v) methanol, 10% (v/v) acetic acid and 50% (v/v) deionized water containing 100 mg/mL Coomassie Brilliant Blue-R-250 for 2 h. Discoloration of gels was done with an aqueous mixture of water, methanol, and acetic acid (5:3:2, v/v/v).

2.2.5. Molecular weight

The apparent weight average molecular weights (Mw) of pea protein samples extracted by different methods were determined by size-exclusion high-performance liquid chromatography (SE-HPLC, Agilent series 1100, Palo Alto, Ca, US) equipped with a TSK G3000 SW column (5 μm, 7.8 mm ID × 30 cm; TOSOH Bioscience, LLC, Japan) at 23°C (J. Yang, et al., 2018). The mobile phase was 0.1 mol/l phosphate buffer (pH 7) with 0.1 mol/l NaCl. The flow rate was maintained at 0.5 ml/min. Signal was detected by UV detector at 220 and 280 nm. The protein concentration was 1 mg/ml and the sample passed through a 0.22 μm filter before injection into the column. The protein standard mix was injected into the same column and analyzed under the same condition as pea protein samples. The retention time of each standard component was obtained from the chromatogram and plotted against the logarithmic molecular mass to obtain a calibration curve. The apparent molecular weights of pea protein samples were calculated from the calibration curve.

2.2.6. Fourier-transform infrared spectroscopy (FT-IR)

The conformation of pea proteins extracted by different methods was analyzed by Fourier-transform infrared spectroscopy (FT-IR). The extracted pea protein was dissolved in D_2O to a protein concentration of 10 mg/ml. The pD of protein solution was adjusted to 7 by 0.1% DCl and NaOD. The protein solution was placed between two CaF_2 windows separated by a 25 μm polyethylene terephthalate film spacer for FT-IR measurement. Infrared spectra were recorded by a Nicolet 6700 spectrophotometer (Thermal Fisher scientific Inc., Pittsburghm PA, US) at wavenumber of 1111–4000 cm^{-1} for 128 scans with a resolution of 4 cm^{-1}. The spectrum was the average of 128 scans. The background of D2O was eliminated by

subtracting the spectrum of D_2O recorded at the same condition. Omnic 8.1 software was used to perform Fourier self-deconvolution on amide I band region (1700 -1600 cm^{-1}) with a bandwidth of 24 cm^{-1} and enhancement factor of 2.5 (Lefevre & Subirade, 1999).

2.2.7. Denaturation Temperature – Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry (DSC TA-60, Shimadzu Corporation, Kyoto, Japan) was used to determine the thermodynamic properties of pea protein isolate (PPI). Approximately 2.5 mg of PPI sample was placed and pressed into a crucible to maintain good heat transfer contact. Then, the sample was carefully put into the heating furnace where the carrier gas was air with a temperature ranging from 20 to 120°C at 10˚C min^{-1} heating rate. A baseline was constructed using an empty crucible over the same temperature range and heating rate (J. Boye, Zare, & Petch, 2010). According to the gelatinization curve of the sample, onset temperature (T_o), end set temperature (T_e), peak temperature (T_d) and enthalpy change (ΔH) can be calculated automatically with the software provided by the equipment.

2.2.8. Solubility

Pea protein samples were mixed with water at a protein concentration of 10 mg/ml. The samples pH was then adjusted to pH 3, 5 and 7, using NaOH 1mol/L and HCl 1mol/L. The mixtures were stirred vigorously for 1 h at room temperature (22°C) and then centrifuged at 4,000g for 30 min at 23°C (J. I. Boye, et al., 2010). The supernatant was collected and freeze-dried. The nitrogen contents of the supernatants and the protein samples were then weighed. The solubility (%) was calculated from the following equation:

$$Solubility\ (\%) = \left(1 - \frac{mass\ of\ insoluble\ protein}{mass\ of\ initial\ protein\ in\ solution}\right) x\ 100$$

2.2.9. Oil and Water holding Capacity

Oil and water holding capacity (OHC and WHC) was determined according to a modification of the method of Ahmedna *et al.* (1999). Protein samples (1 g of protein) were dispersed in 40 ml of canola oil in 50 mL falcon tubes for OHC or 40 ml of water for WHC, and vortexed (VWR Vortex Mixer, VWR International) for 30 s on maximum speed. Samples were left to stand for 6 h at room temperature. Samples were centrifuged (VWR Clinical 200 centrifuge,

VWR International) at 2000 x g for 30 min, followed by the discard of the supernatant and the weighing of the pellets. OHC and WHC was calculated as follows:

$$OHWor\ WHC\ (g\ water/oil\ \ per\ dry\ sample) = \left(\frac{final\ pellet\ weight - initial\ protein\ mass}{initial\ protein\ mass}\right)$$

2.2.10. Emulsifying Capacity

Emulsions of pea protein were prepared by mixing 9 g of canola oil with 21 ml of pea protein solutions (1 %, pH 7) by a high speed homogenizer (T18 Ultra Turrax, IKA, Wilmington, US) at 25,000 rpm for 5 min. Emulsifying capacity (EA) was measured according to the method described by Ahmedna (1999):

$$(EI)\ Emulsifying\ capacity = \left(\frac{H_c}{H_t}\right) x\ 100$$

where Hc represents the height of the cream and Ht represents the height of the whole emulsion.

2.2.11. Foaming Capacity and Foaming Stability

The foaming properties of pea proteins were measured at different pH: 3, 5 and 7, by a modified method of Ahmedna et. al (1999). High speed homogenizer (T18 Ultra Turrax, IKA, Wilmington, US) was used to mix pea protein solution (10 mg/ml, pH 7) at 12,000 rpm for 2 min. The volumes before and after mixing were measured by graduated cylinders. The percentage of volume increased was calculated as foaming capacity (FC):

$$FC\ (\%) = \frac{V_1 - V_o}{V_0} x100$$

where V_0 and V_1 represent the volume of the protein solution and the volume of foam after mixing, respectively.

The volume of the foam was measured again after storage at 22 °C for 30 min. The foam stability (FS) was determined as the remained foam volume percentage.

$$FS\ (\%) = \frac{V_2}{V_1} x\ 100$$

where V_2 represents the volume of foam after storage for 30 min and V_1 represents the initial volume of foam after mixing.

2.2.12. Gelling Properties

The thermal gelation property of pea proteins was indicated by the least gelling concentration which was determined according to the method of Boye et al. (2010) (Boye, Aksay, Roufik, Ribéreau, Mondor, Farnworth, et al., 2010). Pea protein samples were mixed with 0.1 mol/l phosphate buffer (pH 7) to prepare suspensions with protein concentration ranging from 12-20% (w/v). The mixtures were stirred vigorously for 3 h before heated at 90 °C for 1 h. The samples were cooled immediately by ice bath and stored at 4 °C overnight. The least gelling concentration was considered as the minimum protein concentration below which no semi-solid gel could be formed. A gel was defined as a weak gel when the gel was semi-solid, but flow may occur on inversion. A gel was considered as a firm gel when the gel was self-support and no flow occurred on inversion.

2.2.13. Gelling texture Profile

Pea protein gels were prepared at 20% protein concentration by thermal gelation as mentioned in session 2.7. The texture profile was determined using an Instron 5967 universal testing instrument (Instron Corp., Norwood, MA, US) equipped with a 50 N load cell. Pea protein gels were compressed twice to 50% of the original height at a constant crosshead speed of 1 mm/min. The compressive stress and springiness were determined from the stress-time curve. The compressive stress is expressed as the maximum compressive force over the cross-section area of the gel at the first compression, showing the firmness of the gel. Springiness shows how well a gel physically springs back after it has been deformed during the first compression. Springiness was calculated by the detected height during the second compression divided by the original compression distance (Li, et al., 2018; C. Yang, Wang, & Chen, 2017).

2.2.15. Nutritive Value

The protein nutritive quality was evaluated based on the Protein digestibility-corrected amino acid score (PDCAAS). Amino acid composition was determined in an acid hydrolysate using

a HPLC system in 2.2.3. The essential amino acid index (EAAI) was calculated based on the content of all essential amino acids compared to a reference protein, being values for human requirements in this case.

2.2.16. Digestibility

A two-step digestion process involving two enzymes including pepsin (3460 units/mg solid or protein) and pancreatin (activity at least equivalent to 1 USP specification) was applied to simulate digestion in the human body as described by Guathier, Vachon, and Savoie (1986) with some modifications. The first digestion was conducted using pepsin at pH 1.9 and 37ºC for 30 min on a sample containing 250 mg protein in 250 mL water suspension. The digestion was stopped by raising the pH to 7.5 with NaOH (1 mol/L). The second digestion was carried out with pancreatic enzymes prepared in sodium phosphate buffer (0.01 mol/L, pH 7.5) at 37ºC for 6 h. An aliquot of the digest was transferred into a centrifuge tube and TCA 20 g/100 mL was added in a ratio of 1:1 (v/v) to stop the enzymatic reaction and to precipitate undigested protein. The supernatant was then discarded, and the precipitate was freeze dried and weighed. The percentage of digestibility was calculated as the difference between the initial weight minus the final precipitate weight divided by 100.

2.2.17. Statistical Analysis

All experiments were carried out at least 3 independent batches. Results were presented in the form of mean ± standard deviation. SAS software (SAS Institute, Inc., Cary, NC) was used for statistical analysis. The statistical evaluations were conducted by analysis of variance (ANOVA) at a 95% confidence level. The mean comparison was performed by Tukey test with the level of 0.05.

3. Results and Discussion

3.1. Protein Content

The protein content was analyzed for the pea grains and the protein extracts. As shown in figure 1(A), the high protein genotype P0540-41, P1141-5085, and P1142-6195 have protein

contents of 26.7 to 29.0%, which are significantly higher than those of normal genotypes Cooper, Earlystar, AAC Lacombe and CDC Greenwater.

Table 1. Detailed information of pea material

Name	**Type**
Cooper	green pea cultivar
CDC Greenwater	green pea cultivar
Earlystar	yellow pea cultivar
AAC Lacombe	yellow pea cultivar
P0540-41	advanced yellow pea line, selected from the cross of MI3391 X Reward
P1141-5085	advanced yellow pea line, selected from the cross of Earlystar X P0540-41
P1142-6195	advanced green pea line, selected from the cross of Cooper X P0540-41

Then, proteins were extracted from pea grains of all genotypes using alkaline solution followed by isoelectric precipitation because pulse proteins have high solubility in alkaline conditions, whereas minimal solubility at their isoelectric point (pI) between pH 4 and 5 (Fernandes, Borges, & Botrel, 2014). This process takes advantage of the similar solubility characteristics of legumin and vicilin and is the most common method of legume protein extraction reported in the literature (J. Boye, Zare, & Petch, 2010). (J. Boye, Zare, & Petch, 2010). All protein extract samples showed about 80% of protein content. When comparing among genotypes, Earlystar (86.18%) and P1141-5085 (85.19%) exhibit higher protein contents than other genotypes, and Cooper (76.51%) exhibits the lowest protein content. This result demonstrates that traditional extraction methods can be used to obtain protein concentrates from new genotypes and still maintain satisfactory results.

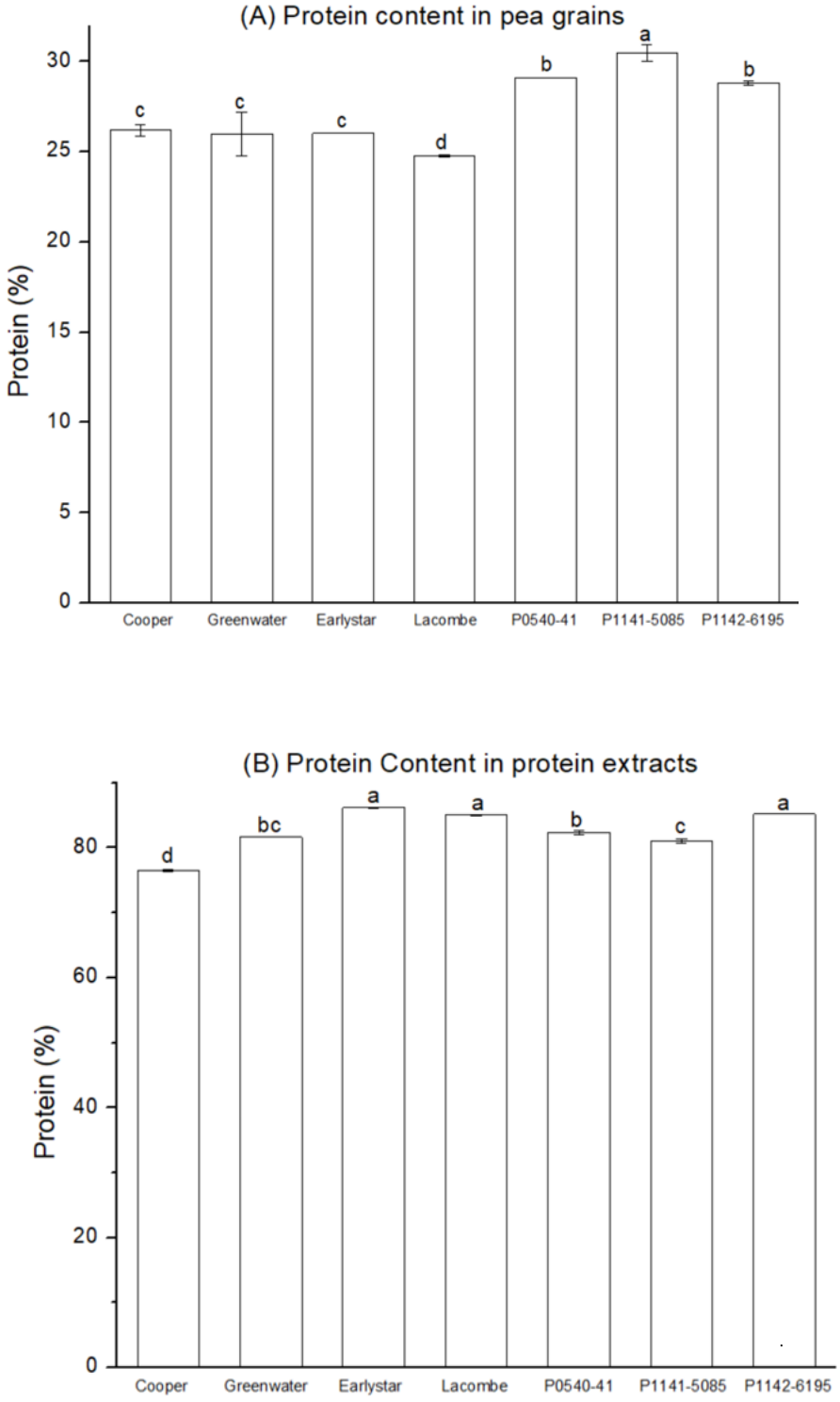


Figure 1. Protein content in (A) pea grains and (B) protein extracts of different genotype. Different letters indicate significant difference ($p < 0.05$) among samples.

