Developing and characterizing functionally enhanced plant proteins for food applications

by

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B.S., Kansas State University, 2014
M.S., Kansas State University, 2016

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Grain Science and Industry
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Abstract

The demand for protein as food ingredient and in the human diet continues to increase due to its nutritional benefits, functional properties, rising protein deficiency, and the growing world population. Plant-based proteins represent a more sustainable source to supplement costly animal proteins. The goal of this study was to improve protein functional properties through different modification approaches, understand the physicochemical properties of the modified proteins, and evaluate their utilization in several food models. Specific objectives were to: 1) investigate the effect of different drying methods, namely freeze drying, spray drying, and vacuum drying on the functional and physicochemical properties of quinoa protein isolate; 2) improve the functional properties of pea protein through chemical and enzymatic modifications; 3) investigate the functional properties of pea protein by modulating protein covalent and non-covalent interactions and understand the physicochemical characteristics of the unfolded pea proteins that are responsible for the functional changes; and 4) evaluate the modified pea proteins as functional food ingredient in meat patty and egg-free mayonnaise applications.

The freeze-dried quinoa protein had the highest emulsification capacity and stability and oil holding capacity (OHC), which was attributed to its higher surface hydrophobicity, while the spray-dried quinoa protein had the highest solubility at pH 7 and water holding capacity (WHC). Gels (8% protein in water, w/w) prepared with the freeze-dried protein had higher elastic and viscous modulus than that of the other drying methods. When comparing pea protein modification through acylation, conjugation, and sequential acylation/conjugation, the sequential modification method demonstrated more beneficial and synergistic effects and greatly enhanced the WHC, OHC, emulsification and gelation properties of pea protein isolate (PPI). The enzyme or/ and conjugation modifications also enhanced the functional properties on pea protein,

including increased WHC, OHC, emulsion capacity, emulsion stability, and gelation. The modified pea proteins had comparable sensory scores as the control pea protein, and these modifications overall did not negatively affect protein sensory properties. For the protein interaction study, both urea and SDS unfolded proteins had significantly higher water holding capacity and oil holding capacity with up to 5.01 and 5.09 g H_2O /g protein, and 3.06 and 2.84 g oil /g protein compared with the control pea protein (4.12 and 1.29 g), respectively. The proteins unfolded with urea or SDS also showed improved emulsification properties. The trypsin hydrolyzed protein exhibited the highest foaming capacity and better gelation properties among all the treatments. Principal component analysis indicated strong associations between protein functional and physicochemical properties and molecular interactions.

The newly developed pea proteins produced through enzyme/polysaccharide conjugation modifications were successfully used applied in meat patties as functional extenders and in mayonnaise as an alternative to egg yolk. Beef patties containing the modified pea protein through sequential deamidation and conjugation (PGG, especially at 5%) showed significantly decreased cooking loss of only 20% and increased moisture and fat retentions compared with the control patty (33% cooking loss). In general, PPI patties exhibited harder texture (e.g., hardness, chewiness, shear force) than the control patty, while PGG patties showed much softer texture than the control. Emulsions prepared with guar gum conjugated PPI (G-PPI) had significantly increased stability, apparent viscosity, and decreased droplet size compared with the PPI emulsions. Several factors, including pH, NaCl concentration, protein concentration, and oil/water ratio significantly affected emulsifying properties of the modified pea protein. The mayonnaise with G-PPI at higher concentrations (6 and 8%) exhibited significantly higher emulsifying properties and viscoelasticity than that made of PPI or egg yolk.

In conclusion, quinoa protein isolate with different functional properties can be obtained by using different processing methods. Functional properties of pea protein can be enhanced through manipulated modification of specific structural domains using different modifiers. The modified pea proteins demonstrated advantageous features when applied in meat patties and mayonnaise products. The modified proteins may also have potential applications in meat analogues, bakery products, and emulsified foods and beverages.

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protein, including increased WHC, OHC, emulsion capacity, emulsion stability, and gelation. The modified pea proteins had comparable sensory scores as the control pea protein, and these modifications overall did not negatively affect protein sensory properties. For protein interaction study, both urea and SDS unfolded proteins had significantly higher water holding capacity and oil holding capacity with up to 5.01 and 5.09 g H_2O /g protein, and 3.06 and 2.84 g oil /g protein compared with the control pea protein (4.12 and 1.29 g), respectively. The proteins unfolded with urea or SDS also showed improved emulsification properties. The trypsin hydrolyzed protein exhibited the highest foaming capacity and better gelation properties among all the treatments. Principal component analysis indicated strong associations between protein functional and physicochemical properties and molecular interactions.

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In conclusion, quinoa protein isolate with different functional properties can be obtained by using different processing methods. Functional properties of pea protein can be enhanced through manipulated modification of specific structural domains using different modifiers. The modified pea proteins demonstrated advantageous features when applied in meat patties and mayonnaise products. The modified proteins may also have potential applications in meat analogues, bakery products, and emulsified foods and beverages.

Table of Contents

List of Figures

List of Tables

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Chapter 1 - Pea protein composition, functionality, modification, and food application: A review

Abstract

The demand for proteins continues to increase due to their nutritional benefits, the growing world population, and rising protein deficiency. Plant-based proteins represent a sustainable source to supplement costly animal proteins. Pea *(Pisum sativum L.)* is one of the most produced plant legume crops in the world and contributes to 26% of the total pulse production. The average protein content of pea is about 20-25%. The commercial utilization of pea proteins is limited, partially due to its less desirable functionalities and beany off-flavor. Protein modification may change these properties and broaden the application of pea proteins in the food industry. Functional properties such as protein solubility, water and oil holding capacity, emulsifying/foaming capacity and stability, and gelation can be altered and improved by enzymatic, chemical, and physical modifications. These modifications work by affecting protein chemical structures, hydrophobicity/hydrophilicity balance, and interactions with other food constituents. Modifiers, reaction conditions, and degree of modifications are critical variables for protein modifications and can be controlled to achieve desirable functional attributes that may meet applications in meat analogs, baking products, dressings, beverages, dairy mimics, encapsulation, and emulsions. Understanding pea protein characteristics will allow us to design better functional ingredients for food applications.

Keywords: plant protein, pea protein composition, functional properties, protein modification, food application

1.1 Introduction:

According to the Food and Agriculture Organization of the United Nations (FAOSTAT, 2015), the United States ranks the fifth-highest country in per capita meat consumption. The average amount of meat consumption in the U.S. has exceeded the healthy level set by the 2015-2020 Dietary Guidelines by 20-60% (Fehrenbach, Righter, & Santo, 2016). Excessive meat consumption can increase risks of cardiovascular disease, stroke, diabetes, and gastrointestinal cancers (Fehrenbach et al., 2016; Micha, Wallace, & Mozaffarian, 2010; Pan et al., 2012; Rohrmann et al., 2013; Sadler, 2004). On the other hand, protein deficiency is a major nutritional problem in developing countries (Batista, Portugal, Sousa, Crespo, & Raymundo, 2005; Ghaly & Alkoaik, 2010). Proteins are essential building blocks of human body (Ghaly & Alkoaik, 2010; López, Galante, Robson, Boeris, & Spelzini, 2018; Wild et al., 2014). For a healthy adult, the Recommended Dietary Allowance (RDA) of protein is 0.8 g protein per kg body weight per day with minimal physical activity (Wu, 2016). Protein malnutrition may result in stunting, physical weakness, vascular dysfunction, and impaired immunity (Wu, 2016). Each year, 60% of the 10.9 million deaths of children under the age of five are reported for suffering malnutrition in the developing countries (WHO, 2002).

Due to the health concerns and high cost of animal proteins, there exists a strong interest in sustainable plant-based proteins. Soy and wheat are the most common plant proteins and have been widely applied to partially replace animal proteins in food applications because of their favorable functional properties. However, due to allergies, some consumers are unable to consume food products containing soy and gluten (Föste, Elgeti, Brunner, Jekle, & Becker, 2015; López-Castejón, Bengoechea, Díaz-Franco, & Carrera, 2020). Thus, developing novel plant proteins is a strategy to solve protein shortage and increase plant protein usage as functional

ingredients in food products (Steffolani et al., 2016; Timilsena, Adhikari, Barrow, & Adhikari, 2016).

Plant proteins are used as food ingredients in many common applications, such as meat, bakery, and alternative dairy products (Owusu‐Ansah & McCurdy, 1991; Sandberg, 2011). In meat products such as patties, hamburgers, and sausages, plant proteins are usually used as extenders for their good oil and water holding capacity, emulsification, and gelation properties (Asgar, Fazilah, Huda, Bhat, & Karim, 2010; Egbert & Payne, 2009; Jones, 2016; Owusu‐Ansah & McCurdy, 1991). In bakery products, plant proteins are used for nutritional improvement, with possible negatively impaired textural properties. For instance, usage of plant proteins may lower bread volume, induce poor crumb texture and sensory properties (Owusu‐Ansah & McCurdy, 1991; Fleming and Sosulski, 1977). On the other hand, the addition of plant proteins in pasta may reduce dough stickiness and improve processability (McWatters, Nielsen, Sumner, 1980). In alternative dairy products, plant proteins can be good natural emulsifiers to replace dairy proteins (Nylander, Arnebrant, Cárdenas, Bos, & Wilde, 2019).

Important functional properties of plant proteins, including solubility, water and oil holding capacity, emulsifying/foaming capacity and stability, viscosity, and gelation, are the intrinsic physicochemical characteristics highly associated with protein behaviors in food items (Adebiyi & Aluko, 2011). The abilities of proteins to form gels or stabilize emulsions, foams, or networks are attributed to their functional properties (Mirmoghtadaie, Shojaee Aliabadi, & Hosseini, 2016). Protein hydration influences proteins' absorption of water and oil, solubility, and thickening characteristics. Protein surface properties such as hydrophilicity, hydrophobicity, or net charge affect protein emulsifying and foaming properties. Protein rheological properties such as gelation, viscosity, and aggregation may be linked to the protein structural changes of

size, shape, and amino acid sequences (Egbert & Payne, 2009; Speroni et al., 2009). To date, the utilization of commercial pea proteins as food ingredients is limited by its less desirable solubility, emulsification, and beany off-flavor (Lam, Can Karaca, Tyler, & Nickerson, 2018; Tamnak, Mirhosseini, Tan, Ghazali, & Muhammad, 2016; Zha, Dong, Rao, & Chen, 2019a). To overcome these limitations, protein could be modified to improve the functional properties according to different application purposes.