3.2. Protein structure characterizations

3.2.1. SDS – PAGE

Proteins extracted from different pea genotypes were analyzed by SDS-Page to further investigate the possible differences in their protein composition.

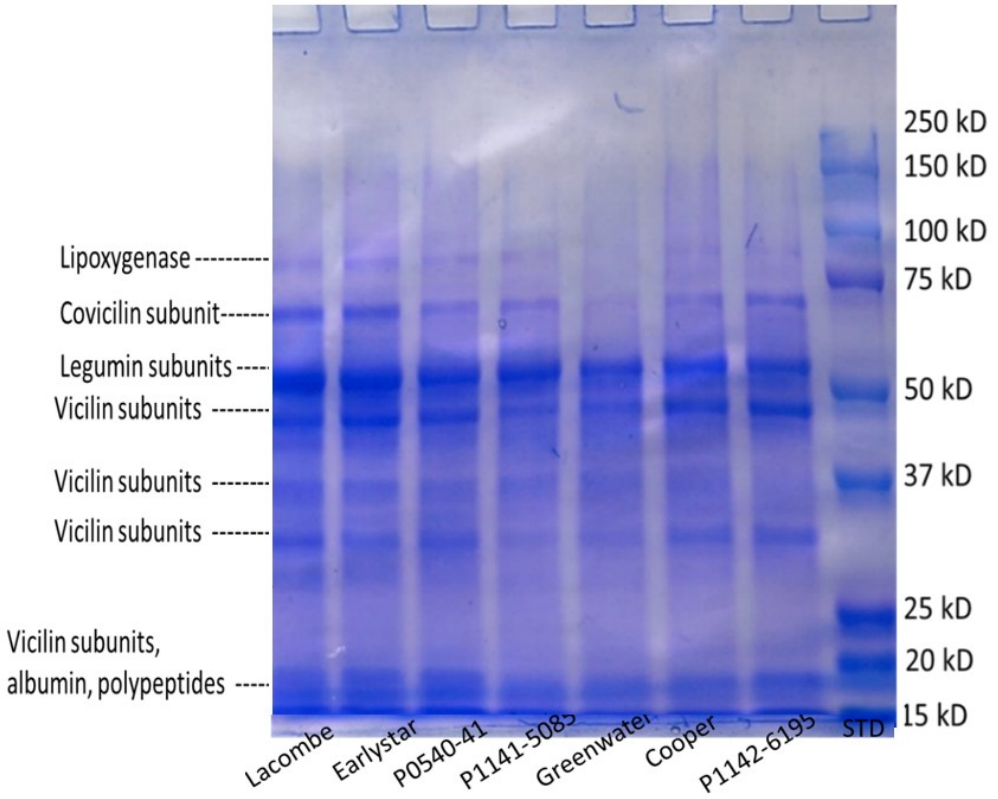


Figure 2. SDS-Page profile of protein extracts from different pea genotype.

The first band from the top with the molecular weight between 100-75kDa is assigned to lipoxygenase. The two bands between 75 and 50 kDa are associated with covicilin and legumin subunits, respectively. The multiple bands between 50 to 20 kDa showed the heterogeneous vicilin subunits. The bands below 20 kDa possibly belong to albumin, polypeptides, and vicilin subunits. These results indicate that globulins are the major protein component in the extracted protein samples. This was expected because globulins are the

major proteins in pea. The intensities of bands are different among samples, indicating that different pea genotype may have various protein composition and 11S to 7S ratio.

3.2.2. HPLC - Size exclusive chromatography (SE)

SE-HPLC was employed to investigate the apparent molecular weight of pea proteins in the extracts. As shown in Figure 2, pea protein chromatographic profile can be separated into 4 fractions according to their apparent molecular weight range. Peaks appeared in I region are attributed to the supramolecular aggregates of legumin (15S) (Croy, A., Tyler, & Boulter, 1980) (Mession, et al., 2013). P1142-6195, Greenwater and P0450 had minor peaks in this region, indicating the existent of protein aggregations, suggesting no or limited aggregates in proteins samples extracted from different genotype. Fraction II, with the apparent Mw of about 452 kDa, is assigned to legumin (Gueguen & Cerletti, 1994) (Mession, et al., 2013). Fractions III and IV correspond, respectively, to vicilin and albumin with subunits, as can be seen in table 2. Legumin and vicilin are the major globulin fractions in pea proteins, with legumin consisting of 20–30% of pea protein while vicilin consists of 20–40% (Gueguen & Cerletti, 1994). The results obtained concur with this, as all samples demonstrated peaks associated with legumin and vicilin.

The ratio between globulins/albumins and/or legumin/vicilin can vary based on species and the methods of protein extraction (Gueguen, 1983). Differences in ratio and structure can lead to functionality differences (Yerramilli, Longmore, & Ghosh, 2017). As described by Yang, Zamani, Liang, & Lingyun (2021), the ratio of 11S to 7S was calculated by the area under curve of fraction II divided by those of fraction III and IV. As showed in Table 3, the 11S/7S ratio of all genotypes ranged from 1.36 to 9.10, being P1141-5085 the highest and P1142-6195 the lowest.

Although studies have shown the effect of 11S/7S ratio on pea protein functionalities, soy protein has been studied, such information is still limited for pulse proteins currently. Mixed results have been documented so far. Some studies have shown that vicilin has better emulsifying and foaming properties including capacity and stability than legumin (Dagorn-Scaviner, Gueguen, & Lefebvre, 1987), while others found legumin has the higher emulsifying and foaming stability compared to vicilin (Liang & Tang, 2013). In addition, the

factors that contribute to such various ratios in the pea production are yet to be defined. In the case of this study, the breeding process may have favored one fraction over the other. This opens the possibility of considering breeding in order to achieve specific ratios, and therefore improving specific functionality for target food applications.

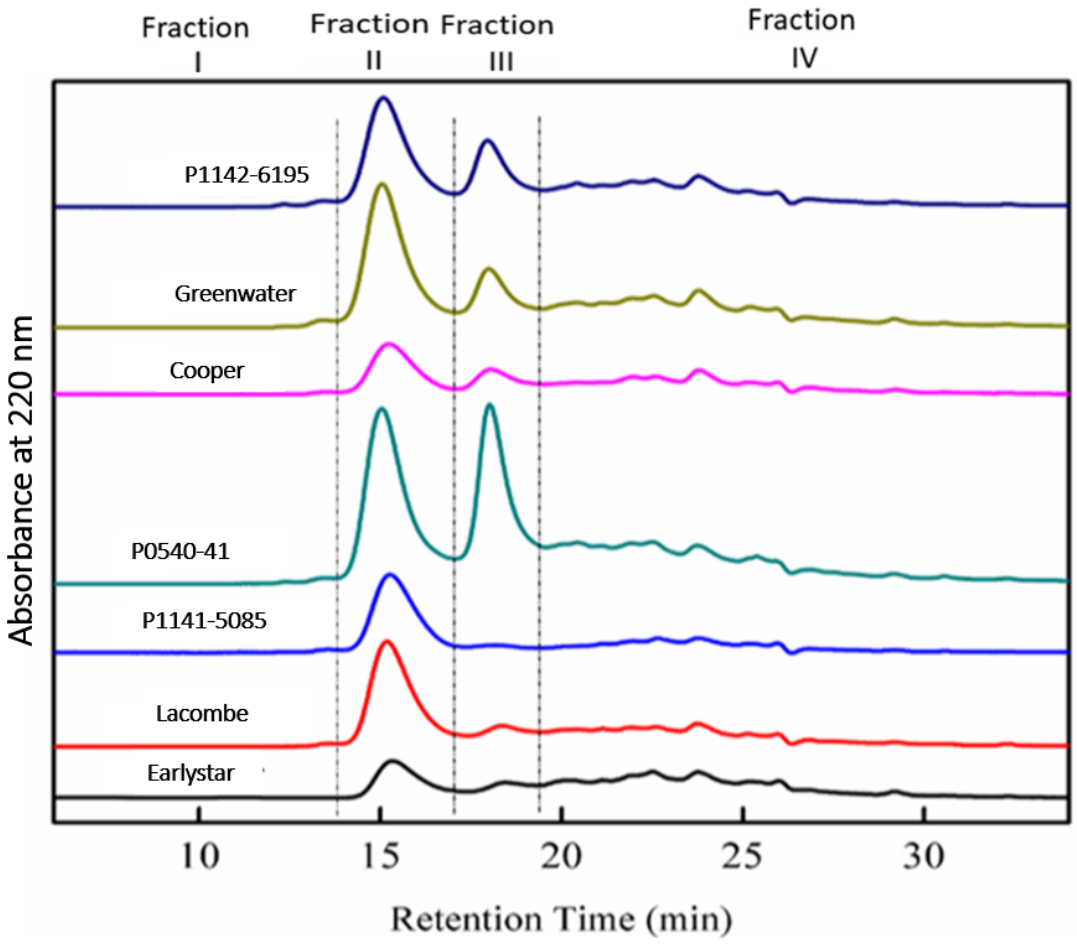


Figure 3. SE-HPLC elution profiles of extracted pea proteins from different genotype. The protein concentrations were 1 mg/ml. All samples passed through 0.22 µm filter before injected into the HPLC column.

Table 2. The peak identification from SE-HPLC profile for pea protein extract samples.

Fraction	**I**	**II**	**III**	**IV**
Retention Time (min)	0–13.0	13.0–15.4	16.6–18.7	18.7–23.6
Mw (kDa)	>660	453	138	57-17

Correspondent fractions	**protein**	>15 and 15S	11S - Legumin	7S – Vicilin and covicilin	Albumin and subunits

Table 3. 11s/7s ratio in protein extracts from different pea genotype.

Pea Genotype	**11S/7S**
Cooper	2.0 ± 0.2^{d}
Greenwater	2.9 ± 0.2^{c}
Earlystar	2.6 ± 0.1^{c}
Lacombe	6.0 ± 1.3^{b}
P0540-41	1.9 ± 0.7^{d}
P1141-5085	8.7 ± 0.7^{a}
P1142-6195	1.5 ± 0.5^{d}

Data are presented as mean ± standard deviation, calculated from three spectra of independent sets of samples. Different letters, (a, b, c, d), indicate significant difference ($p < 0.05$).

3.2.3. Fourier-transform infrared (FTIR) spectroscopy

Fourier-transform infrared (FT-IR) spectroscopy in the mid-infrared region is widely used in protein secondary structure determination through analysis of the amide I band (stretching vibrations of C=O in the peptide bond) (Jackson and Mantsch 1995). The sensitivity of amide I to conformational changes makes it possible to study not only protein folding and unfolding, but also aggregation processes. FT-IR spectroscopy has been successfully applied to monitor structure of isolated soy, buckwheat, oat globulins and wheat gliadins under the effect of pH, salts, and thermal treatments (Choi and Ma 2005; Secundo and Guerrieri 2005).

β sheets were shown to be the main components in pulse proteins and these secondary structure elements were found to play a major role in decreasing protein digestibility (Carbonaro, Maselli, & Nucara, 2012). The decrease in protein digestibility as a function of the amount of β conformations can be explained by the high hydrophobic character of these

structures, which involves aromatic residues (tyrosine, phenylalanine and tryptophan) and β-branched amino acids (threonine, valine and isoleucine).

The FT-IR spectra of the pea protein are displayed in figure 4. The pea proteins possessed two major peaks centered at 1630 and 1650 cm^{-1}, which are assigned to β-sheets and α-helix/random coils, respectively. For all seven pea protein samples, the major secondary structure component is β-sheets. This result is consistent with those reported for protein extracted by isoelectric precipitation from field peas (Shevkani, Singh, Kaur, & Rana, 2015). The peak at 1610 cm^{-1} assigned to vibration of amino acid residues. Lacombe, Earlystar and P0540-41 had a shoulder at about 1690 cm-1, which assigned to β-sheets/turns. The FT-IR spectra revealed that pea proteins from different genotypes have similar secondary structure profile. This demonstrates that the increase in protein content does not seem to affect the secondary structure when compared to the regular protein genotype.