Chemical, enzymatic, and physical modifications are the common methods to modify proteins. Chemical modification is achieved by modifying the side groups of amino acids, including the ε-amino group of lysine; the thiol group of cysteine; the carboxyl group of glutamic acid and aspartic acid; the hydroxyl group of tyrosine, threonine, and serine; the imidazole group of histidine; the indole group of tryptophan; or the thioether group of methionine (Ustunol, 2015). The involved reactions include glycosylation, acylation, alkylation, and deamination, which would alter protein structures and functionalities depending on the reaction conditions and degree of modifications. Enzymatic modification mostly involves hydrolyzing the protein by breaking down the peptide bonds, which consequently reduces the protein molecular size and improves solubility and interfacial activity (Martínez, Sánchez, Ruíz-Henestrosa, Rodríguez Patino, & Pilosof, 2007; Tamm, Herbst, Brodkorb, & Drusch, 2016; Ustunol, 2015). Another enzymatic approach is to covalently cross-link proteins by enzymes such as transglutaminase and improve protein gelation (Klompong, Benjakul, Kantachote, & Shahidi, 2007; Mirmoghtadaie et al., 2016). Physical modifications include thermal, extrusion, and high-pressure treatments, to name a few. Thermal treatment alters proteins' hierarchy structure and causes protein denaturation, which results in protein aggregation through disulfide bonds and hydrophobic and electrostatic interactions (Barac, Stanojevic, Jovanovic, & Pesic,

2004). The extrusion process may induce protein crosslinking and aggregation and reduce protein solubility due to the exposure of hydrophobic groups on the protein surface (Mirmoghtadaie et al., 2016). High-pressure treatment has the advantage of avoiding thermal degradation and preserving food due to the absence of heating processes. This approach could change protein functional properties by disrupting the hydrophobic and electrostatic interactions (Chapleau & de Lamballerie-Anton, 2003; Mirmoghtadaie et al., 2016).

Though there have been a few reviews on pea protein functional properties, extractions, and applications (Burger & Zhang, 2019; Ge et al., 2020; Lam et al., 2018; Lu, He, Zhang, & Bing, 2020), this review is more comprehensive by discussing pea protein composition and structures, functional properties, modification methods, and specific food applications. This research provides the fundamental knowledge of pea protein structures and functional improvements for potential food applications. Since pea or modified pea proteins have not been widely studied, we also included some references and food applications of other plant proteins to inspire further study of pea protein.

1.2 Pea protein

Pea *(Pisum sativum L.)* is one of the most popular legume crops in the world, contributing to 26% of the total pulse production in 2014 (Rawal and Navarro, 2019). Peas originated from Southwest Asia and the Mediterranean region. Canada is the largest yellow pea producer, producing 30% of the world's annual dry pea stock (Pownall, Udenigwe, & Aluko, 2010; Sijtsma et al., 1998; Zhan, Shi, Wang, Li, & Chen, 2019). Pea seeds contain high protein content, carbohydrate, dietary fiber, fat, minerals, and vitamins (Gharsallaoui, Saurel, Chambin, & Voilley, 2012; Liang & Tang, 2014; Sandberg, 2011). The average protein content of pea is about 20-25%, depending on cultivars, genetics, and environmental factors (Burger & Zhang,

2019; Sandberg, 2011; Zhan et al., 2019). The protein content in pea is strongly related to the amount of nitrogen, phosphorus, and *S*-triazine applications and also soil conditions (Owusu‐Ansah & McCurdy, 1991). Pea protein contains high levels of lysine, threonine, and tryptophan (Adebiyi & Aluko, 2011; Boye et al., 2010; Burger & Zhang, 2019; T. Xiong et al., 2018) and has 18-25% of water soluble albumin and 55-65% of salt extractable globulin (Table 1) (Burger & Zhang, 2019; Owusu‐Ansah & McCurdy, 1991; Sandberg, 2011). The water soluble albumin consists mainly of enzymic and metabolic proteins (Burger & Zhang, 2019). The globulin protein consists of two major components, which are legumin (11S) and vicilin (7S), as well as a small quantity of convicilin with a 180-210 kDa molecular weight (Burger & Zhang, 2019; Owusu‐Ansah & McCurdy, 1991; Zhan et al., 2019). During seed development, legumin synthesizes faster than vicilin; therefore, legumin is presented in larger quantities than vicilin in the mature seed (Owusu‐Ansah & McCurdy, 1991).

Legumin is a hexameric protein with a molecular weight of 320-410 kDa and is comprised of six heterogeneous pairs of subunits that are held by non-covalent interactions (Lu et al., 2020) (Table 1). Each of the pair consists of an acidic (40 kDa) and a basic (20 kDa) chain linked by disulfide bonds. Most of the legumin acidic subunits are located on the surface of the protein, while the hydrophobic core consists of basic subunits (Owusu‐Ansah & McCurdy, 1991). By contrast, vicilin is a glycosylated trimeric cluster containing three subunits (48-50 kDa each) with a lower molecular weight of 150 kDa (Lu et al., 2020).

While legumin has high content of sulfur-containing amino acid such as methionine, vicilin is rich in isoleucine, leucine, phenylalanine, and lysine (Owusu‐Ansah & McCurdy, 1991) (Table 2). Legumin is less soluble in salt solutions, contains higher amounts of nitrogen and sulfur, and is difficult to coagulate at higher temperatures (Gueguen, Chevalier, And, &

Schaeffer, 1988). Generally, legumin stays in its native form at pH 7 – 9, but it is prone to dissociate at extremely high pH conditions. By contrast, vicilin is soluble at pH 4.8 (Casey, 1982). The ratio of vicilin to legumin in pea protein varies from 0.5 to 1.7 depending on the species and extraction methods (Burger & Zhang, 2019; Mession, Sok, Assifaoui, & Saurel, 2013; Owusu‐Ansah & McCurdy, 1991). Different ratios of vicilin and legumin could result in structural and functional differences, and higher content of overall globulins tend to bring about better emulsifying properties (Makri, Papalamprou, & Doxastakis, 2005). In addition, the nutritional quality of pea protein may be improved by increasing the proportion of legumin to vicilin (Owusu‐Ansah & McCurdy, 1991).

Pea protein isolate is typically obtained via wet processing. The process starts with solubilization, followed by isoelectric precipitation (Owusu‐Ansah & McCurdy, 1991). Specifically, after pea flour is dispersed in water, the pH is adjusted to 9-10 to solubilize the protein, followed by centrifugation of the slurry to separate the protein and carbohydrates. The pH of the supernatant is then adjusted to the isoelectric point (4.3-4.5) to precipitate the protein. Finally, the precipitated protein extract is neutralized and spray dried or lyophilized to protein powders (Swanson, 1990). The final yield of protein is determined by the processes, solubilizing agent, and pH of solubilization and precipitation (Owusu‐Ansah & McCurdy, 1991). Gueguen and Cerletti (1994) reported that solubilizing agents potassium hydroxide and sodium hydroxide resulted in similar protein yields, while calcium hydroxide may solubilize 10% less pea protein due to the salting-out effect of calcium ions. They also pointed out that protein isolates precipitated below pH 5.3 had lower protein content but higher lipid content, compared with the sample precipitated at pH 5.3 (Gueguen, 1983). Some bond lipids were also co-extracted from

this extraction method, as they were not extractable in hexane because of their tight binding to proteins (Owusu‐Ansah & McCurdy, 1991).

Pea protein has a beany off-flavor due to the presence of lipoxygenase. Ma et al. (2011) reported that beany flavor volatiles such as alcohols, aldehydes, and ketones were formed partially by lipoxygenase-catalyzed oxidation of unsaturated fatty acids. Gao et al. (2020) found that pea protein extracted at pH 9 had the lowest beany flavor, as it had the lowest lipoxygenase activity. Zha et al. (2019b) indicated that conjugating pea protein with gum arabic through Maillard reaction could mitigate the beany flavor. Other drawbacks of pea protein include its gritty texture and a feeling of lumps stuck in the throat during swallowing, which may be caused by the aggregation of pea protein through hydrophobic interactions, hydrogen bonding, and van der Waals forces, electrostatic, and steric interactions (Fang, Xiang, Sun-Waterhouse, Cui, & Lin, 2020). Further research is necessary to eliminate or reduce the off-flavor of pea protein.

1.3 Functional properties

Some functional properties, including water holding capacity, oil holding capacity, emulsifying properties, and foaming properties of pea protein are summarized in Table 3.

1.3.1 Water holding capacity

Water holding capacity (WHC), also known as hydration capacity, water binding capacity, or water absorption capacity, refers to the ability of proteins to retain water (Shevkani et al., 2015), or the capacity of per gram protein to absorb water (Boye et al., 2010). It is a critical parameter to determine protein function in terms of water retention, swelling, and gelation. It is also important in food formulations, affecting hydration with dry ingredients (Foegeding & Davis, 2011; Xiong, 2014). Poor water holding capacity may cause liquid loss during processing and lead to textural changes of final products (Lam et al., 2018). Water

binding is usually resulted from a combination of ion-dipole, dipole-dipole, and hydrophilic interactions (Lam et al., 2018). A lot of factors are associated with water holding capacity, such as protein extraction methods, protein concentration, pH, temperature, as well as the balance of hydrophilic and hydrophobic amino acid residues.

Wang et al. (2020) reported that the water holding capacity of pea protein ranged from 1.18 to 2.60 g water/g protein. They found that alcohol-washed and air-classified pea protein had significantly higher water holding capacity than untreated protein. However, water holding capacity was dramatically decreased when increasing the concentration of ethanol or isopropanol up to 80 % during protein pretreatment, which may be attributed to charge reduction on the protein surface. Feyzi et al. (2018) reported water holding capacity values of pea protein from 2.15 to 2.70 g water/g protein, with no significant differences among different extraction methods. Milad et al. (2019) found that pea protein extracted by ultrafiltration-diafiltration precipitation exhibited higher water holding capacity of 2.83 g water/g protein than those by isoelectric precipitation and salt extraction methods, which were 2.24 and 2.39 g water/g protein, respectively. It was believed that isoelectric precipitation resulted in higher hydrophobic/hydrophilic ratio than the other methods and greater surface hydrophobicity of proteins than salt extraction. This may be responsible for protein denaturation during acid precipitation and cause interactions between protein and non-protein compounds, thus decreasing protein's solubility and water holding capacity (Krause, Schultz, & Dudek, 2002). In addition to pea protein isolates, Ribéreau et al. (2018) studied pre-germinated and micronized pea flour and found a 7-16 % increase in water holding capacity compared with untreated pea flour. This may be because both pretreatments altered protein structures and enhanced imbibition, swelling, and

retention of water. Furthermore, the micronization process can induce water entrapment in protein due to protein denaturation and starch damage during the process.

1.3.2 Oil holding capacity

Oil holding capacity is another important functional property that may affect food texture and quality. It is defined as the ability of protein to absorb and retain fat through interactions with lipids in food (Foegeding & Davis, 2011; Xiong, 2014). The interaction of lipid and protein occurs via the binding of aliphatic chains of lipids and the nonpolar side chains of amino acid residues, so proteins with higher hydrophobicity have greater propensities to interact with oil. Oil holding capacity is especially important when protein is used as an ingredient in meat, beverage, and salad dressing applications and is related to other functionalities such as emulsifying properties. It is affected by protein sources and structures, processing conditions, droplet size, and the distribution and stability of lipids.

Several studies reported variable oil holding capacities of pea protein isolates, ranging from 0.67 to 3.30 g oil/g protein among pea varieties and different treatments (Feyzi et al., 2018; Milad et al., 2019; Lam, Warkentin, Tyler, & Nickerson, 2017; Y. Wang et al., 2020). Lam et al. (2017) reported that the oil holding capacities of six pea cultivars ranged from 3.0 to 3.3 g oil/g protein without significant differences. Feyzi et al. (2018) also found no significant difference in oil holding capacity among different pea protein extraction methods. However, Milad et al. (2019) demonstrated that pea protein extracted from ultrafiltration-diafiltration precipitation exhibited similar oil holding capacity (2.21 g oil/ g protein) as that from salt extraction (2.16 g oil/ g protein), while isoelectric precipitation resulted in a lower oil holding capacity (0.67 g oil/g protein). The nonpolar side chains of amino acids play an important role in oil holding capacity, thus better oil holding capacity is attributed to higher content and/or availability of hydrophobic

amino acid residues. Furthermore, compared with that of untreated protein, the oil holding capacity of alcohol-washed, air-classified pea protein was reported to be lowered from 0.96 to 0.67-0.80 g oil/g protein (Wang et al., 2020).

1.3.3 Solubility

Protein solubility is measured by the amount of protein dissolved in water at specific testing condition. It is considered a critical functionality in the food industry and affects proteins' emulsifying, foaming, and water holding capacities (Ghribi et al., 2015; Lam et al., 2018). Protein solubility mainly depends on the proportion and distribution of hydrophilic and hydrophobic groups on the surface of protein molecules, which may be affected by intrinsic factors (amino acid composition and distribution, isoelectric point, molecular flexibility, and charge) and extrinsic factors (pH, temperature, and ionic strength) (Lam et al., 2017). When protein is dissolved in water, hydrophilic amino acids tend to orient toward the aqueous interface, while hydrophobic residues are in the interior of protein molecules to minimize free energy. Exposure of hydrophobic residues to the protein surface would reduce protein solubility (Lam et al., 2018). When pH value is below or above the isoelectric point, protein solubility would increase due to electrostatic repulsion by net negative or positive charge at protein surface. Protein exhibits the lowest solubility at the isoelectric point because it carries zero net charge, and no electrostatic repulsive force occurs. At this point, protein aggregation may be formed by hydrophobic interactions between protein molecules, which could result in protein precipitation and decrease protein solubility.

According to the study reported by Lam et al. (2017), pea protein solubility ranged from 62.5 to 75.2% at pH 7 among different varieties, with significant differences between cultivars and environments. Wang et al. (2020) reported that alcohol-washed, air-classified pea protein

had significantly lower solubility than untreated protein, with solubility decreasing from 85.4% to 42.6-52.7% and 20.6-38.0%, respectively, when washed with ethanol and isopropanol. This was because the lower polarity of solvents induced protein partial denaturation. During the washing stage, the ratio of albumin and globulin increased, while prolamin protein was washed away (Bader, Oviedo, Pickardt, & Eisner, 2011; Chang, Stone, Green, & Nickerson, 2019). Milad et al. (2019) found that pea protein had the lowest solubility at pH 4 – 5. Pea protein extracted by ultrafiltration-diafiltration precipitation exhibited relatively higher solubility compared with pea protein extracted by isoelectric precipitation and salt extraction, while isoelectric precipitation extraction led to the lowest solubility (Milad et al, 2019). This result could be explained by the higher surface charge and lower surface hydrophobicity of protein produced by ultrafiltration-diafiltration precipitation, and higher surface hydrophobicity of protein obtained by salt extraction. Therefore, different degrees of protein unfolding resulted in different protein solubility from various extraction methods. Drying methods also affect pea protein solubility. Freeze-dried pea protein was reported to exhibit better solubility than vacuum-oven-dried protein, indicating more availability of hydrophilic amino acid in the freeze-dried protein during hydration and more protein denaturation as a result of vacuum oven drying (Feyzi et al. (2018)).

1.3.4 Emulsifying properties

Protein can naturally be an emulsifier due to its amphiphilicity. Emulsification property enhances proteins' role as food ingredient by controlling the quality and texture of products in many applications (Karaca, Low, & Nickerson, 2011). Two types of food emulsions are commonly seen in the food industry: oil in water (O/W) emulsions which create a creamy texture and water in oil (W/O) emulsions which exhibit greasy properties. Protein has been widely used

as an emulsifier due to its ability to adsorb at the oil and water interface and form stabilized layers around oil or water droplets (Dickinson, 2010; Kimura et al., 2008). During emulsion formation, proteins or their aggregates are adsorbed at the surface of droplets and reoriented at the interface, with their hydrophobic side chains moving to the oil phase while the hydrophilic moieties face the water phase, thus minimizing the interfacial tension to prevent coalescence or flocculation (Burger & Zhang, 2019; Stone, Avarmenko, Warkentin, & Nickerson, 2015). An emulsion is mostly stable at low ionic strength and when the pH is away from the isoelectric point of the protein. This is when the interactions between proteins are weak and protein is adsorbed into droplets, promoting it to form an interfacial film and avoid coalescence. On the other hand, an emulsion is the least stable at the isoelectric point and at high ionic strength, which is because the dispersed phase is close to each other so that electrostatic repulsion is weakened and the attractive force between the droplets is in turn relatively strengthened (Lam et al., 2018). The emulsifying properties of proteins are characterized by emulsifying capacity (EC) and emulsifying stability (ES). Emulsifying capacity is the ability of a protein to adsorb at the interfacial area of oil and water to form emulsions, and it depends on the shape, charge, and hydrophobicity of protein molecules; emulsifying stability is the stability of an emulsion over a certain time, which depends on the magnitude of these interactions such as electrostatic repulsion, van der Waals forces, etc. (Karaca et al., 2011; Ma et al., 2011).

Lam et al. (2017) reported that pea protein had an emulsion stability from 95.1 to 96.1% at pH 7 from different cultivars with no significant differences among cultivars and environmental factors. Wang et al. (2020) demonstrated that the emulsion stability of pea protein significantly decreased from 40 to 28.13% with ethanol wash, especially with 80% ethanol, but isopropanol wash caused no difference in emulsion stability. Milad et al. (2019) found that pea

protein extracted by ultrafiltration-diafiltration precipitation exhibited the highest emulsion capacity and stability compared with that extracted by isoelectric precipitation and salt extraction.

1.3.5 Foaming properties

Foaming is the ability of a protein dispersion to form a stable foam when air is mixed in. Because of the high free energy at the gas-liquid interface, foam is thermodynamically unstable and may undergo coalescence to reduce the interfacial area (Lam et al., 2018). Solubilized proteins diffuse and adsorb to the gas-liquid interface, with hydrophobic groups being oriented to the gas phase and hydrophilic regions to the liquid phase, thereby reducing surface tension and forming a cohesive film around the gas bubbles (Wierenga & Gruppen, 2010). Characteristics of protein foaming properties include foaming capacity (FC) and foaming stability (FS). Foaming capacity refers to the amount of interfacial area that could be created by a protein, and it is positively related to protein hydrophobicity/hydrophilicity ratio. Foaming stability is the ability of a protein to stabilize a foam against stress during a certain time (Lam et al., 2018).

Lam et al. (2017) reported that the foaming capacity of pea proteins ranged from 167.4 to 243.7% and significantly differed among cultivars and with different environmental factors. In addition, compared with salt extraction and ultrafiltration-diafiltration precipitation, isoelectric precipitation was more effective at increasing pea proteins' foaming capacity and stability by increasing globulin molecules, which adsorb at the interface and reduce the interfacial tension (Milad et al., 2019). Makri et al. (2005) pointed out that to achieve good foaming capacity, proteins should adsorb quickly at the air-water interface after the rapid conformational change and rearrangement of protein at the interface. Moreover, Ribéreau et al. (2018) reported that foaming capacity and stability of pea flour treated by micronization and pre-germination were

significantly decreased compared to those of untreated pea flour. The reduction may be attributed to the partial denaturation and aggregation of proteins in treated flours.

1.3.6 Gelation

Gelation is a critical functional property of globular proteins to modify the texture, quality, and sensory attributes of food products (Foegeding & Davis, 2011). A protein gel is defined as a three-dimensional network that entraps large amount of water. Protein gelation involve two stages: conformational changes or protein denaturation and the subsequent protein association or aggregation into the three-dimensional matrix structure. A protein's gel forming ability and viscoelastic property largely depend on the hydrogen and covalent bonds and electrostatic and hydrophobic interactions (Adebiyi & Aluko, 2011). Protein gelation can be affected by heat treatment, pH, salt, pressure, and other constituents (Lam et al., 2018).

Milad et al. (2019) investigated gelation concentrations of pea proteins that were prepared by different extraction methods. They found that the lowest least gelation concentration (10%, w/v) was achieved for the pea protein extracted by ultrafiltration-diafiltration precipitation, followed by that from isoelectric precipitation (12%, w/v) and salt extraction (14%, w/v). This was attributed to the intensity of intermolecular interaction from ultrafiltration-diafiltration precipitation being stronger than that from isoelectric precipitation and salt extraction (Milad et al., 2019). In addition, Adebiyi and Aluko (2011) compared the gelling properties of different pea protein fractions, including water-, salt-, alkaline-, and ethanol-soluble fractions. In their study, the ethanol-soluble fraction was not evaluated due to its complete insolubility in water. Water-soluble and salt-soluble fractions were found not be able to form a firm gel. However, the alkaline-soluble fraction was able to form a firm gel with the least gelation concentration of 10%. Since the alkaline-soluble fraction represents the majority of pea

protein fractions, its gelling property could represent the gelation ability of pea protein (Adebiyi & Aluko, 2011). Agboola et al. (2010) reported that pea protein isolates formed a paste texture rather than a cohesive gel in the room condition, which was possibly due to insufficient intensity of intermolecular interaction. Moreover, Shand et al. (2007) found that the addition of NaCl could enhance pea protein gel formation, presumably by increasing intermolecular hydrophobic interactions, decreasing electrostatic repulsion, and altering water structure around protein molecules to enhance protein hydration and gel network formation.