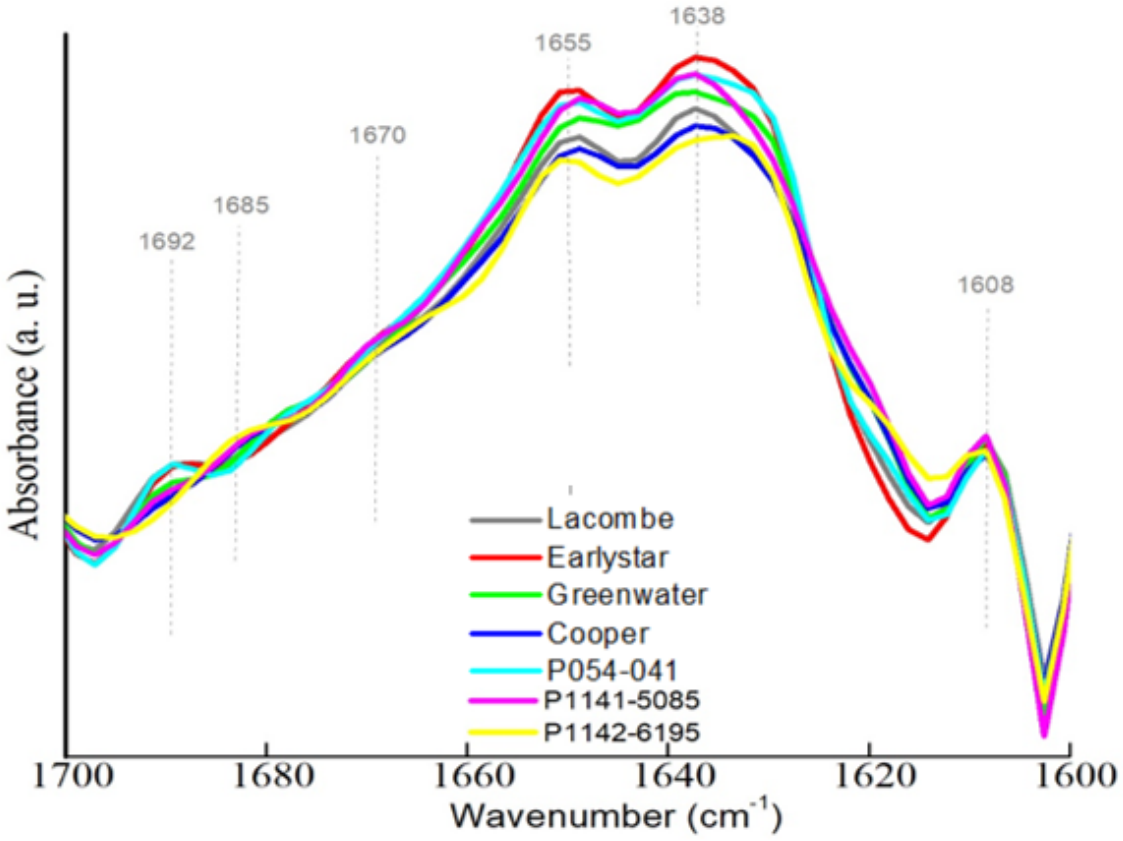


Figure 4. FT-IR spectra of protein samples extracted from different pea genotype.

3.2.4. Denaturation Temperature – Differential Scanning Calorimetry (DSC)

DSC was exploited to study the thermal stability of the protein concentrate samples obtained from different pea genotype. Thermal stability is defined as the temperature range which structural integrity is maintained. Outside this thermal span, denaturation occurs (Argos, et al., 1979). Denaturation temperature is defined as the temperature required to denature a protein and high denaturation temperature indicate high thermal stability for a globular protein (Choi & Ma, 2005). The denaturation temperature (T_d) of the pea protein samples ranged from 87.5 – 105 °C. Samples P1141-5085 and Cooper present higher denaturation temperature with the values of 105.0 and 101.8 °C, respectively when compared to other genotypes. The pea protein samples from different genotype have T_d values comparable to those reported for pea globulins by Shand et al. (2007) and Sun and Arntfield (2010). Another previous research reported T_d value of 78.5 – 82.4ºC for commercial pea protein isolates (Mession, Sok, Assifaoui, & Saurel, 2013).

Pea legumin hexamers (11S) are of higher thermal stability than vicilin trimers (7S), attributable to both structure compactness of legumin molecules and the presence of interchain disulfide bridges between the acidic and basic polypeptides constitutive of the $L_{\alpha\beta}$ subunits (Marcone, Kakuda, & Yada, 1998). Studies evaluate the T_d of legumin to be around 82.3°C, while vicilin ranges from 69.7 – 82.1°C, depending on the vicilin fraction (Kozhevnikov, Danilenko, Braudo, & Schwenke, 2001) (O'Kane, Happe, Vereijken, Gruppen, & van Boekel, 2004). The discrepancies regarding thermal parameters of pea globulins were particularly related to the pea cultivar and the legumin-to-vicilin ratio (O'Kane, Happe, Vereijken, & Gruppen, 2004) (O'Kane, Vereijken, Gruppen, & van Boekel, 2005) (Sun & Arntfield, 2012). Mession et. al (2015) investigated the heat denaturation of pea protein, legumin subunits and vicilin subunits. The authors reported a narrow range of denaturation temperature at 75 – 77 °C for pea protein and legumin samples, while the vicilin sample was at least 6°C lower. These findings, along with the results obtained on the molecular weight of the pea protein genotypes, may explain why P1141-5085 demonstrated a higher T_d than the other samples, as P1141-5085, also displayed the highest 11s/7s ratio.

P054041 and P1142-6195 had a relatively lower T_d, which may be related to their relatively lower 11S/7S.

Although this is an initial study, the knowledge generated here can encourage plant geneticists to develop high protein genotypes, modulating them to be thermal stable for different industrial processing and applications. Denaturation is an important factor when considering food processing. For example, when producing pea protein enriched food products such as bread and pasta, where a degree of denaturation is required to achieve certain viscoelastic properties, a lower T_d would be beneficial. Lower T_d can also be beneficial for pea protein gelling properties because the gelling involves protein unfolding by heating, followed by aggregation to form a three-dimensional gel network, in which the unfolding would be achieved more rapidly. In other cases, such as the production of pea protein enriched beverages, good thermal stability would be favorable to avoid protein denature and aggregate that can trigger precipitation. In this sense, the ability of modulating T_d of pea protein genotypes for specific processing or products would define a new range of breeding possibilities.

Figure 5. Denaturation temperatures of protein extract samples from pea grains of different genotype.

Pea Variety	**Denaturation Temperature (°C)**
Cooper	101.18 ± 0.45^{a}
Greenwater	95.265 ± 0.04^{b}
Earlystar	94.12 ± 0.12^{b}
Lacombe	87.58 ± 0.10^{d}
P0540-41	95.22 ± 0.70^{b}
P1141-5085	105.02 ± 0.21^{a}
P1142-6195	91.245 ± 0.67^{c}

Data are presented as mean ± standard deviation, calculated from three spectra of independent sets of samples. Different letters, (a, b, c, d), indicate significant difference ($p < 0.05$).

3.3. Protein solubility and functionality evaluations

3.3.1. Solubility

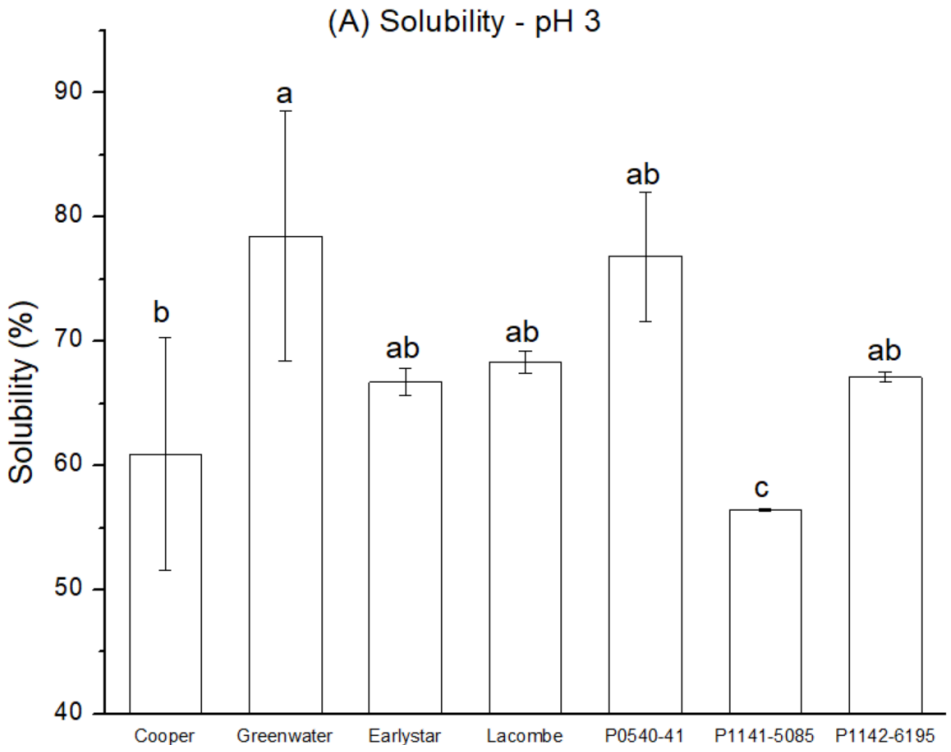
Protein solubility can be defined as the equilibrium between protein–protein (hydrophobic) and protein–solvent (hydrophilic) interactions (Hoang, 2012). Some of the major factors influencing protein solubility are solvent pH, ionic strength, temperature, and organic solvent components (Can Karaca, Low, & Nickerson, 2011). The surface properties of proteins, in particular the amount and distribution of hydrophilic and hydrophobic amino acid moieties on the surface, can impact how a protein behaves in solution (Hall, 1996). The hydrophilic amino acid residues tend to orient toward the water, whereas most of the hydrophobic residues are buried in the interior of the protein to minimize free energy (Damodaran, Parkin, & Fennema, 2008).

Samples were tested at three different pH: 3, 5 and 7 solutions. In general, pea protein isolates exhibit the lowest solubility between pH 4 and 6 irrespective of the pea cultivar since the isoelectric point of the pea globulins (legumin and vicilin) is around 4.5 (J.I. Boye, Askay, Roufik, Ribéreau, Mondor, Farnworth, et al., 2010; Hall, 1996; A. C. Y. Lam, Can Karaca, Tyler, & Nickerson, 2018). At pH values above and below the pI, solubility is increased due to electrostatic repulsion brought by positive and negative net charges on the protein chain that prevents protein from aggregation and precipitation (Taherian, Mondor, Labranche, Drolet, Ippersiel, & Lamarche, 2011). A protein exhibits the lowest solubility at its isoelectric pH since it carries a zero-net charge, thereby minimizing electrostatic repulsive forces (Withana-Gamage, Wanasundara, Pietrasik, & Shand, 2011). For all samples tested, lower solubility (10 – 22%) was found to be at pH 5, compared to other pHs tested. Under these conditions, hydrophobic interactions between neighboring proteins can lead to aggregation, and once the aggregates are sufficient in size and number, precipitation occurs (Hall, 1996).

At pH 3 in this study, solubility for all protein extract samples is considerably high, ranging from 56 to 78 %, with those from P0540-41 and Greenwater being the highest (76.84 and 78.47 %, respectively). At pH 7, solubility results are similar, with highest value over 80%, obtained for protein extracted from Earlystar (85.34%). Yin, Zhang, & Yao (2015) and Chao & Aluko (2018) also found the solubility of pea protein is strongly pH-dependent, reporting

the higher solubility above pH 6.0 and below pH 4.0 (about 80%), while the lowest solubility was reported to be between 4 and 6 (less than 30%).

When comparing high protein and regular protein containing genotypes, it can be observed that the higher protein content does not influence on protein solubility based on statistical analysis. This can be beneficial in industrial practices since solubility is a critical physicochemical property for protein processing and applications. Nevertheless, it should be mentioned that more studies on high protein genotypes are needed for more comprehensive conclusions.