1.4 Protein modifications

Commonly used protein modification methods include protein-polysaccharide conjugation, enzymatic modification, acylation, deamidation, and physical modification, as summarized in Table 4.

1.4.1 Enzymatic modification

The most common method of enzymatic modification is to hydrolyze the protein with protease, which involves breaking down the polypeptide bonds with additional water molecules. Protease hydrolysis can affect protein functional properties, for example, increasing the solubility of proteins by hydrolyzing them to smaller peptides (Adler-Nissen, 1986; Ustunol, 2015). In general, hydrolyzed proteins have altered water binding, foaming, and emulsifying properties. Many studies (Mahdavi‐Yekta, Nouri, & Azizi, 2019; Nongonierma, Le Maux, Dubrulle, Barre, & FitzGerald, 2015; Pownall et al., 2010; Shen, Hu, & Li, 2020) reported that bioactive peptides derived from controlled hydrolysis exhibited strong antioxidant activity and could inhibit lipid oxidation. In addition to protease hydrolysis, cross-linking of proteins with microbial transglutaminase is another commonly used enzymatic modification. This reaction involves the acyl transfer between the ε-amino group of lysine residues and the amide group of

glutamine residues (Ustunol, 2015). Microbial transglutaminase is a polypeptide consisting of 331 amino acids with an isoelectric point of 8.9. It is active at pH 4-9 with an optimum pH range of 6-7 and an optimum temperature of 50 °C (Ustunol, 2015). It can induce cross-linking of proteins and enhance gelation, creating good gel stability at high temperatures. Such cross-linked proteins are used in restructured meat products for the purpose of binding meat pieces (Ustunol, 2015).

Klost and Drusch (2019) reported that trypsin hydrolysis significantly enhanced pea protein solubility at pH 4 to 6, which was due to the smaller protein size and increased amount of terminal carboxyl and amino groups. However, when pH was at 3 and 7, hydrolysis decreased solubility. This was because the increased exposure of hydrophobic residues caused aggregation and sedimentation of peptide fractions and impacted the electrostatic properties of hydrolysates (Klost & Drusch, 2019). When hydrolysis increased from degree of hydrolysis (DH) of 0 to 4%, there was a shift in the protein's isoelectric point from 4.91 to 4.37. From this, the authors deduced that the peptide fractions that aggregated and experienced lower solubility at pH conditions away from the isoelectric point may consist of more basic amino acids than acidic ones. Since trypsin hydrolysis occurred at pH 8 at the C-terminus of basic amino acids, small and basic peptides from pea protein were probably close to the isoelectric point (Klost & Drusch, 2019). This resulted in a larger fraction of acidic peptides in the soluble fractions, which required more protons to bind with dissociated carboxyl groups, thus lowering the isoelectric point (Klost & Drusch, 2019). In addition to protein solubility, enzymatic hydrolysis may affect protein emulsifying properties. Tamm et al. (2016) found that pea protein hydrolyzed by trypsin had improved emulsion stability, presenting smaller oil droplets compared with that hydrolyzed by Alcalase. Sijtsma et al. (1998) also showed that hydrolysis by Protamex significantly improved

pea protein solubility, emulsifying properties, and foam expansion. Similar results were also found by Barać et al. (2011) using pea protein hydrolyzed by chymosin.

Transglutaminase cross-linking can improve protein gelation properties. Shand et al. (2008) and Sun and Arntfield (2011) demonstrated that pea protein treated with transglutaminase exhibited enhanced gel properties, evidenced by increased gel strength, elasticity, and lower least gelation capacity. Sun and Arntfield (2011) reported that addition of transglutaminase lowered the least gelation concentration of a salt-extracted pea protein from 5.5 to 3 % (w/v). Increasing transglutaminase concentrations increased gel strength, which was confirmed by the increased magnitudes of both elastic (G´) and viscous (G´´) modulus. In addition, slowing down heating and cooling rates allowed the formation of stronger gel by giving protein molecules more time to rearrange. Moreover, higher pea protein concentrations at the same level of transglutaminase resulted in higher G´ and G´´ (Sun & Arntfield, 2011).

1.4.2 Protein-polysaccharide conjugation/complexation

Protein-polysaccharide conjugation is usually generated by heating the mixture of protein and carbohydrate below the denaturation temperature with controlled water activity via covalent bonds (Ustunol, 2015). It is a glycosylation reaction between the amine group of protein and carbonyl group of a reducing sugar, representing the early stage of the Maillard reaction. Glycosylation can affect the balance of hydrophilicity and hydrophobicity of proteins and enhance water-protein interactions (Baniel, Caer, Colas, & Gueguen, 1992). Protein-polysaccharide complexation is a pH-dependent, electrostatic attractive interaction between the positively charged protein surface and negatively charged polysaccharide (Lan, Chen, & Rao, 2018). Many studies reported that interactions between protein and polysaccharides improved protein functional properties, such as emulsifying and water holding properties (Gharsallaoui,

Yamauchi, Chambin, Cases, & Saurel, 2010; Tamnak, Mirhosseini, Tan, Ghazali, et al., 2016; Tamnak, Mirhosseini, Tan, Tabatabaee Amid, et al., 2016; Zha, Dong, et al., 2019a).

Wei et al. (2020) studied the stabilization of pea protein dispersions using various polysaccharides, including corn fiber gum, high-methoxy pectin, carboxymethyl cellulose, and konjac glucomannan. They found that all these polysaccharides effectively improved the stability of pea protein dispersions due to electrostatic repulsions, steric hindrance, and high viscosity of the continuous phase. In addition, pH and concentrations of polysaccharides also affect the stability of protein dispersions (Wei et al., 2020). Guo et al. (2019) reported that pea protein complexed with propylene glycol alginate had improved solubility, emulsifying activity, and increased denaturation temperature compared with native pea protein or propylene glycol alginate alone. They also pointed out that the pea-protein-propylene complex exhibited the best functionalities at the mass ratio of 3: 1 (w/w) at pH 4. Moreover, several studies have shown functional property improvements of pea-gum arabic complexes (Liu, Elmer, Low, & Nickerson, 2010; Zha, Dong, et al., 2019b, 2019a; Zha, Yang, Rao, & Chen, 2019c). Zha et al. (2019a) reported that pea protein-gum arabic conjugates possessed better emulsifying properties, which was confirmed by their smaller droplet size, higher surface charge, and stronger steric hindrance. The emulsifying property was also affected by pH, temperature, and ionic strength. Similar findings were found by Liu et al. (2010). They reported that the emulsion stability and foaming property of pea protein were greatly improved by complexing with gum arabic. Zha et al. (2019b) and Zha et al. (2019c) further investigated the effect of pea-gum arabic complexes on flavor attributes. They reported that the beany flavor was remarkably diminished after incubating the mixture at 60 °C with a longer incubation time, because key aromatic compounds were produced during the Maillard reaction process, such as pyrazines, thiophenes, ketones, and

Strecker aldehydes (Zha, Dong, et al., 2019b). In addition, several studies reported that pea-pectin conjugates exhibited good rheological behavior and physicochemical properties in oil-in-water emulsions (Gharsallaoui et al., 2010; Lan et al., 2018; Tamnak, Mirhosseini, Tan, Ghazali, et al., 2016; Tamnak, Mirhosseini, Tan, Tabatabaee Amid, et al., 2016; Warnakulasuriya, Pillai, Stone, & Nickerson, 2018).

1.4.3 Acylation

Acylation is a chemical modification that covalently links an acyl group to the protein amino groups (Shen & Li, 2021). The ε-amino group of lysine is the most active and can be readily acylated (Ustunol, 2015). The two most common acylation methods are acetylation and succinylation, and they work by introducing acetic and succinic anhydride, respectively (Ustunol, 2015). Acetylation decreases proteins' positive charges by substituting the positively charged ε-amino groups with hydrophobic acetyl groups; succinylation introduces anionic succinate residues, which are covalently linked to the ε-amino groups of lysine residues, to change proteins' net charge from positive to negative and also to change their electrostatic and conformational properties (El-Adawy, 2000). Acylation usually occurs at pH 8 in a protein slurry and leads to protein unfolding and exposure of hydrophilic residues of polypeptides, hence improving emulsifying properties (Johnson & Brekke, 1983). This modification has also been reported to greatly improve protein solubility and gelation (Ustunol, 2015).

Johnson and Brekke (1983) reported that acetylated and succinylated pea proteins had significantly improved water holding capacity, solubility, and emulsifying properties. In their study, acylation was found to lower the protein isoelectric point by increasing negative charges and replacing the ε-amino groups of lysine with neutral acetyl groups through acetylation or with negatively charged carboxyl groups through succinylation (Johnson & Brekke, 1983). Adawy

(2000) found that succinylated mung bean protein had higher solubility than acetylated one. During succinylation, longer side chains were introduced, thus producing more electrostatic repulsions in the protein. This also alters protein conformation to diminish protein-protein interactions while enhancing protein-water interactions. The addition of carboxyl groups by succinylation enhanced the interaction between protein and the aqueous phase of emulsions. As a result, protein solubility increased, and a protein layer was formed around fat droplets to facilitate association with the aqueous phase, thereby enhancing interactions at the protein-oil interface (El-Adawy, 2000). Similar results were found in succinylated oat protein isolates (Mirmoghtadaie, Kadivar, & Shahedi, 2009), acetylated and succinylated mung bean protein isolates (El-Adawy, 2000), African yam bean protein (Arogundade et al., 2013), flax protein isolates (Wanasundara & Shahidi, 1997), and rapeseed protein isolates (Wang, Zhang, Zhang, Ju, & He, 2018).