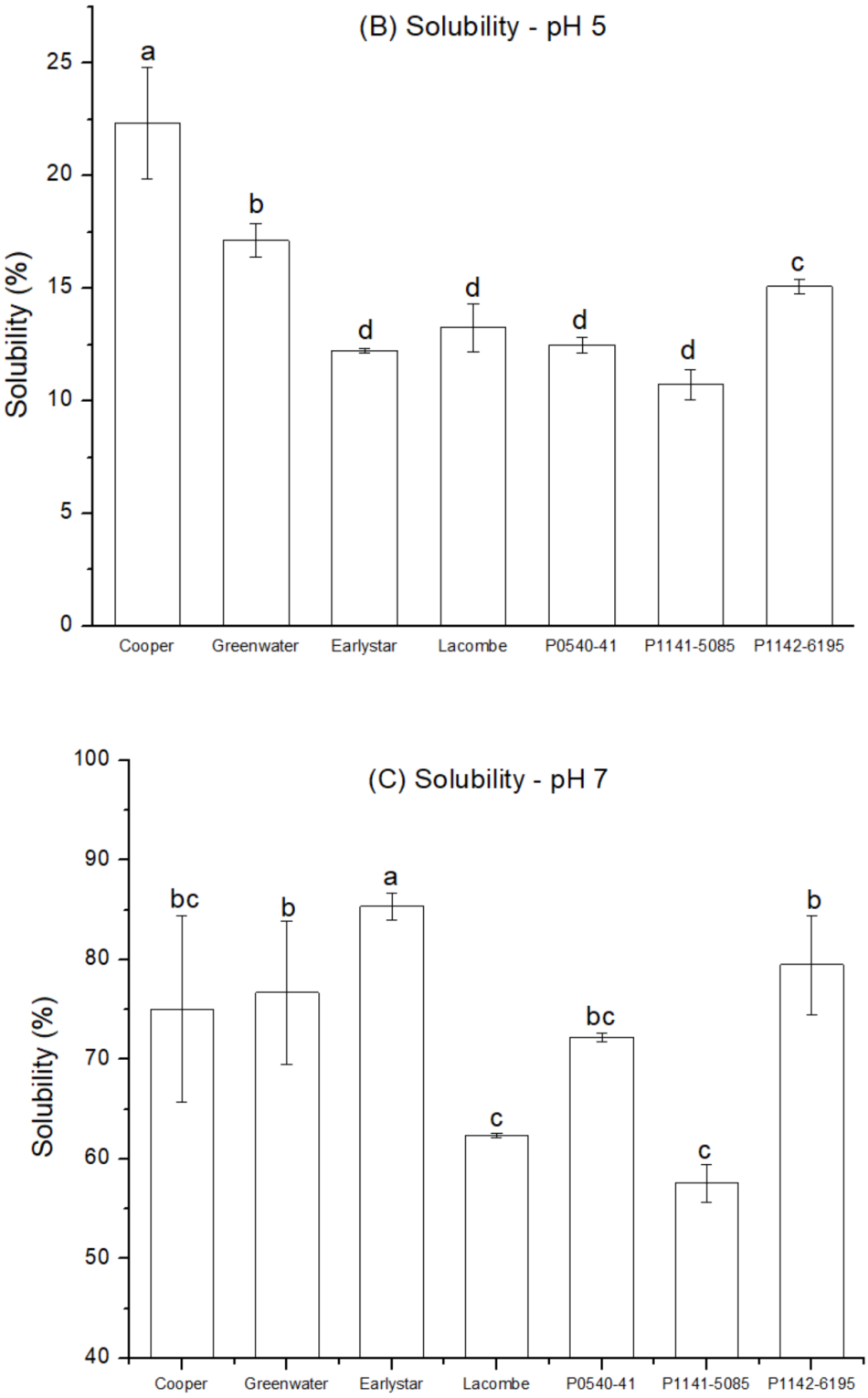


Figure 5. Solubility of the protein samples extracted from different pea genotype at pH 3 (A), 5 (B) and 7 (C). Different letters, (a, b, c), indicate significant difference ($p < 0.05$).

3.3.2. Oil and Water holding Capacity

Water-holding capacity (WHC) is defined as the amount of water that can be absorbed per gram of protein material (Lam, Can Karaca, Tyler, & Nickerson, 2018), or the ability of proteins to retain water against gravity (Zayas, 1997). Because conventional food products can comprise more than 50% water, poor WHC can further trigger liquid loss during processing and unfavorably alter the texture of products (J. Boye, Zare, & Petch, 2010). Water binding occurs through a combination of ion–dipole, dipole–dipole, dipole-induced dipole, and hydrophobic interactions (Shevkani, Singh, Kaur, & Rana, 2015). The association between water and protein is affected by the protein matrix structure, especially pore size (Hall, 1996). The amino acid composition of a protein is also one determinant of WHC. Water molecules bind to the charged groups, backbone peptide groups, amide groups, hydroxyl groups, and nonpolar residues of amino acids, where each group varies in its capacity to bind water molecules (Damodaran, Parkin, & Fennema, 2008).

The water holding capacities for the pea protein concentrates ranged between 1.2 and 6.0 g/g. When compared to soy protein, which ranged from 4.0 to 7.5 g/g, pea protein in this work showed a relatively lower WHC. In literature pea flour (15.2% protein) and pea protein flours (~50% protein) showed WHC values of 0.72-1.03 g/g (Sosulski & Youngs, 1979) (Wang, Bhirud, & Tyler, 1999). When extracted, pea protein (80% protein) shows WHC of approximately 4.1 g/g (Boye, et al., 2010). This agrees to what was observed in this study. Among the pea genotype, Earlystar and P1141-5085 have significantly lower WHC than the other tested samples, while Greenwater and Cooper have the highest WHC.

WHC is an important factor to consider when choosing food ingredients. Flours with high WHC could be good ingredients in bakery applications, such as bread formulations, since a higher WHC enables bakers to add more water to the dough, thus improving the handling characteristics and maintaining freshness in bread (Ladjal & & Mohamed, 2015). In bakery products, adding 5% of pea protein has resulted in less loss of moisture during baking (Pico, Reguilón, Bernal, & Gómez, 2019). Furthermore, WHC is a critical property of proteins in viscous foods, e.g. soups, dough, custards and baked products, because these are supposed to imbibe water without dissolution of protein, thereby providing thickening effect and viscosity (Sreerama, Sashikala, Pratape, & Singh, 2012). In this research, the pea protein concentrates

from high protein genotype seem to have relatively lower WHC than those from some normal genotype, which is worthy of investigation in the future.

Oil-holding capacity (OHC) is defined as the amount of oil that can be absorbed per gram of protein (Hall, 1996). Lipids and proteins interact through the binding of the aliphatic chains of lipid to the nonpolar side chains of amino acids; therefore, proteins with higher hydrophobicity tend to have a greater oil holding capacity (Damodaran, Parkin, & Fennema, 2008; Lin & Zayas, 1987). OHC values can be influenced by the matrix structure of a protein, the type of lipid present, and the distribution and stability of lipids. The stability of lipids is affected by both droplet size and distribution and the presence of emulsifying agents (Withana-Gamage, Wanasundara, Pietrasik, & Shand, 2011). All genotypes tested showed good OHC, ranging from 3.1 to 6.0 g/g. These results were higher than what was previously reported for pea protein isolates by Boye et al. (2010) (~ 1.2 g/g), Fuhrmeister and Meuser (2003) (0.87 g/g) and Fernandez-Quintela et al (1997) (1.2 g/g). Lacombe displayed significantly lower OHC than the other genotypes. Similar to WHC, Greenwater and Cooper showed better OHC than the other genotypes. It is interesting to observe that both these samples have similar 11s/7s ratio. However, there is no evident relationship between WHC/OHC and 11S/7S ratio according to statistical analysis in this study. Thus 11s/7s might influence the pea protein water and oil binding capacities in a confounded way with involvement of other impacting factors, which is worthy of investigation in the future.

Understanding the OHC is important as it relates to the emulsifying capacity of a protein and is an important characteristic when pea protein ingredients are used in meat binder applications (Sanjeewa, 2008). Also, fats act as a flavor retainer and increase the mouth feel of food (Elkhalifa & Bernhardt, 2010). In this sense, high protein line does not necessarily show different OHC when compared to normal protein genotypes, demonstrating their potential industrial use.

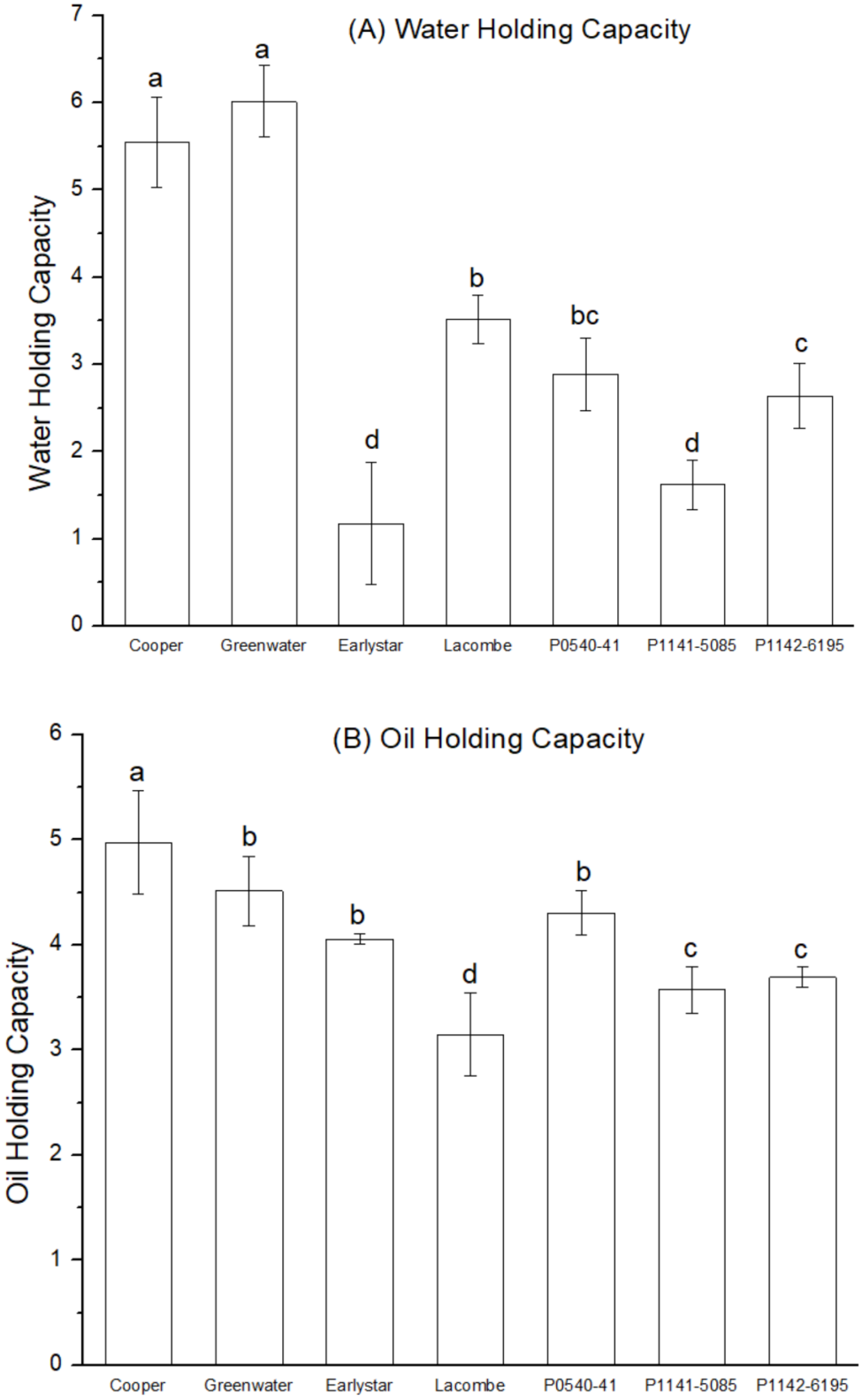


Figure 6. Water (A) and Oil (B) holding Capacity of different pea genotypes. Different letters, (a, b,

c), indicate significant difference ($p < 0.05$).

3.3.3. Emulsifying Capacity

An emulsion is the dispersion or suspension of two immiscible liquids created by mechanical agitation, resulting in a dispersed phase of submicron droplets suspended within a continuous phase (Hall, 1996). In foods, emulsions are of either oil-in-water (O/W) type, such as milk and mayonnaise, or water-in-oil (W/O) type, such as butter and margarine (A. C. Y. Lam, Can Karaca, Tyler, & Nickerson, 2018). Emulsions are thermodynamically unstable because such an arrangement increases the interfacial area, thereby increasing the interfacial free energy of the system (Hall, 1996). Over time, O/W emulsions are prone to the phenomena of creaming, flocculation, and coalescence as the system attempts to minimize its free energy (Alzagtat & Alli, 2002).

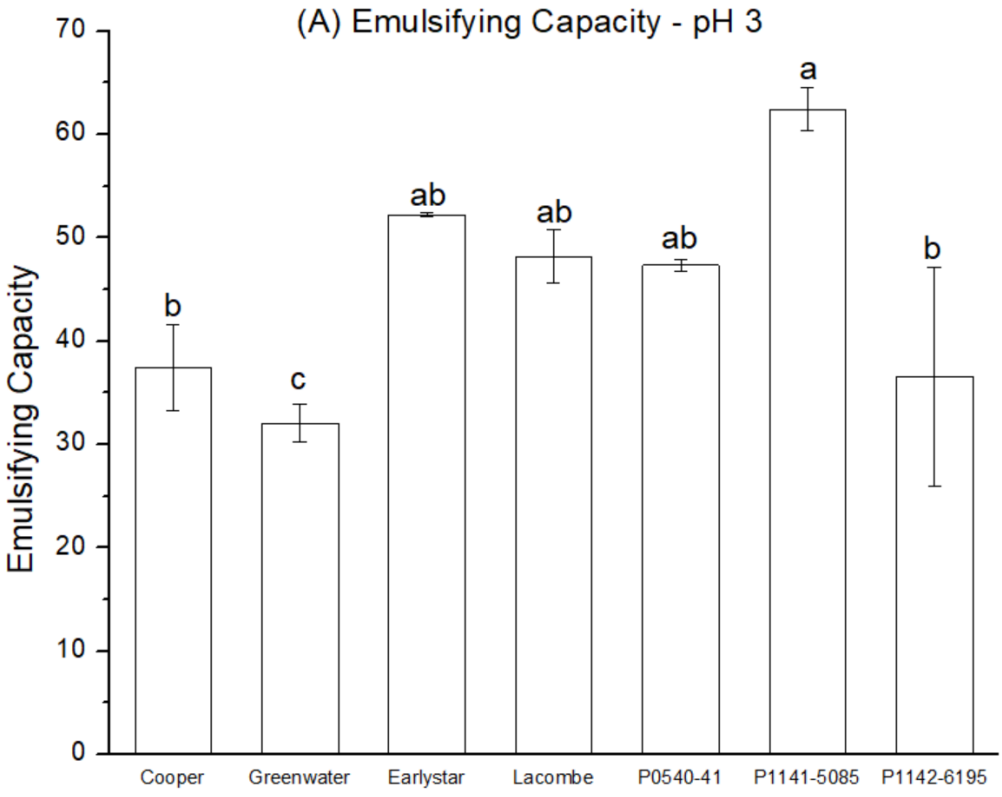
Emulsifying ingredients can come from dairy and egg proteins are used for milk, creams, salad dressings, mayonnaise, soups, margarine, and butter (Lam & Nickerson, 2013). With the consideration of sustainability, emulsifying ingredients from plant proteins are favorable in food formulations. Proteins adsorb to the interface to minimize the interfacial tension between the two phases (A. C. Y. Lam, Can Karaca, Tyler, & Nickerson, 2018). They align at the interface according to their amphiphilic nature and conform to train, loop, and tail configurations to form a viscoelastic interfacial film. Trains lie along the interface, whereas loops and tails protrude into the continuous phase to facilitate repulsion (Alzagtat & Alli, 2002; Walstra, 2003).