1.4.4 Deamidation

Deamidation refers to the conversion of amide groups (glutamine/ asparagine) of protein to carboxyl groups (glutamic acid/ aspartic acid) by using acid, alkali, heat, or enzymes (Fang et al., 2020). Deamidation increases carboxyl groups, which shifts the isoelectric point to the acidic side and exposes hydrophobic regions (Ustunol, 2015). Deamidation can improve protein solubility and emulsifying and foaming properties, but excessive deamidation may also impair protein functional properties (Fang et al., 2020). The rate of deamidation depends on the ratio of asparagine to glutamine and amino acid composition (Hamada & Swanson, 1994). Among chemical, physical, and enzymatic methods for protein deamidation, enzymatic deamidation appears to be superior due to its high efficiency, safety, mild conditions, and fewer side reactions. Chemical deamidation may cause severe protein denaturation and hydrolysis of

peptide bonds, which can induce undesirable changes of protein structures and affect functional properties (Kunarayakul, Thaiphanit, Anprung, & Suppavorasatit, 2018). Amyloid-protein aggregation could be also affected by chemical deamidation (Osaki & Hiramatsu, 2016; Wang, Shu, Frieden, & Gross, 2017). The most common enzymes used in protein deamidation include transglutaminase, glutaminase, and protein-glutaminase.

Glutaminase is a food-grade commercial enzyme that can catalyze the glutamine and asparagine residues of protein with slight hydrolysis (Fang et al., 2020). Fang et al. (2020) reported that glutaminase deamidation improved solubility and reduced the beany flavor, grittiness, and lumpiness of pea proteins. Protein-glutaminase is an important deamidation enzyme that is isolated from soil bacterial *Chryseobacterium proteolyticum*. It has no side reaction and low susceptibility to hydrolysis compared with other enzymes (Kunarayakul et al., 2018). Functional changes with protein-glutaminase deamidation were reported for various food proteins, such as increased protein solubility and foaming properties in coconut proteins (Kunarayakul et al., 2018) and decreased gel formation in heat-induced whey protein aggregates (Miwa, Yokoyama, Nio, & Sonomoto, 2013), which was resulted from the increase of the electrostatic repulsion between carboxylic acid groups and the reduction of disulfide bond formation.

1.4.5 Physical modification

Physical modifications play a critical role in altering protein structures and functionalities, with thermal treatment (Cerdán‐Leal et al., 2020; Chao & Aluko, 2018; Kaspchak et al., 2017; Mession et al., 2013; Outi E Mäkinen, Emanuele Zannini, Peter Koehler, 2016), ultrasound (Jiang et al., 2017; Li et al., 2018; Mir, Riar, & Singh, 2019; Vera, Valenzuela, Yazdani-Pedram, Tapia, & Abugoch, 2019; Xiong et al., 2018), and high pressure (Chao, Jung,

& Aluko, 2018) being the most widely used methods. Thermal treatment causes dissociation of protein quaternary structures, protein denaturation, and protein aggregation through disulfide, hydrophobic, and electrostatic interactions (Mirmoghtadaie et al., 2016; Shen et al., 2021). Chao & Aluko (2018) investigated the effect of thermal pretreatment on the emulsifying properties of pea protein at various pH conditions, finding an improvement in emulsion capacity at pH 7.0 and deterioration at pH 3.0.

The radicals and superoxides generated during the ultrasound process may promote cross-linking of protein molecules, while the hydrogen peroxide produced during cavitation may oxidize free sulfhydryl groups to undesirable sulfinic acid; therefore, the ultrasound technique can significantly alter protein structures and protein functional properties (Soria & Villamiel, 2010). Xiong et al. (2018) found that high-intensity ultrasound treatment increased the foaming ability and stability of pea protein from 145.6 to 200.0%, and 58.0 to 73.3%, respectively. The high-intensity ultrasound applied in the study was capable of inducing partial protein unfolding, leading to rapid protein adsorption at the freshly formed air-water interface, forming a viscoelastic film, and improving foam stability. However, the bubble size of foam increased with an extension of time, and the protein was desorbed from the interface with more hydrophobic groups being exposed to the surface. As a result, foam-induced protein aggregation may be formed.

High pressure treatment may disrupt protein hydrophobic and electrostatic interactions, while inducing new bond formation to enhance protein aggregation or precipitation as well as gelation properties (Mirmoghtadaie et al., 2016). Do Carmo et al. (2016) illustrated that pea protein treated with supercritical carbon dioxide showed improved foam stability, which was

attributed to the affinity of carbon dioxide to hydrophobic moieties enhancing the surface properties of pea protein while stabilizing air/water interfaces.

Wang et al. (2017) reported that pea protein hydrolysates irradiated by electron beams had improved protein emulsifying and foaming properties. This physical modification is considered a clean, safe, and efficient technique to improve protein functional properties.

1.5 Applications

Some applications of pea and other plant proteins, including meat analogues and meat extenders, bakery applications, sauces, beverages, dairy products and analogues, encapsulating wall materials, and Pickering particles, are summarized in Table 5.

1.5.1 Meat analogues and meat extenders

In recent years, plant-based meat analogues made using extrusion texturization techniques are becoming popular. Texturization is the conversion of a protein to a fibrous structure. It alters protein textural properties through the induction of protein denaturation, cross-linking, and realignment of protein molecules (Ustunol, 2015). The pea-protein-based meat developed by Beyond Meat is popular among consumers, including vegetarians. Osen et al. (2015) and Osen et al. (2014) investigated using pea proteins as raw materials for fibrous meat-like alternatives and observed that the resulting fibrous structure was closely associated with different extrusion conditions. Since few published studies are available regarding the texture properties of plant-based meat products using pea proteins, we review plant-based meat using other plant proteins in the following paragraphs, which can still provide useful information for developing pea protein based meat analogues and products.

Chiang et al. (2020) compared the textures of sausages made from soy protein/wheat gluten with different moisture content and found that when moisture content increased, hardness

of the sausages decreased, suggesting that higher moisture content creates softer texture. This could be attributed to the addition of water in the formulation and manufacture process disrupting the sausage intact structure and reducing hardness and chewiness. Sausage made from soy protein/wheat gluten showed a higher adhesiveness than control chicken breast due to the fact that wheat gluten creates sticky consistency and higher adhesion. In addition, sausage with soy protein/wheat gluten was more susceptible to oxidation compared with the control sausage, which could be caused by the longer storage time after the extrusion and frozen process and higher level of oxygen in the package that decreased the product's ability to maintain its storage stability (Zhang, Xiao, & Ahn, 2013). Kamani et al. (2019) reported that the full substitution with plant protein in sausage minimized cooking loss and shrinkage and greatly improved emulsion stability, while control meat sausage had better elasticity and gel strength. This was because the soy protein used in the study is hydrophilic and could strongly retain moisture and fat to form an adhesive gel matrix and consequently stabilize the emulsion. It was acting as a fat-encapsulating agent that prevented oil separation during cooking, thus reducing cooking loss. Hatamikia et al. (2019) conducted a study to investigate the functional properties of plant-based protein burgers based on various *Vicia ervilia* (L.) Willd. protein isolates produced by different extraction methods. They found that the protein produced by salt-dialysis had higher water holding capacity in comparation to that produced by alkaline solubilization/acid precipitation, because of a higher degree of denaturation during the extraction and the reduced purity and solubility of the protein. Moreover, the plant-based burger did not undergo tangible changes after six months of storage, which confirmed that the protein isolate of *Vicia ervilia* could be used to produce novel products with a longer shelf life.

Plant proteins have also been widely used as extenders in meat products because of their oil and water holding capacities and emulsifying properties and relatively lower cost (Dakhili, Abdolalizadeh, Hosseini, Shojaee-Aliabadi, & Mirmoghtadaie, 2019; Owusu‐Ansah & McCurdy, 1991). Kassama et al. (2003) investigated the textural properties of beef patties containing soy protein flour (SPF) and texturized soy protein (TSP), and found that increasing soy protein concentration in both SPF and TSP enhanced the water holding capacity of meat patties and reduced cooking loss. Besides, patties with SPF and TSP exhibited softer and more cohesive textures than the control beef patty (Kassama et al., 2003). Akesowan (2010) studied light pork burgers fortified with 2 % soy protein isolates, which showed significantly improved textural characteristics, including cohesiveness, springiness, and chewiness. Similar findings were observed in beef sausage with texturized vegetable protein (Hidayat, Wea, & Andriati, 2017) and buffalo meat sausage with soy protein (Ahmad, Rizawi, & Srivastava, 2010).

Saturated fats have a harder texture at room temperature than unsaturated fats. Replacing meat with texturized proteins reduces fat content and results in softer texture of the final products. The rehydration effect of soy protein during meat formulation as well as its water holding capacity could also contribute to the softer texture and juiciness of meat products. In addition, texturized protein produced by extrusion cooking of defatted protein can give an elastic structure and imitate spongy chewy texture (Wild et al., 2014). Zhang et al. (2013) indicated that the increased hardness of meat patties might be associated with emulsion destabilization caused by the separation of fat and water. Protein oxidation may also impact hardness through the formation of carbonyls and the induction of protein cross-linking via disulfide bonding. Li et al. (2020) reported that treating low-sodium pork meat with high pressure and 2% soy protein significantly improved the emulsion stability of the meat batter due to the ability of the added

protein to form gels with good water and oil holding capacities. In addition, adding soy protein to pork meat batters and treating them with high pressure delayed thermal denaturation, reduced pre-gel formation generated by the denaturation of myosin tail, and significantly changed the protein secondary structure by decreasing the α-helix structure and increasing the β-sheet, β-turn, and random coil structures. Despite these advantages of plant-based meat products, pea protein based meat products are still under investigation and their physicochemical, functional, and sensory properties need to be further studied.

1.5.2 Bakery applications

The addition of pea protein or other modified proteins in bakery products improves nutritional value, textural properties, and sensory properties. Morales-Polanco et al. (2017) reported that crackers baked with dehulled oat flour and pea protein had higher nutritional content and lower hardness. Shah and Singhal (2019) found that eggless cake batter with succinic anhydride modified pea protein possessed good viscosity, lower cake bulk density, along with improved cell number and softness. These improvements were attributed to the alteration of emulsifying and foaming properties due to the hydrophobic modification of the protein. Pico et al. (2019) conducted a study to investigate effects of plant proteins (rice and pea proteins) and animal proteins (egg white and whey proteins) on the crust quality of gluten-free bread. They found that bread containing 10% of pea protein and whey protein exhibited a less crispy texture. They also mentioned that higher moisture content or water activity reduced the jaggedness of the deformation curve and that high water activity caused a transition from the glassy to the rubbery state, both of which led to a less crispy texture.

Campbell et al. (2016) investigated the textural properties of wheat bread and sponge cake fortified with cowpea protein that had been thermally denatured and glycated. It was

concluded that the bread dough with the denatured protein had significantly increased water holding capacity, leading to softer texture of the bread. Wheat bread with unmodified cowpea protein showed higher crumb hardness compared with bread containing other modified proteins (Campbell et al., 2016). This is because unmodified cowpea protein was more susceptible to thermal denaturation and loss of solubility than glycated protein during heat treatment and the water holding capacity of protein was highly related to its solubility. In addition, replacing 20 % of whole egg with glycated cowpea protein (3.5% w/w in dry bases) in sponge cake did not affect sensory acceptability, whereas using unmodified and thermally denatured cowpea protein resulted in significantly lower acceptance (Campbell et al., 2016).

López-Alarcón et al. (2019) reported that cupcakes containing heat-denatured and lyophilized quinoa proteins exhibited greater firmness and water activity and an extended shelf life than cupcakes containing unmodified protein. The modified proteins were found to have a more porous surface to retain water and consequently led to less free water for microorganism growth and strong interactions between protein-carbohydrates (López-Alarcón et al., 2019). In addition, lyophilization could modify the nanostructure of protein, improving the distribution of water molecules, causing lower water mobility with high viscosity, and thus decreasing the growth of microorganisms on cupcakes and extending their shelf life (López-Alarcón et al., 2019). Masure et al. (2019) illustrated that gluten-free batter containing soy protein had lower surface activity and stability and resulted in a lower bread volume with inhomogeneous crumb structure compared with batter containing egg white protein. The volume and crumb structure of gluten-free bread were mainly determined by the balance between gas cell opening and crumb structure setting. The rate and total carbon dioxide release of batter with soy were higher than

those of bread with egg white, which created less efficient gas cell stabilization of bread containing soy protein.

Apart from traditional bakery products, plant proteins could also be applied in steamed bread to improve nutrition and quality. Liu et al. (2016) added 1% of soy protein hydrolysates to improve dough fermentation and acidification, which was confirmed by the dough's increased gas holding capacity. In addition, soy protein hydrolysates reduced the time needed for steam dough to develop and stabilize, as less energy was required for the formation of the gluten dough network. Du et al. (2016) found that extruded soy protein improved the volume and interior structure of Chinese steam bread. The extruded soy protein formed a larger polymer network with the gluten protein, which acted as the nitrogen source of yeast and increased the S-S bonds of the gluten network.

1.5.3 Dressings

Mayonnaise is one of the oldest dressings or sauces, normally used in sandwiches or salad in North America. It is a semi-solid oil-in-water emulsion made from egg yolk, vinegar, and oil. The stability of mayonnaise depends on many factors, including the amount of oil and egg yolks, viscosity, mixing, water quality, and temperature. For health reasons, replacing egg yolks with cholesterol-free ingredients or plant proteins has been widely investigated. Very few studies have reported the usage of pea proteins in mayonnaise or other dressings or sauces, but other comparable analogues have been used. Papalamprou et al. (2006) observed higher stability and improved rheological behavior in salad dressing emulsions containing lupin seed globulins protein, compared with that containing lupin seed albumin or the 1:1 mixture of the two isolates. This was attributed to the "bridging" effect of the globulin aggregates strengthening the droplet-droplet interactions of the emulsion gel network. Creaming properties depended mainly on the

concentration of lupin seed proteins, regardless of the isolate type. Aluko and McIntosh (2005) found that mayonnaise made with hydrolyzed canola protein exhibited a reddish-brown color as the concentration of the protein increased, which may be caused by the oxidization of phenolic compounds during protein extraction. This result implied that the addition of ascorbic acid or bisulfite reducing agents during extraction may help to prevent the oxidation of canola polyphenols. In addition, larger amounts of egg yolk (up to 50% w/w) can be replaced with hydrolyzed protein than native protein (up to 15% w/w) without disrupting the stable emulsion, confirming that limited enzymatic hydrolysis can unfold the protein's globular structure and increase hydrophobicity, which resulted in intensified interactions among oil droplets and the enhancement of emulsion properties.

Ouraji et al. (2020) reported that mayonnaise made with equal amounts of faba bean protein and egg yolk (0.375 %) had the finest mean particle diameters and better texture properties, which was attributed to the reduction of surface tension and the formation of a flexible protein film around the dispersed oil droplets to prevent coalescence and flocculation. As a result, the finer dispersed emulsion presented higher physical stability, monodispersity, and elasticity. Moreover, Alu'datt et al. (2017) found that mayonnaise made with broad bean protein or the mixture of either broad bean/chickpea protein or broad bean protein/lupin flour was superior in lightness. In addition to functional properties, Garcia et al. (2009) optimized the taste, mouthfeel, and overall liking of mayonnaise using the formulation of 37% rice bran oil, 6% soy protein, and 57% water.

The inclusion of hydrocolloids in the mayonnaise formulation containing plant protein could enhance the stability of the interfacial film around droplets and consequently prevent coalescence. Diftis et al. (2005) reported that the conjugation of soy protein with dextran

improved the viscoelastic properties and creaming behavior of salad dressing emulsions because the steric repulsion effects between droplets were enhanced by conjugation. Similar findings were observed by Ghoush et al. (2008), who reported that the combination of egg yolk and protein (0.1% iota-carrageenan + 4% wheat protein) at 25:75 mass ratio significantly increased emulsion stability compared with whole egg yolk. The stability of mayonnaise emulsion decreased as the storage temperature elevated, which was because of the rapid flocculation or coalescence of small droplets at higher temperatures.

1.5.4 Beverages

Beverages fully or partially made with plant-based protein can help prevent cardiovascular disease, cancer, and osteoporosis (Gerliani, Hammami, & Aïder, 2019) as well as animal-protein allergies such as lactose intolerance and milk protein allergies. A study conducted by Štreimikytė et al. (2020) investigated the formulation of protein-based beverages for the elderly who had dysphagia. Mixtures of milk and pea protein showed viscous liquid behaviors and could be more rapidly hydrolyzed under gastrointestinal conditions than milk protein alone. This finding was attributed to the aggregation of pea protein in the stomach and its better intestinal bioavailability than casein. The coagulation properties were also affected by different proteins during digestion.

The beany off-flavor of legume proteins is poorly accepted by consumers, and it is associated with lipid oxidation. Trikusuma et al. (2020) demonstrated that ultra-high temperature processing greatly changed the volatile aroma composition of the pea-protein-based beverage. The dominant pathway of pea protein oxidation was through lipoxygenase, which is the most abundant enzyme in legume seeds. Lipoxygenase converted polyunsaturated fatty acids into unsaturated fatty acid hydroperoxides, which were unstable and could be further degraded to

carbonyls such as aldehydes and ketones. These chemicals were further oxidized to alcohols and acids, which were aroma active and could alter the flavor of pea protein beverages (Trikusuma et al., 2020). Similarly, Chen et al. (2020) demonstrated that rice protein could induce the formation of alcohol esters, contributing to the improvement of sensory properties of Chinese rice wine.

Proteins used in beverages should satisfy specific requirements, such as high solubility, good emulsifying properties, and low viscosity. Protein modifications help to achieve these properties. Physical modification and protein-polysaccharide conjugation are the most common protein modification approaches used in foods and beverages for their chemical-free and "clean labels" claim. Manassero et al. (2019) reported that the high hydrostatic pressure (HHP) treatment improved the physical stability of peach juice made with soy protein and 20 mmol L^{-1} $CaCl_2$ compared with unpressurized juice, while the addition of calcium in the unpressurized juice destabilized the dispersion and induced protein aggregation. The HHP treatment caused parts of protein aggregates to remain in the suspension and significantly increased the viscosity of calcium added juice. This result was attributed to the interactions between soy protein, pectin, and calcium. HHP was also reported to enhance the activity of pectin methylesterase but decrease that of polygalacturonase to form a three-dimensional gel structure. Gerliani et al. (2019) investigated the protein-carbohydrate extract obtained from soybean meal by electro-activation and found that different protein extracts resulted in significantly different beverage functionalities. Alteration of treatment parameters, such as alkaline concentration, time, and protein slurry concentration could improve protein solubility, foaming property, and/or water holding capacity. Protein contents and fractions in the extracted materials were responsible for the differences in the foaming properties, and beverage with higher protein content showed better

foaming ability. Boostani et al. (2017) reported that beverages made with spray-dried, soy-protein-dextran conjugation had higher solubility, wettability, and dispersibility than beverages made with unmodified protein, due to increased thermal stability and solubility of glycated proteins. In addition, the conjugated protein had significantly higher DPPH antioxidant radical scavenging activity (20.5%) than unmodified protein (4.7%), which was attributed to the products of the Maillard reaction affecting the antioxidant potential.

Legume proteins have been investigated for their wine fining efficiency in comparison to commercial gelatins. The fining treatment, carried out for wine clarification and stabilization without disturbing the color of the wine, involved the addition of substance that could bind target compounds and form insoluble aggregates that were later removed from the wine (Marangon, Vincenzi, & Curioni, 2019). A few studies reported using the insoluble proteins from pea, lentil, and soybean as fining agents, because these proteins effectively sped up clarification (Granato, Ferranti, Iametti, & Bonomi, 2018; Granato, Nasi, Ferranti, Iametti, & Bonomi, 2014; Marangon et al., 2019). Lentil protein was the best at removing monomeric and dimeric flavonols, while pea and soy proteins were similarly effective at reducing aroma compounds compared to commercial agents. Generally, the dose of legume-based fining agents ranged from 5 to 30 g/hL, though it was dependent on the vinification stage and treatment conditions (Marangon et al., 2019).

1.5.5 Dairy products and analogues

Low-fat and nonfat dairy products are growing in popularity. Low-fat yogurt usually exhibits poor textural properties such as weak structure and whey separation (Drake, Chen, Tamarapu, & Leenanon, 2000). The use of plant proteins or combinations of plant and milk proteins bring added nutritional value to yogurt, while improving its emulsion stability,

functional properties, and biological activity. To date, few studies have reported using pea proteins in non-dairy products; however, other plant-based proteins have been successfully applied. Drake et al. (2000) reported that dairy yogurt fortified with soy protein exhibited higher viscosity than the control with similar sensory thickness. The fortified yogurt showed a darker color because of Maillard browning during processing and storage. Yogurt with soy protein maintained the fermented dairy aroma and flavor, but such sensory attributes decreased as the concentration of soy protein increased. The elevated content of soy protein along with the decreased concentration of nonfat dried milk resulted in a decline of lactose, which may reduce the sweetness of yogurt. Soleymanpuori et al. (2014) showed that yogurt enriched with soy protein possessed improved water holding capacity compared with the control during extended storage. They explained that soy protein was acting as an inert filler in the yogurt gel matrix on day 1, but experienced a gradual hydration on the following days that enhanced the product's water holding capacity. Milk treatment with transglutaminase had higher water holding capacity at day 1. This was attributed to the formation of covalent cross-linkages with the gel structure achieved by the enzyme treatment, which intensified the 3-D network of yogurt gel to prevent the liquid from flowing out. In addition, enzymatically treated yogurt had higher viability of probiotics, which was attributed to the gel formed by transglutaminase creating a good microenvironment for starter bacteria growth. Similar findings were observed in the study of Pham & Shah (2009), who reported that 4% of soy protein supplemented to yogurt increased the biological activity of starter bacteria.