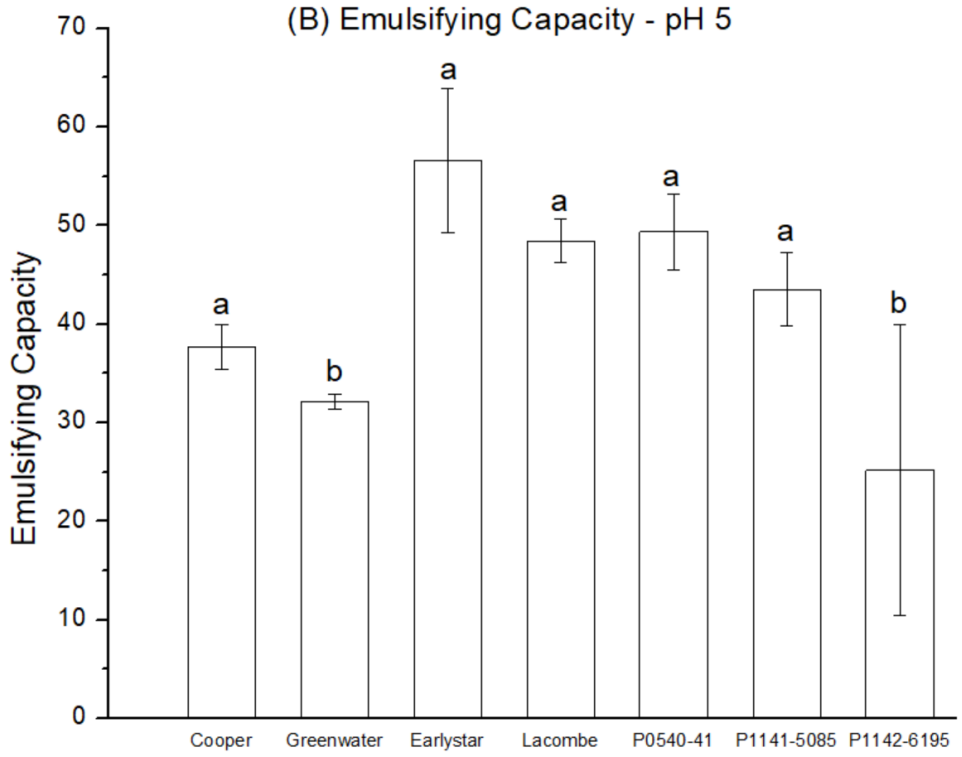
Proteins differ in the minimum amount required for monolayer coverage of oil droplets and in the rate of adsorption to the oil–water interface (Damodaran, Parkin, & Fennema, 2008) due to their different structures. The surface hydrophobicity and charge of a protein and its molecular flexibility determine the surface properties of a protein ingredient because relatively hydrophobic and flexible proteins can rapidly reorient to the interface to form a viscoelastic film (R. S. H. Lam & Nickerson, 2014).

In this study, at pH 7 (Figure 7C), Earlystar, Lacombe, P0540-41 and P1141-5085 have higher emulsifying capacity, ranging from 44 to 52%. This trend was maintained at pH 3

(range 47 – 62%) and pH 5 (range 43 – 56%). In addition, the pea protein samples show good emulsifying capacity at all the pH tested with those at pH 3 and 5 slightly than at pH 7, suggesting they may have potential as emulsifiers in food formulations with a broad pH range including mild acidic conditions. The pH dependence of pea protein emulsifying capacity was also reported in previous literature, but the trend was different. In the work of Lam et al., pea protein stabilized emulsions were more stable away from the pI of a protein when the electrostatic repulsive forces are greater to prevent flocculation and coalescence (A. C. Y. Lam, Can Karaca, Tyler, & Nickerson, 2018). The difference might be related to protein concentrates obtained from pea grains of different genotypes.

The 11s/7s ratio may partially influence the emulsifying capacity, as P1141-5085, Lacombe and Earlystar demonstrated relatively high emulsifying capacity at all tested pHs as well as. the highest 11S/7S ratio. Liang and Tang (2013) found that 11S had higher surface hydrophobicity than 7S which might contribute to their fast absorption to the interface, resulting in a better emulsifying property (Liang & Tang, 2013). Perrechil, Ramos, & Cunha (2015) studied the effect of protein heat treatment on the synergistic functionality of soybean 7S and 11S fractions in oil-in-water emulsions. The authors reported that emulsions stabilized by 7S showed smaller droplets than those containing 11S due to the higher surface activity, whereas 11S may have form stronger surface membrane at the oil droplet surface (Zhang, Li, Tatsumi, & Isobe, 2005). It is possible that the emulsions formed by protein containing a higher amount of 11S tended to form gel, promoting the formation of more viscoelastic protein layer at the oil droplet surface to slow down flocculation and coalescence. However more research is required to study the surface behaviors of pea protein 11S and 7S to better understand their roles in emulsion formation and stabilization, such as their capacity to reduce surface tension and formation of viscoelastic surface film.


(A) Emulsifying Capacity - pH 3
Emulsifying Capacity
70
60
50
40
30
20
10
0
b
c
ab
ab
ab
a
b
Cooper
Greenwater
Earlystar
Lacombe
P0540-41
P1141-5085
P1142-6195


(B) Emulsifying Capacity - pH 5
Emulsifying Capacity
70
60
50
40
30
20
10
0
a
b
a
a
a
a
b
Cooper
Greenwater
Earlystar
Lacombe
P0540-41
P1141-5085
P1142-6195

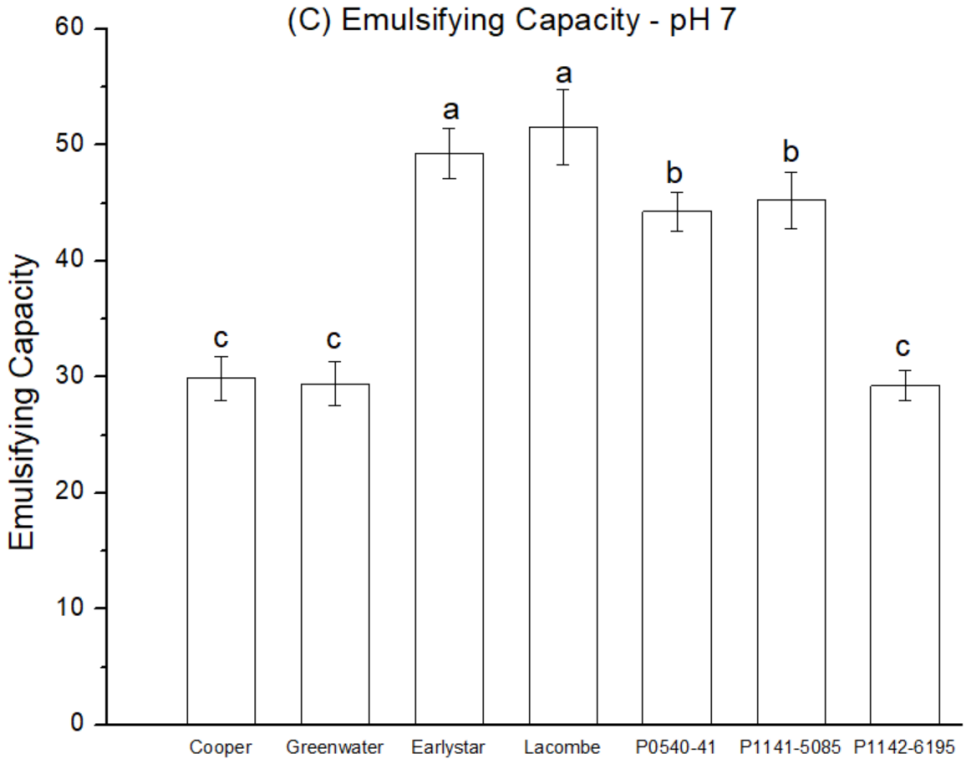


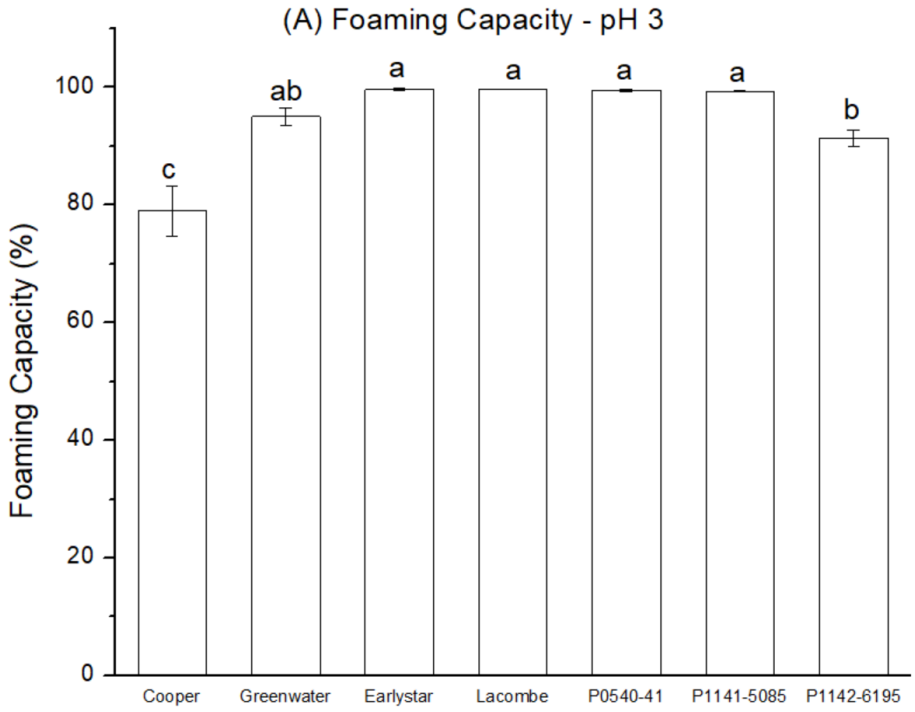
Figure 7. Emulsifying Capacity of different genotype at pH 3 (A), 5 (B) and 7 (C). Different letters, (a, b, c), indicate significant difference ($p < 0.05$).

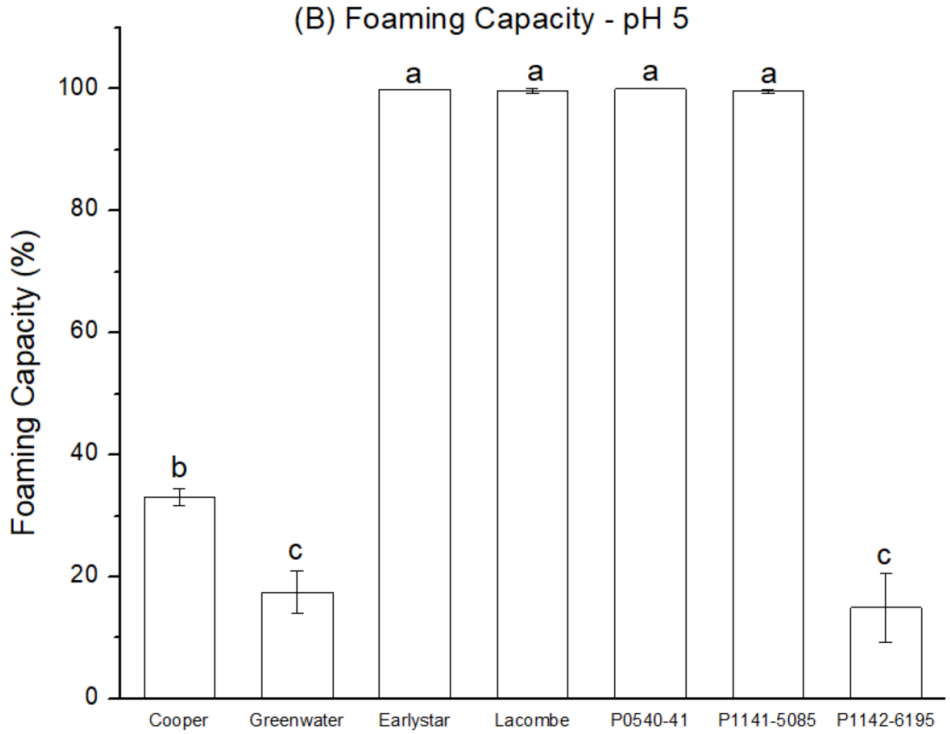
3.3.4. Foaming Capacity and Foaming Stability

Foams are dispersions of gas bubbles within a liquid (usually water) or solid continuous phase and can be generated by sparging (forcing gas into the liquid phase through an aperture), whipping (beating atmospheric air into the liquid phase), shaking, or pouring (such as a glass of beer) (McClements, 2004). Due to high free energy at the gas–liquid interface, foams are thermodynamically unstable and undergo coalescence and disproportionation to reduce the interfacial area (R. S. H. Lam & Nickerson, 2014). Disproportionation (Oswald ripening) is the diffusion of gas from small to large bubbles due to higher pressure within the former (Hall, 1996). Solubilized proteins diffuse and adsorb to the gas–liquid interface, which reduces surface tension. They then unfold and orient hydrophobic regions to the gas

phase and hydrophilic regions to the liquid phase to assume train and loop formations. Ideally, the protein should adsorb rapidly to the gas–liquid interface and possess high molecular flexibility for quick reorientation (Dickinson, 2010).