Sengupta et al. (2019) investigated the emulsion stabilized by soy protein nanoparticles as potential ingredients in non-dairy yogurt. They reported that with increased particle concentrations, the fortified emulsion had a smaller droplet size and higher stability against

coalescence and creaming. With an increased number of repeated cycles during solubilization, crystallization, and ultrasonication, more soy protein nanoparticles aggregated and adsorbed at the interface of oil droplets to form a gel-like network of oil droplets that inhibited creaming. In addition, the viscosity of yogurt increased as soy protein nanoparticles decreased in size, which was because of the intermolecular attraction between various soy protein nanoparticles and lipids of soy yogurts. The yogurt had excellent water holding capacity to retain a large quantity of water and enable a slow release of water with decreased syneresis. In another study conducted by Brückner-Gühmann et al. (2019), yogurt developed by the fermentation of oat protein concentrate underwent acidification and exhibited increased gel properties due to the production of lactic acid during fermentation. The porous network structure of fermented oat protein concentrate confirmed by scanning electron microscopy suggested the capability of oat protein as an alternative source for yogurt-type products.

1.5.6 Encapsulating wall materials

Microencapsulation has been widely used to entrap biological compounds in a carrier and protect food against oxidation and degradation during storage. Microencapsulation protect sensitive compounds, control the release of core agent, mask unpleasant taste and odor, and protect biological ingredients from undesirable light, moisture, and oxygen, thus extending products' shelf life (Li et al., 2015). The most common techniques used for encapsulation include spray drying, ionic gelation, and complex coacervation (Rios-Mera et al., 2019). The spray drying method is inexpensive and fast but utilizes high temperature, which may cause thermal degradation of active encapsulated compounds. Ionic gelation is the gelation of biopolymers during an extrusion process and does not involve heat treatment. This method renders the longest shelf life of encapsulated compounds due to its provision of an impermeable

barrier against oxygen. Complex coacervation occurs between oppositely charged biopolymers (proteins and polysaccharides) through electrostatic interactions and results in smaller particle sizes (Rios-Mera et al., 2019).

Kamaldeen et al. (2020) reported that different ratios of soy protein isolates to cassava starch contributed to the different hygroscopicity of ionic encapsulated carrot powder beads, with higher proportion of cassava starch in the soy protein isolate film solution resulting in a higher hygroscopicity. This may be attributed to the differences in the molecular weights of cassava starch and soy protein as they form a matrix to encapsulate carrot powder beads. The addition of plasticization to the film was expected to increase hygroscopicity due to an increase in molecular mobility within the film. Čakarević et al. (2020) investigated the encapsulation efficiency of phenols from beetroot juice encapsulated by freeze- and spray-dried pumpkin protein. Higher encapsulation efficiency was found in freeze-dried than spray-dried samples, suggesting that drying techniques played a role in encapsulation efficiency. Furthermore, they found that beetroot juice encapsulated in pumpkin protein had higher antioxidant activity than the proteins alone after *in vitro* digestion, which indicated that pumpkin protein could be a good carrier agent in the encapsulation of bioactive compounds in the gastrointestinal tract.

Apart from native proteins, modified proteins also exhibit excellent properties in encapsulation applications. Nesterenko et al. (2014) found that in comparison to microencapsulation with unmodified proteins, microencapsulation with acylated soy protein brought about reduced oil droplet size due to increased surface activity of soy protein, while acylation modification enhanced the amphiphilic characteristic of the protein. On the other hand, encapsulation with protein modified by cationization resulted in decreased viscosity, which may be attributed to the enhanced hydrophilic properties of protein during cationization that increased

the mobility of proteinic chains in the aqueous media, thus decreasing the viscosity of encapsulated samples. Xue et al. (2019) reported that the encapsulation of grapefruit essential oil in emulsion-based edible films prepared by soy protein-gum acacia conjugates showed lower water vapor permeability compared with the control, because of the smaller droplet size of emulsions that resulted in a uniform distribution of the oil phase in the film structure. The reduced water vapor permeability was also related to the surface hydrophobicity and compact microstructure. Higher tensile strength was also achieved because of thick or multiple layers of conjugates at the interface that led to the formation of a stronger film network. Furthermore, films containing the grapefruit essential oil showed better water-repelling ability and a higher glass transition temperature than those containing oregano, lemon, fruit of *Amomum tsaoko* Crevost et Lemaire, indicating that the grapefruit essential oil had stronger molecular interactions with the soy protein-gum acacia matrix during encapsulation. A similar result was found by Li et al. (2015) in the encapsulation of tomato oleoresin using soy protein-gum acacia conjugates as carrier agents. Besides functional properties, the soy protein-gum acacia conjugate also exhibited better biocompatibility compared with the control emulsifier (Tween 80). Moreover, Rios-Mera et al.(2019) reported that the encapsulation of fish oil by complex coacervation formed by transglutaminase induced cross-linked soy protein and that inulin enhanced the resistance of microparticles and increased oil holding capacity due to improved thermal stability.

1.5.7 Pickering particles

Pickering emulsions are emulsions stabilized by solid particles. These surface-active particles remain stable in the water and oil system and have a contact angle (wettability). There is an increasing interest in Pickering emulsions because they are "surfactant-free", flocculation recovery and suitable for the environmental responsiveness in high internal phase emulsions

(Qin, Luo, & Peng, 2018). Particle wettability, particle size, and surface charge are the most important characteristics governing the formation of Pickering emulsions. Particle wettability is characterized by the three-phase contact angle (θ), which measures the distribution of particles at the oil-water interface. A particle could stabilize oil-in-water emulsions if the contact angle is below 90 ° (hydrophilic particles), while water-in-oil emulsions may form when the contact angle is above 90 ° (hydrophobic particles). For Pickering emulsions, particles should have a contact angle close to 90 °, so that they could be equally immersed in both the dispersed and continuous phases to form a stable film at the oil-water interface (Albert et al., 2019; Huang et al., 2019). A smaller particle size is preferred in Pickering emulsions because they can adsorb better at the oil-water interface (Zhu, 2019). Surface charge is the distribution of ions on the particle surface, which may affect the interfacial region of a solution, and it could be induced by both dispersed and continuous phases (Hu et al., 2016).

Zhang et al., (2020) developed pea protein microgel particles as a Pickering stabilizer and investigated the emulsion properties that were affected by pH and ionic strength. They found that salt enhanced emulsion viscosity and shear-thinning properties. The highest degree of particle aggregation of microgel formed at pH 5 due to the activation energy barrier in particle-particle interaction, which resulted in higher adsorption efficiency and higher viscosity. Liang and Tang, (2014) reported that pea protein isolates exhibited a good Pickering stabilization effect for oil-in-water emulsions at pH 3. Furthermore, Shao and Tang, (2016) demonstrated that gel-like pea protein Pickering emulsions at pH 3 could be used as a potential intestine-targeted and sustained-release delivery system for β-carotene. They found that the release of β-carotene during the intestinal digestion of the emulsion could be altered by oil fractions. The emulsion at the oil fraction of 0.6 showed a much slower release of β-carotene and higher stability against

degradation during digestion than the emulsion at the oil fraction of 0.3. This might be due to the change of interfacial layers resulted from the different actions of pepsin during gastric digestion. Similar findings were reported by Wang et al., (2020) and Nikbakht Nasrabadi et al., (2019), who demonstrated that rapeseed and flaxseed proteins, respectively, could be used as stabilizers in Pickering emulsions.

Other modified proteins also exhibit excellent properties in Pickering emulsion applications. Qin et al., (2018) reported that quinoa protein nanoparticles treated with ultrasound improved the wettability and surface hydrophobicity of Pickering emulsions, thus enhancing emulsification efficiency, especially at higher quinoa protein concentrations. The ultrasound treatment altered disulfide bonds and hydrophobic interactions. In another study, soy protein conjugated with jackfruit filum pectin by photocatalysis exhibited good wettability and led to exhibit good freeze-thaw stability in stabilized emulsions (Jin et al., 2019). This was because the photocatalysis treatment caused the exposure of hydrophobic residues on the protein surface and strengthened the affinity of soy protein to jackfruit filum pectin. Improvement in freeze-thaw stability was caused by interactions between proteins and polysaccharides, which enabled the formation of thick interfacial membranes that were difficult to penetrate by fat crystals and created steric repulsions between emulsion droplets. In addition, Ju et al., (2020) developed a novel Pickering emulsion stabilized by soy protein-anthocyanin complex nanoparticles. The fabricated Picking emulsion showed better emulsion oxidative stability, which was attributed to beneficial biological activities of anthocyanins, including anti-inflammation, anti-oxidation, and inhibition of lipid peroxidation.

1.6 Conclusions

Plant proteins are gaining increasing attention due to the growing world population, rising protein deficiency, and their versatile environmental, functional, nutritional, and health benefits. Pea is one of the most widely consumed and cultivated legumes. Different from soybean and wheat, pea involves low allergic effects or few GMO concerns. Current utilization of commercial pea protein as a food ingredient is limited partly due to its less desirable techno-functional properties. Various modification methods (enzymatic, chemical, and physical) have been used to alter pea protein chemical structures and enhance functional properties for different food applications. Pea proteins, along with other types of plant proteins have been investigated in different applications, such as meat products and analogues, bakery products, dressings, beverages, dairy products and analogues, encapsulating wall materials, and Pickering emulsions, although research studies utilizing pea protein are still relatively limited. How to apply pea proteins without affecting foods' textural and sensory properties needs to be further investigated. Future studies are also needed to explore how different pea protein fractions and modifications affect functional properties for specific applications. Comprehensive understandings of protein characteristics would provide meaningful insights to create and develop better functional ingredients for food applications.

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Table 1-1 Classification of pea proteins and their molecular characteristics.