Foaming capacity (FC) is the amount of interfacial area that can be created by the protein (Wierenga & Gruppen, 2010). It is positively correlated to the surface hydrophobicity of proteins, and can be enhance enhanced by protein partial denaturation to increase surface activity (Damodaran, Parkin, & Fennema, 2008; A. C. Y. Lam, Can Karaca, Tyler, & Nickerson, 2018).





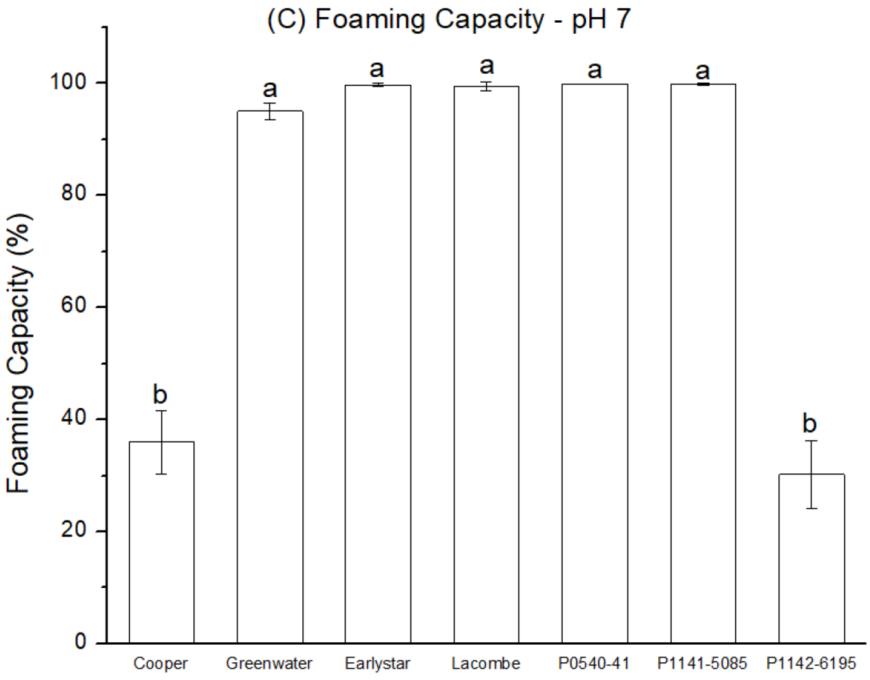


Figure 8. Foaming Capacity of protein extracts from different pea genotype at pH 3(A), 5(B) and 7(C). Different letters, (a, b, c), indicate significant difference ($p < 0.05$).

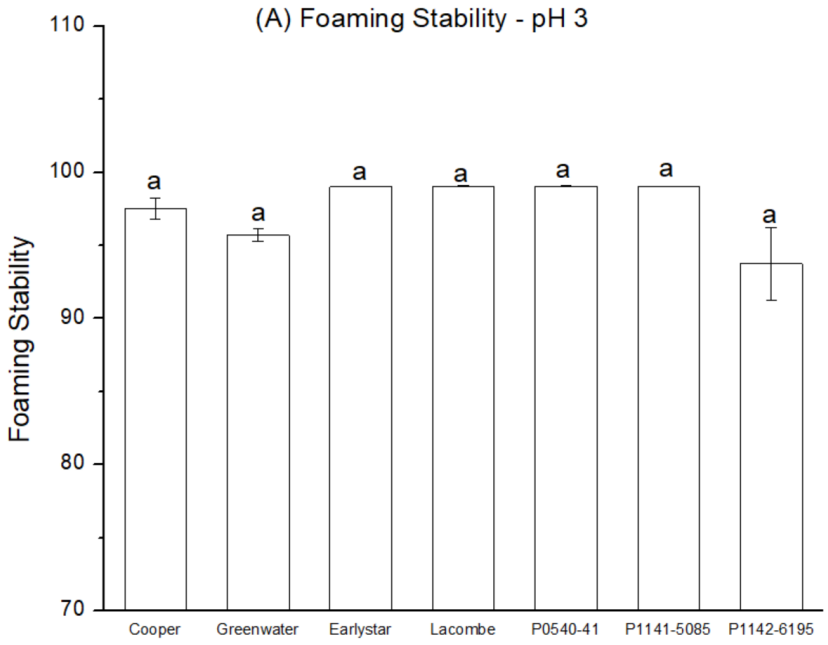
Pulse proteins such as pea protein and lentil protein concentrates were found to be more suitable to generate stable foams than many other plant proteins (Jarpa-Parra, et al., 2015) (Mohanan, Nickerson, & Ghosh, 2020). The extracted pea protein samples exhibit good foaming capacity at pH 3. Protein concentrates from Lacombe (99.6%), Earlystar (99.5%), P0540-41 (99.4%) and P1141-5085 (99.3%) are higher than those from Cooper (79%), Greenwater (95%) and P1142-6195 (91%) in foaming capacity. These four genotypes (Lacombe, Earlystar, P0540-41 and P1141-5085) have better results than Cooper, Greenwater and P1142-6195 in all tested pHs. Chao et al. (2018) observed the highest foaming capacity of a pea protein isolate at pH 3.0, with a maximum value of 81%, and lower values at pH 5.0 and pH 7.0 (38% and 62% respectively). Foaming capacities found in this study agreed with what was reported for two commercial pea protein isolates (99% and 96%) (Soral-Smietana, Swigon, Amarowicz, & Sijtsma, 1998). Cooper and P1142-6195 had relatively low foaming capacity at all tested pHs and had the lowest 11S/7S ratio as compared to other samples. However, the correlation between 11S/7S ratio and pea protein foaming capacity has not been clearly reported. More systematic study is needed to reveal how the 11S/7S ratio may impact pea protein foaming capacity.

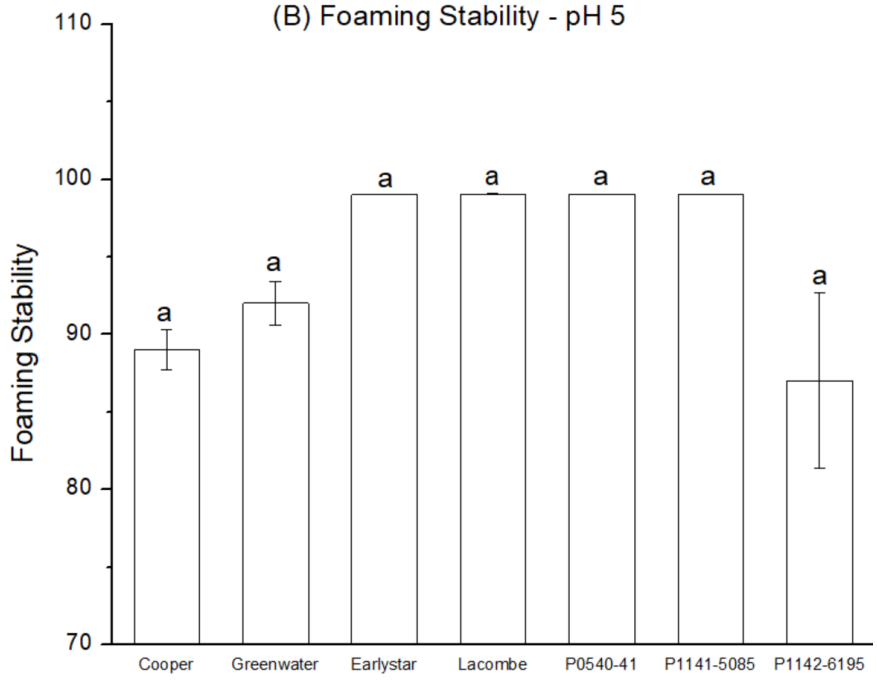
Foam stability (FS) is the ability of a protein to stabilize a foam against stresses (Damodaran, 2005). Stable foams tend to be resistant to gas diffusion, drainage and thinning of lamella fluid, and mechanical shock. Accordingly, stable protein-based foams should possess interfacial films that are cohesive through hydrogen bonding and electrostatic and hydrophobic interactions. Intermolecular associations should result in a network structure of high surface elasticity to allow for some deformation (Damodaran, Parkin, & Fennema, 2008; J. E. Kinsella & Melachouris, 1976). Foams are reported to be more stable at the isoelectric pH of a protein. Because of minimal electrostatic repulsion, protein–protein interactions and adsorption to the interface are maximized, which promotes viscous film formation and steric stabilization (Wierenga & Gruppen, 2010).

In this study, most of the extracted pea protein samples show high foaming stability regardless of the pH and pea genotype as more than 94% of the foams were remained after 30 min.

Stone et. al (2015) studied the functional atribuites of pea protein isolates. Overall, the foaming stability (FS) for the lab produced pea protein isolates ranged from ~ 48.9% to 77.8% depending on the extraction method and cultivar. The results obtained in this study are higher than those described previously. It is possible to say that the lab extracted protein maintained better native protein and therefore, higher functional properties than the industry processed samples.

The incorporation and retention of air is of great importance for numerous foods, such as angel food cake, meringue, marshmallows, and whipped cream as it changes its texture through imparting body and smoothness (Campbell & Mougeot, 1999) (Kinsella, 1981). Thus, foams are desired structures with reduced density and unique rheological properties (Lau & Dickinson, 2005). Some authors have described the use of pea protein as an excellent additive in baked goods, such as crackers (Morales-Polanco, Campos-Vega, Gayt´an-Martínez, Enriquez, & Loarca- Piña, 2017), gluten-free bread (Matos, Sanz, & Rosell, 2014) and eggless cake (Lin, Tay, Yang, Yang, & Li, 2017). From the results obtained in this study, it seems that pea genotype may impact the protein foaming capacity more than foaming stability. Nevertheless, no clear correlation between foaming capacity (FC) / stability (FS) and 11s/7s ratio was found. Thus, further research is worthy of investigation in order to better understand. Again, high protein genotype do not necessarily show different foaming properties when compared to normal protein genotypes. Considering the good stability of pea protein stabilized foams, high protein genotype have high potential to be used as foaming ingredients in food formulations.


(A) Foaming Stability - pH 3
Foaming Stability
110
100
90
80
70
a
a
a
a
a
a
a
Cooper
Greenwater
Earlystar
Lacombe
P0540-41
P1141-5085
P1142-6195


(B) Foaming Stability - pH 5
Foaming Stability
110
100
90
80
70
a
a
a
a
a
a
a
Cooper
Greenwater
Earlystar
Lacombe
P0540-41
P1141-5085
P1142-6195

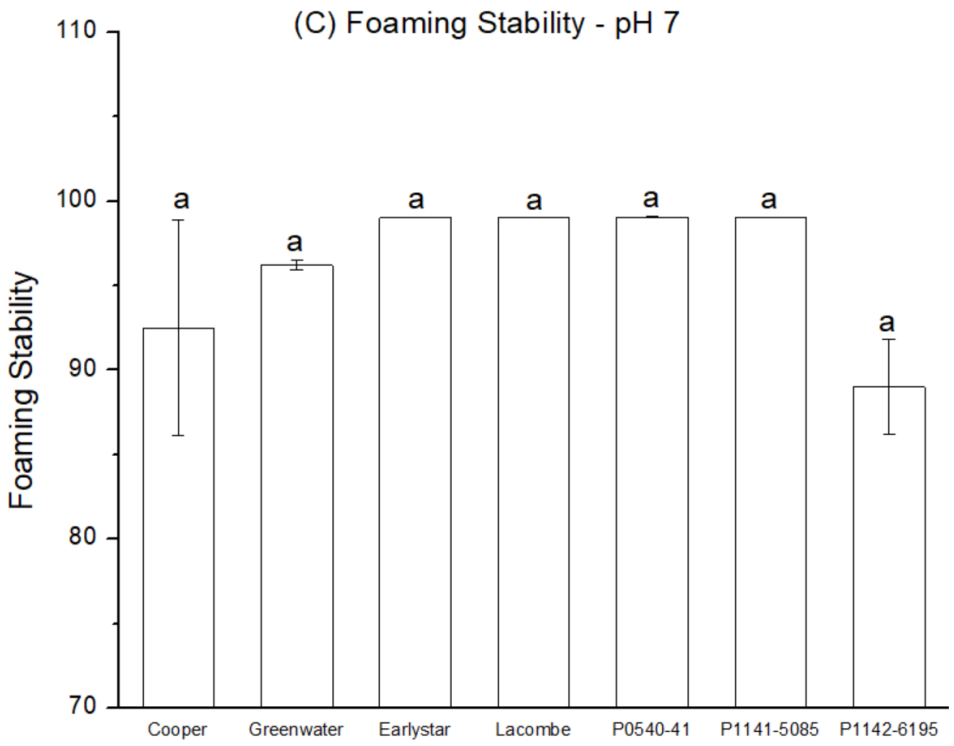


Figure 9. Foaming Stability of protein extracts from different pea genotype at pH at pH 3(A), 5(B) and 7(C). Different letters, (a, b, c), indicate significant difference ($p < 0.05$).

3.3.5. Gelling Capacity and gel mechanical properties

A protein gel is defined as a three-dimensional network assembled from protein molecules that can hold a large amount of water (Kinsella, 1981). Gelation mechanism of globular proteins consists of two stages, conformational change or denaturation of protein molecules, followed by association or aggregation into a three-dimensional matrix structure that traps water, fat, and other food ingredients (Lam, Can Karaca, Tyler, & Nickerson, 2018).

Protein gelation can be induced by heat treatment, pH, salts, pressure or shearing, and the presence of various solvents (A. C. Y. Lam, Can Karaca, Tyler, & Nickerson, 2018). Heat-induced gelation of pea proteins has been studied by several researchers and has been reported to be affected by many factors such as cultivar, extraction procedure, heterogeneity of the protein, solvent parameters, and heating procedure (Yang J. , Zamani, Liang, & Chen, 2021) (Culbertson, 2005; Mession, Blanchard, Mint-Dah, Lafarge, Assifaoui, & Saurel,

2013; O'Kane, Happe, Vereijken, & Gruppen, 2004; Shand, Ya, & Wanasundara, 2007; Sun & Arntfield, 2011). In this study samples were tested on their gelling capacity at 3 different concentrations, while maintaining the same heat treatment (1h heating at 90°C). The Results are summarized in table 4. Forming gels at a lower protein concentration can be advantageous for pea protein applications as a gelling ingredient. All the tested protein samples formed gels with 18% protein. Cooper, Lacombe, P1141-5085 and P1142-6195 could not form gel with 16% of protein while P0450 could form gels of medium strength at the same protein concentration. Proteins from Earlystar, P0450 and P1142 formed strong gel at 20% protein concentration. From the above results, Earlystar and P0540-41 had better gelling capacity and their gels had higher compressive stress, while, at the same time, showing relatively lower 11S/7S. On the other hand, Lacombe and P1141 had lower gelling capacity and higher 11S/7S ratios. This result suggests that pea protein gelling capacity and gel strength may be negatively correlated to protein 11S/7S ratio. O'Kane reported that the cysteine in legumin could form disulfide bonds during gelation, which was a factor that prevent gel network strengthening (O'Kane, Vereijken, Gruppen, & van Boekel, 2005). Moreover, the N-terminal extensions and carbohydrate moieties of vicilin could prevent the random protein-protein interactions after denaturation, which leading to the more homogeneous and interconnected gel network (O'Kane, Happe, Vereijken, & Gruppen, 2004) (Yang J. , Zamani, Liang, & Chen, 2021). It was also described that the higher 11s/7s ratio had negative impacts to the gel structure, in the sense of the formation of more porous gels (Wu, Hua, Chen, Konga, & Zhang, 2016).

Moreover, the gelling capacity is affected by the heating time used during processing. Prolonged heating at 90°C for 1h led to strong gels for Cooper, Greenwater, Earlystar P054-041 and P1142-6195, whereas only soft and medium gels were formed when heated at 90°C for 30 min. As shown in Table 4, Cooper, P1141-5085 and P1142-6195 demonstrated higher denaturation temperature and this should be considered when programing future studies.

As seen in figure 7, Cooper, Earlystar, P054-041, P1141-5085 and P1142-6195 could only form firm strong gels at 20% concentration. Thus, 20% protein gels were used to evaluate the texture profiles. Compressive stress is the peak force that occurs during the first compression, corresponding to the hardness of the gel. As seen in figure 8, the protein

extracts from different pea line had diverse results. Protein gels from Earlystar demonstrated to withstand the highest compressive stress of 8 and 7 kPa, drastically higher than the gels prepared by protein extracts from P1142-6195, P1141-5085, Cooper and P0540-41 respectively. As shown in Fig. 9, the springiness values of gels prepared from different pea protein samples also varied. The springiness value of all gel samples was ranging from 60 to 80 %, meaning that all the gels can return to about 20 to 40% of the original height after the deformation generated by the first compression.

Protein gelling properties are very important for their food applications, since several foods are marketed in the form of gel that offers convenience to the consumers. Examples include jam, jelly, confectionery products, desserts, quick-set gels, and other gel products based on fruits and vegetables (Banerjee & Bhattacharya, 2012).

Pea protein isolates have been reported to form weaker and less elastic gels compared to soy protein isolate when processed under the same conditions (O'Kane, Vereijken, Gruppen, & Van Boekel, 2005; Jingqi Yang, Zamani, Liang, & Chen, 2021).Thus developing strong gelling capacity from pea protein is necessary to allow their wide applications as alternatives to soy in food formulations. It is interesting to notice that proteins from some high protein pea genotypes show significantly improved gelling properties. This may suggest the opportunity to improved pea protein gelling capacity by breeding effort.

Table 4. Gel formation at different concentrations for protein extracts from different pea genotype.

	16% - heating at 90°C for 1 h	18% - heating at 90°C for 1 h	20% - heating at 90°C for 1 h	20% - heating at 90°C for 0.5h
Cooper	Liquid	Medium Gel	Medium Gel	Medium Gel
Greenwater	Soft Gel	Soft Gel	Soft Gel	Soft Gel
Earlystar	Soft Gel	Medium Gel	Strong Gel	Medium Gel
Lacombe	Liquid	Soft Gel	Soft Gel	Soft Gel

P054-041	Medium Gel	Medium Gel	Strong Gel	Medium Gel
P1141-5085	Liquid	Medium Gel	Medium Gel	Medium Gel
P1142-6195	Soft gel	Soft Gel	Strong Gel	Medium Gel

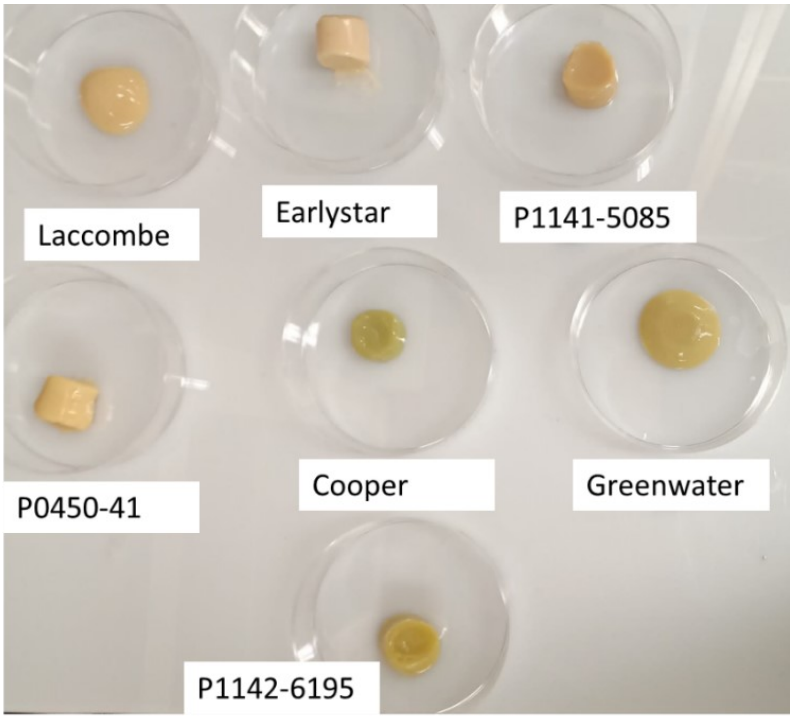


Figure 10. Photos of gels prepared using pea protein extracts from different pea genotype.

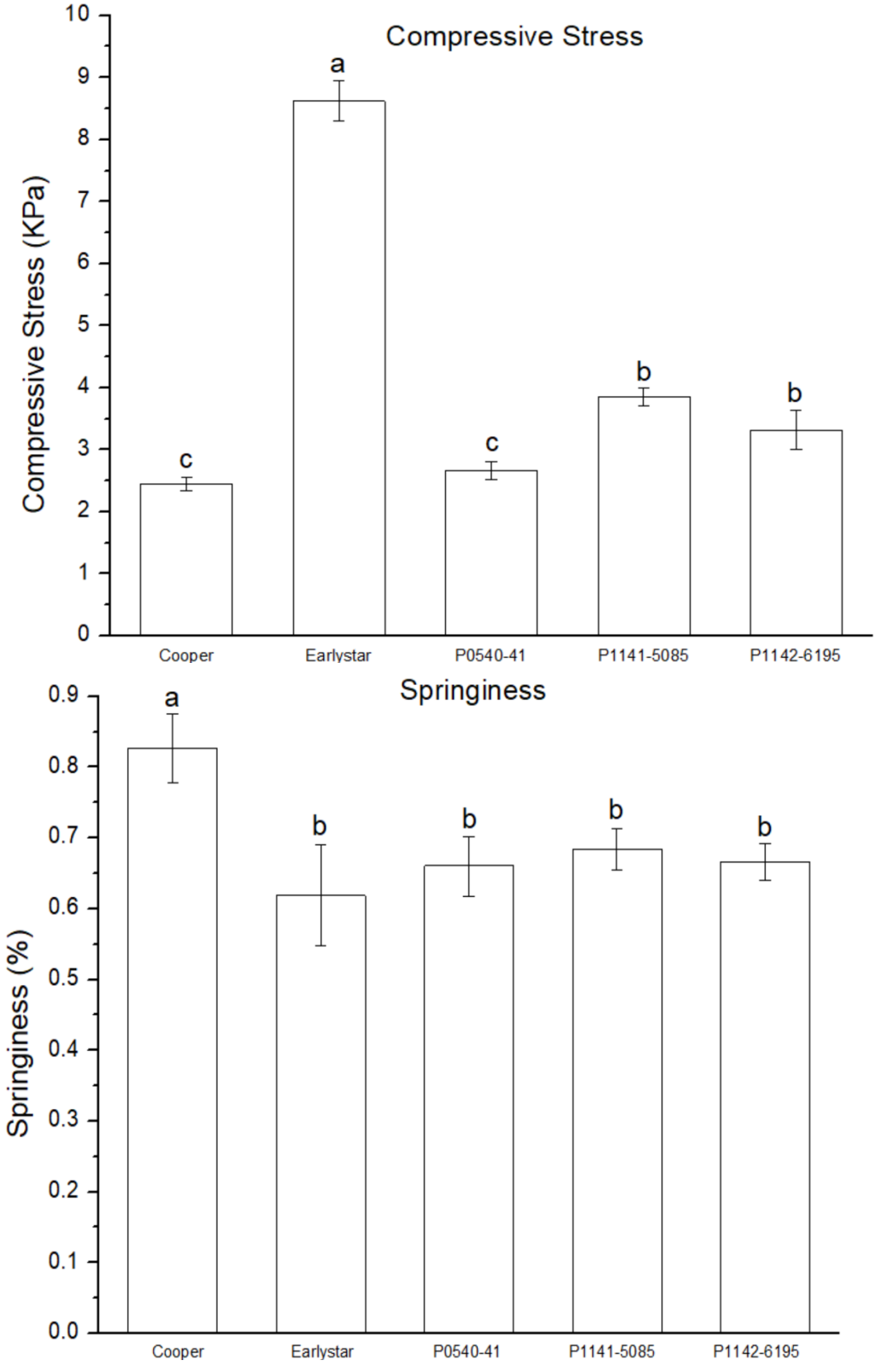


Figure 11. Compressive stress and springiness of pea protein gels.

3.4. Digestibility

Proteins from legumes, such as pea (*Pisum sativum L.*), are a good source of lysine and other biologically active components such as antifungal bioactive peptides or dietary lectins with health-promoting properties (Nguyen, Gidley, & Sopade, 2015). Besides the amino acid contents, the bioavailability of the protein, which is in part governed by the digestion rate and extent, is a key determining factor of protein quality and postprandial protein gain (Dangin, et al., 2001). The digestion of a particular protein may also depend on the protein processing conditions such as pH, and interactions with other food components (Sarkar, Goh, & Singh, 2010) (Sarkar A. , Goh, Singh, & Singh, 2009) (Singh & Sarkar, 2011). Habiba et al (2002) studied the changes in anti-nutrients' content, protein and amino acid solubility, digestibility of vegetable pea after different cooking methods (ordinary cooking, pressure cooking and microwave). Overall, cooking improved the *in vitro* protein digestion rates by decreasing the levels of various anti-nutrients, such as phytic acid and trypsin inhibitor etc. However, traditional cooking was also postulated to result in lesser extent of digestibility. For example, high temperatures or prolonged exposure to heat has been reported to result in losses in the essential amino acids due to Maillard reactions (Satterlee & Chang, 1982), and thus might reduce the overall digestibility of the proteins due to denaturation.

During the analysis of the protein nutritive value, one limitation of this study is that the amino acid composition did not account for cysteine. This is mainly because the cysteine is derivatized during hydrolysis. Thus the amino acid profiles of the protein extracts were included in the Appendix 1.

When comparing previous studies to the results obtained in this study, it is noticeable that all pea genotypes had good digestion percentage, above 75% through *in vitro* test. Lacombe and P0540-41 have significantly higher percentage, 86.3 % and 88.45 %, respectively, while P1142-6195 and Cooper demonstrated lower digestion percentages – 75.8 % and 76.44 %. The minor components in protein extracts may also impact protein digestibility such as fibers and phenolic compounds. In a summary, all tested pea genotypes can be considered for food products due to their high digestion and nutritious values.

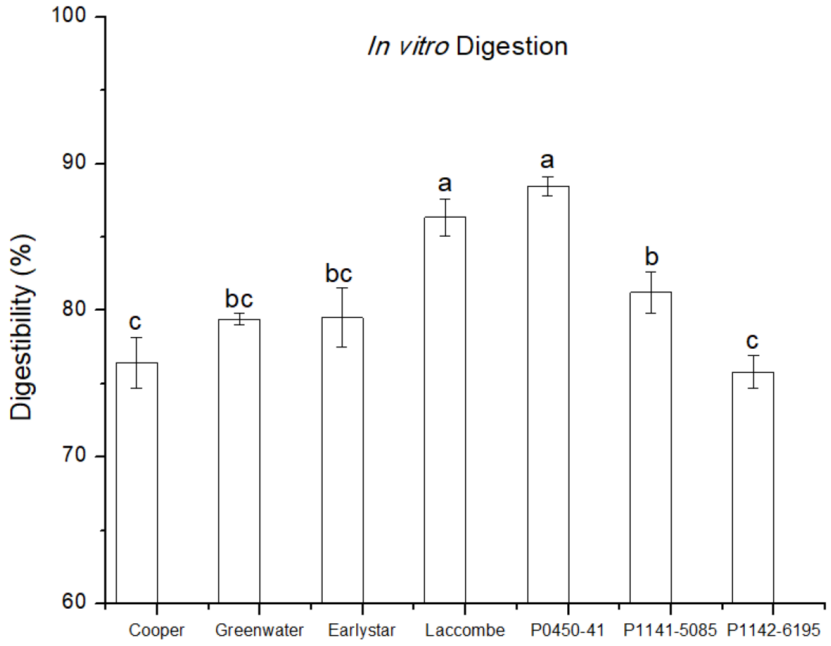


Figure 12. Digestion (%) of protein extracts from different pea genotype.

4. Conclusions

Although a lot of research and effort has been focused on exploring the functional properties of pea proteins, this is the first of its kind to compare the structure and functional properties of pea protein extracted from high protein and regular pea genotypes.

The different genotypes display different protein compositions. P1141- 5085 demonstrates the highest 11s/7s ratio which can be presumed correlated to a high denaturation temperature. On the other hand, the remaining two high protein genotype (P0540-41 and P1142-6195) had the lowest 11s/7s ratio, and this may have an impact on some functional properties, such as foaming, emulsifying and gelling capacities. Normal and high protein genotypes had similar solubility. P0540-41 and P1141-5085 have excellent emulsifying capacity. Especially, Earlystar can form better gels among all 7 genotypes. Overall, protein from P0540-41 seems to present better functionality than the protein extracts from the other tested genotypes.

Pea protein samples from high protein genotypes can have comparable functional properties to normal genotypes, with significantly increased protein content. This can benefit the food industry as there is an increasing awareness about the benefits of protein-rich diet and plant-based alternatives; rising world demand for protein due to rapid population growth; growing consumer interest in foods that promote health and well-being, and adoption of environmental stewardship and protection of animal welfare (National Research Council of Canada, 2019).

It also interesting to notice that 11S/7S ratio may be related to pea protein functional properties. Once the correlation has been identified by future studies, new variety development strategy may be provided to modify protein physicochemical properties (e.g., denaturation temperature, solubility) as well as their functionalities (e.g., gelling, foaming and water/oil holding capacities) in order to target specific protein functional properties for specific industrial needs in food applications.

For this reason, the obtained results are interesting to incite future studies with more pea genotypes to compare the influence on their protein functionality. It is known that functionalities are directly impacted by protein composition and structures. Thus, more systematic studies to understand the structure-function properties of protein from different pea genotypes will be required. The generated information can provide useful information to help breeding work to develop new pea genotypes targeting not only increased protein content but improved specific functionality to allow wider applications of pea proteins in food formulations.

Chapter 3 – Conclusion and Recommendations

1. Summary and Conclusion

The food industry has been shifting trends on use of animal proteins due to a variety of factors, which include consumer´s perception of health, ethical and religious purposes, as well as environmental factors (Barac, et al., 2010). Because of this, plant-based alternatives have gained particular interest.

Pulses are inexpensive source of proteins and other nutrients such as resistant starch, dietary fiber, vitamins, minerals, and polyphenols (Boye, Zare, & Petch, 2010) (Dave Oomah, Patras, Rawson, Singh, & Compos-Vega, 2011). Among the pulse family, field pea (*Pisum sativum*) is a widely produced legume (36% of total pulse production) (Dahl, 2012) that has low allergenicity, high nutritional value, availability, and low cost (Shevkani, Singh, Kaur, & Rana, 2015) (Stone, Karalash, Tyler, Warkentin, & Nickerson, 2015). Canada has become a leading producer of field pea, accounting for 35% of global pulse trade each year (Bekkering, 2011). To maintain the competitive advantage in the global market, Canada government has significantly invested in developing new field pea genotypes that resist lodging and disease, are adapted to shorter growing season and improved nutritive quality (Bekkering, 2011). Though a lot of research has been focused on the applications of pea protein, the effect of genotype on the functional properties of pea proteins has not been clearly investigated.

In this collaborative study with Agriculture and Agri-Food Canada (AAFC), pea grains from seven genotypes were provided by AAFC Lacombe Research and Development Centre. These genotypes are classified as high protein containing genotype and regular protein containing genotype. The protein from these seven different pea genotypes were extracted by alkaline solution followed by acidic precipitation. These protein extracts were then studied for their structure-function properties.

P0540-41 and P1141-5085, and P1142-6195 have protein contents of 26.4 %, 26.7 % and 29.0%, respectively, which are significantly higher than those of normal genotypes including

Cooper, Earlystar, AAC Lacombe and CDC Greenwater (25.8 %, 25.5 %, 25.6 % and 24.7 %, respectively). Pea protein concentrates were successfully extracted from pea grains of all seven genotype with the protein content ranging from 76.5 % to 86.2 %. Protein extracts from Earlystar (86.18%) and P1141-5085 (85.19%) exhibited relatively higher protein contents, while those from Cooper (76.51%) exhibited relatively the lower protein content. Then the protein content, composition and structures (e.g. amino acid profile, secondary structure, denaturation temperature, molecular weight) were systematically characterized using an array of advanced analytical tools including SDS – PAGE, Fourier-transform infrared (FT-IR) spectroscopy, HPLC - Size exclusive chromatography (SE), Differential Scanning Calorimetry (DSC).

Globulins are major storage proteins in pea grains with legumin (11S) and vicilin (7S) as the major globulin fractions (Gueguen & Cerletti, 1994). It is interesting to notice that the protein extracts from different pea genotype possess a wide range of 11S/7S ratio from 1.5 to 8.7. While P0540-41 and P1142-6195 show similar 11S/7S results (1.9 ± 0.7 and 1.5 ± 0.5, respectively), P1141-5085 demonstrated an extremely high ratio of 8.6 5± 0.65, which is also high when compared to regular protein containing genotypes with the 11S/7S ratio range of 2.0 to 5.95. This may be explained due to breeding process that have favored one protein fraction over the other. The denaturation temperature (Td) of the pea protein samples ranged from 87.5 – 105 °C. Protein samples from P1141-5085 and Cooper present higher denaturation temperature with the values of 105.0 and 101.8 °C, respectively when compared to other genotypes. Protein extracts from P054-041 and P1142-6195 had relatively lower Td. The pea protein denaturation temperature variation in this study could be partially related to the protein 11S/7S ratio because legumins have higher denaturation temperature. However, such correlation needs to be investigated by further studies. The FT-IR spectra revealed that pea proteins from different genotypes have similar secondary structure profile, all having β-sheet as the major secondary structure component. This demonstrates that the increase in protein content does not affect the secondary structure when compared to the regular protein genotype.

Pea protein is commonly known to be lacking in sulfur containing amino acids including methionine and cysteine. Because of this, it is often consumed along with cereal grains, as

they have a complementary essential amino acid profile in that cereal proteins are generally deficient in lysine but contain higher levels of sulfur amino acids. When it comes to digestibility, the protein concentrates from Lacombe and P0540-41 have higher digestibility values of 86.3 % and 88.45 %, respectively, while P1142-6195 and Cooper demonstrated lower digestion percentages of 75.8 % and 76.44 %, respectively. The minor components in protein extracts may also impact protein digestibility such as fibers and phenolic compounds.

The physicochemical and functional properties tested were solubility, oil and water holding capacity, emulsifying capacity, foaming capacity and stability, gelling capacity and mechanical gel strength. Solubility, emulsifying capacity and foaming capacity and stability were tested at 3 different pHs: 3, 5 and 7. These pHs were chosen as they represent the range in which most food products are produced in.

Solubility is a critical function for processing and manufacturing. At pH 3, solubility of protein extracts for all tested genotypes is considerably high, ranging from 56 – 78 %. Those from P0540-41 and Greenwater showed higher solubility values (76.84 and 78.47 %, respectively). High solubility was also observed at pH 7, with the highest value over 80%, obtained by protein extract from Earlystar (85.34%). Lowest solubility was observed at pH 5 for all the protein extracts which was expected because a protein exhibits the lowest solubility at its isoelectric pH.

In general, the pea protein extracts show good foaming and emulsifying properties, and their foaming stability values are especially high. Among the protein extract samples, those from Cooper and P1142-6195 had relatively low foaming capacity at all tested pHs and the lowest 11S/7S ratio as compared to other samples. However, the correlation between 11S/7S ratio and pea protein foaming properties has not been clearly established. The pea protein extracts showed slightly higher emulsifying capacity at pH 3 than at pH 5 and 7, suggesting they have better potential as emulsifiers in acidic conditions than at neutral or alkali pH. Among protein extracts from different pea genotype, those from P1141-041, Lacombe and Earlystar demonstrated relatively high emulsifying capacity at all tested pHs. The 11S/7S ratio may partially influence the emulsifying capacity, as legumin and vicilin ratio impacts the protein molecule flexibility. It is known that a protein with more flexible conformation can better adsorb at the oil droplet surface to form the emulsions.

It is interesting to notice that the pea protein gelling properties are influenced by the genotypes. The protein samples from Earlystar and P0540-41 possess better gelling capacity and their gels showed significantly increased mechanical strength. The pea protein gelling capacity and gel strength may be negatively correlated to protein 11S/7S ratio which is worthy of investigation in the future. Improving pea protein gelling properties is important because developing strong gelling capacity from pea protein is necessary to allow their wide applications as alternatives to soy in food formulations. The result from this research suggests new strategy to improved pea protein gelling capacity by breeding effort.

Overall, this study shows that increasing protein content in new pea genotype does not necessarily sacrifice the protein functional properties and nutritive quality, when compared to the regular pea genotype. Considering all the tested functional properties, among all the pea genotype tested, the high protein line P0540-41 presents a better source to generate pea protein ingredient with good overall functional properties to support food applications.

2. Significance of Research

This research is the first of its kind to compare not only the nutritive value of peas, but also the structure and functional properties of pea proteins between high protein genotype and regular protein genotype. Although, it is an initial study with few samples, the results obtained demonstrate that high protein genotype are comparable, and, in some instances superiors to regular protein genotype in terms of protein functionality and nutritive value. This opens the possibility of using breeding in order to achieve not only high protein content, but also to target specific protein physical-chemical and functional properties for desirable industry processing and food applications.

3. Future work and recommendations

Future recommendations would include raising the number of samples, including more high protein genotypes. This would be beneficial because a larger sample size would be more representative of high pea protein genotype. This would lead to a better understanding of how variety impacts protein composition and structure, subsequently their physical-chemical and functional properties.

In addition, genotypes from different harvest years and different regions need to be studied in order to understand how combination of these factors can profoundly influence the pea protein development and their functionality and nutritive quality.

In this study, the protein extraction method chosen was the alkaline extraction followed by isoelectric precipitation, due to its high yield and easy processing. In this sense, it was assumed that the high protein genotypes had similar isoelectric point. However, this may not be the case. Therefore, it is suggested to experimentally test if the isoelectric point shifts in high protein genotypes in the future. Also, other extraction methods may favor these high protein genotypes, so it may be interesting to study the influence of the extraction method on protein yield and purity these genotypes.

During the analysis of the protein nutritive value, one limitation of this study is that the amino acid composition did not account for cysteine. This is mainly because the cysteine is derivatized during hydrolysis. Thus, it is recommended to analyze the cysteine content by an alternative method in the future such as the phenyl isothiocyanate (PITC) derivatization method that is inexpensive and can be automated for daily mass spectra analysis. *In vitro* model was used to study the protein digestibility in this study. *In vitro* models posses many advantages such as simplicity and cost efficiency, less or no ethical issues. But the limitation of *in vitro* method is lack of important metabolizing enzymes (such as cytochrome P450 enzymes) and absence of intestinal motility. Thus *in vivo* methods will need to be applied to evaluate the protein digestibility in the future. In such way, the protein PDCAAS value and protein efficiency ratio (PER) can be quantified for protein extracts from different pea genotypes. PDCAAS and PER are required in USA and Canada, respectively, to more accurately evaluate the protein nutritive value

In summary, more systematic studies to understand the structure-function properties of protein from different pea genotypes will be required. The generated information can provide useful information to help breeding work to develop new pea genotypes targeting not only increased protein content but improved specific functionality to allow wider applications of pea proteins in food formulation.

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Appendix 1. Amino Acid Composition of pea protein extracts

	Protein Pea Genotype (g/100g protein)							Requirements by age groups (FAO, 2007)		
Essential Amino Acids	Cooper	Greenwater	Earlystar	Lacombe	P054-041	P1141-041	P1142-6195	1 - 3	4 -18	≥18
Histidine	1.5	2.1	2.1	2	1.9	2.4	1.2	1.6 - 1.8	1.6 - 1.8	1.5
Valine	2.7	3.3	3.4	2.8	3.1	3.7	3	4.1	4	3.9
Methionine + Cysteine	1.4	**6.4**	**6.7**	0	**6.6**	1.5	0.5	2.5	2.3	2.2
Isoleucine	2.6	3.2	3.1	2.6	3	3.4	3	3.1	3	3
Leucine	4.2	5.6	5.6	4.9	5.4	6.1	5.2	6.3	6.1	5.9
Phenylalanine + Tyrosine	5.1	6.2	6.6	5.3	6	7	5.6	4.1 - 5.6	4	3.8
Lysine	4.1	5	5.1	4.3	4.9	5.5	4.8	4.8 - 5.2	4.7	4.5
Threonine	2.1	2.6	2.6	1.5	2.4	2.9	2.1	2.5 - 2.7	2.4	2.3