Class	Content	Solubility	Protein	Svedberg unit	MW, kDa	No. of subunit	MW of subunit, kDa
Globulin	55-65%	salt solution	legumin	11S	320-410	6	60-65
			vicilin	7S	150	3	48-50
			convicilin	8S	180-210	3	70
Albumin	18-25%	water solution	albumin	2S	53	2	25
					48	2	24
Prolamin	4-5%	alcohol solution	prolamin	n/a	n/a	n/a	n/a
Glutelin	3-4%	insoluble	glutelin	n/a	n/a	n/a	n/a

*n/a, not available (Lu et al, 2019; Croy et al, 1984).

Table 1-2 Amino acid composition (g/100 g protein) of pea seed meal and its protein fractions.

Amino acid	Seed meal	Legumin[a]	Vicilin[b]	Albumin[c]	Prolamin+glutelin[d]
Essential					
Methionine	2.6	1.2	0.8	2.2	1.3
Cystine	0.0	0.0	0.0	0.0	0.0
Lysine	8.5	6.4	12.4	12.5	8.2
Histidine	3.8	3.0	3.6	4.4	3.7
Arginine	6.0	3.9	4.6	10.4	5.1
Tyrosine	3.9	3.4	3.9	4.8	5.6
Leucine	6.0	6.3	9.7	4.3	7.0
Isoleucine	3.5	3.3	4.6	3.5	4.2
Threonine	6.3	6.5	7.1	5.4	8.9
Valine	3.9	3.8	4.4	4.5	5.6
Phenylalanine	4.3	3.9	6.1	3.8	4.7
Non-essential					
Asparate	11.5	11.2	16.8	16.0	10.1
Glutamate	15.2	16.2	22.7	18.9	12.0
Serine	4.8	4.5	6.2	5.4	5.1
Glycine	4.4	3.5	3.7	7.0	5.0
Alanine	3.8	3.3	3.5	6.3	4.2
Proline	6.0	5.1	5.5	5.5	10.3

(Rubio et al., 2014)

a: Borate buffer (pH 8) extract, adjust pH to 4.5, collect the sediment, dialysis, and freeze-dry;

b: Borate buffer (pH 8) extract, adjust pH to 4.5, collect the supernatant, dialysis, centrifuge, collect the sediment, freeze-dry;

c: Borate buffer (pH 8) extract, adjust pH to 4.5, collect the supernatant, dialysis, centrifuge, collect the supernatant, percipient in 608 g/L $(NH_4)_2SO_4$, centrifuge, collect sediment, dialysis, freeze-dry;

d: Collect the residue after borate buffer (pH 8) extraction, freeze-dry.

Table 1-3 Functional properties of pea proteins.

*ND: not determined.

Sources	**Treatments**	**Protein content (%)**	**Water holding capacity (g/g)**	**Oil holding capacity (g/g)**	**Emulsifying capacity (EC) /stability (ES) (%)**	**Foaming capacity (FC)/stability (FS) (%)**	**References**
Commercial air-classified pea protein-enriched flour	Untreated	55.5	1.18	0.96	ES-40.00%	ND	(Wang et al., 2020)
	20% ethanol wash	62.4	2.53	0.80	ES-40.31%	ND	
	50% ethanol wash	61.4	2.60	0.77	ES-29.06%	ND	
	80% ethanol wash	58.2	2.00	0.76	ES-28.13%	ND	
	20% isopropanol wash	64.2	2.39	0.72	ES-46.88%	ND	
	50% isopropanol wash	64.3	2.54	0.67	ES-45.31%	ND	
	80% isopropanol wash	59.3	2.07	0.73	ES-37.19%	ND	
Pea Agassiz	Dehulled, milled, defatted, solubilized at pH 9, and precipitated at pH 4.5	90.9	ND	3.3	ES-95.80%	FS-74.90	(Lam et al., 2017)
Pea CDC Dakota		91.0	ND	3.2	ES-95.70%	FS-74.10	
Pea CDC Golden		91.1	ND	3.2	ES-95.10%	FS-75.00	
Pea CDC Striker		92.5	ND	3.1	ES-95.70%	FS-75.20	
Pea -Tetris		91.7	ND	3.3	ES-96.00%	FS-75.30	
Pea Cooper		89.7	ND	3.1	ES-96.10%	FS-73.50	
Commercial pea protein isolate	None	83.5	ND	1.5	ES-79.3%	FS-56.6	(Lam et al., 2017)

Grass pea seeds	Solubilize at pH 9.7 and precipitate at pH 4.5	80.0	2.24	0.67	EC-36.73% ES-18.03 min	FC-109.78 FS-121.35	(Milad et al., 2019)
	Salt extraction containing 6.4% KCl	77.9	2.39	2.16	EC-36.21% ES-12.90 min	FC-106.23 FS-118.34	
	Ultrafiltration-diafiltration precipitation	90.5	2.83	2.21	EC-38.63% ES-18.86 min	FC-105.06 FS-119.79	
Grass pea seeds	Optimized extraction based on protein content	92.5	2.70	1.37	EC-35.80% ES-29.75%	FC-41.00 FS-100.00	(Feyzi et al., 2018)
	Optimized extraction based on extraction yield	87.5	2.15	1.19	EC-87.50% ES-28.65%	FC-87.00 FS-78.00	

Table 1-4 Functionality improvement of pea and other proteins through modifications.

Modification methods	Functional changes	References
Protein-polysaccharide conjugation/complexation		
Pea protein glycosylated with galactose, lactose, and galacturonic acid	Improved protein solubility and foaming properties	(Baniel et al., 1992)
Pea protein with soluble soybean polysaccharide	Improved emulsifying and foaming properties	(Zhan et al., 2019c)
Pea protein with high methoxyl pectin	Improved thermal stability and solubility	(Lan et al., 2018)
Pea protein with pectic polysaccharides	Improved solubility and coacervation	(Warnakulasuriya et al., 2018)
Pea protein with pectin	Improved emulsifying and encapsulation properties	(Tamnak et al., 2016)
Pea protein with high methoxyl pectin	Improved emulsifying properties	(Gharsallaoui et al., 2010)
Pea protein with pectin	Improved emulsifying properties	(Tamnak et al., 2016)
Pea protein with gum Arabic	Improved solubility and emulsifying properties; diminished beany flavor	(Zha et al., 2019b, 2019a; Zha et al., 2019c)
Pea protein with gum Arabic	Improved emulsifying and foaming properties	(S. Liu et al., 2010)
Pea protein with propylene glycol alginate	Improved solubility and emulsifying properties	(Guo et al., 2019)
Pea protein with corn fiber gum/ high methoxyl pectin/ carboxymethyl cellulose/ konjac glucomannan	Improved emulsion physical stability	(Wei et al., 2020)
Enzymatic modifications		
Pea protein hydrolyzed by trypsin	Improved solubility and emulsion stability	(Klost & Drusch, 2019)
Pea protein hydrolyzed by trypsin and Alcalase	Improved emulsifying properties	(Tamm et al., 2016)
Pea protein hydrolyzed by Protamex	Improved emulsifying properties, foam expansion, and solubility	(Sijtsma et al., 1998)
Pea protein hydrolyzed with chymosin	Improved solubility and emulsifying and foaming properties	(Barać et al., 2011)

Salt extracted pea protein catalyzed by transglutaminase	Improved gel strength	(Sun & Arntfield, 2011)
Quinoa protein catalyzed by transglutaminase	Reduced film surface roughness and improved physical stability	(Escamilla-García et al., 2019)
Quinoa protein with high-intensity ultrasound combined with transglutaminase	Improved edible film thickness, decreased elongation percentage, and increased tensile strength	(Antonia Vera, Tapia, & Abugoch, 2020)
Pea protein deamidated by glutaminase	Improved solubility and reduced beany flavor	(Fang et al., 2020)
Coconut protein deamidated by protein-glutaminase	Improved solubility, emulsifying stability, and foaming capacity	(Kunarayakul et al., 2018)
Whey protein deamidated by protein-glutaminase	Decreased heat-induced aggregation; increased water holding capacity	(Miwa et al., 2013)
Rice/ pea/ blend deamidated by glutaminase	Decreased solubility and increased aggregation	(Marco et al., 2007)
Myofibrillar/pea protein mixture induced by transglutaminase crosslinking	Improved gelation	(Sun & Arntfield, 2012)
Chemical modification		
pea protein acetylation and succinylation	Improved solubility, water holding capacity, and emulsifying properties	(Johnson & Brekke, 1983)
Rapeseed protein acylation	Improved water holding capacity and gelation	(Wang et al., 2018)
Flax protein acylation	Improved emulsifying properties and solubility	(Wanasundara & Shahidi, 1997)
African Yam Bean acylation	Improved solubility, water holding capacity, and emulsifying properties	(Arogundade et al., 2013)
Mung bean protein acylation	Improved water/oil holding capacity and foaming and emulsifying properties	(El-Adawy, 2000)
Oat protein succinylation	Improved solubility and emulsifying properties	(Mirmoghtadaie et al., 2009)
Amyloid β protein deamidation	Improved aggregation; slower fibrillation formation	(Osaki & Hiramatsu, 2016)

Curli amyloid protein deamidation	Slower and less amyloid fibril formation	(Wang et al., 2017)
Rice bran protein-alkaline deamidation	Improved solubility	(Guan et al., 2017)
Physical modification		
Thermal treatment of pea protein	Increased protein aggregation and decreased solubility	(Mession et al., 2013)
Ultrasound treatment of pea protein	Improved solubility, smaller particle size, and increased surface hydrophobicity	(Jiang et al., 2017)
Ultrasound treatment of pea protein	Improved foaming properties	(Xiong et al., 2018)
High pressure treatment of pea protein	Improved foaming stability at pH 7, but reduced foaming and emulsifying properties at pH 3	(Chao et al., 2018)
High pressure with supercritical carbon dioxide treatment of pea protein	Improved foam stability and surface properties	(Do Carmo et al., 2016)
High hydrostatic pressure of pea protein	Reduced solubility	(Chao & Aluko, 2018)

Table 1-5 Food applications of pea and other plant proteins and related functional improvement.

Plant proteins	Processes	Functional improvement	References
Meat/meat analogues			
Soy protein flour/protein	Added at various levels of flour/ protein (0, 2, 3.5 and 5% kg/kg total mass) to beef patty	Improved water holding capacity and reduced cooking loss; softer and more cohesive texture of beef patty	(Kassama et al., 2003)
Soy protein/texturized vegetable protein	Directly added or substitution of meat from 10-40%	Improved textural characteristics of burgers, beef sausage, and buffalo meat sausage	(Ahmad et al., 2010; Akesowan, 2010; Hidayat et al., 2017)
Soy protein	Added at 2-4% protein to meat batter	Improved meat batter emulsion stability	Li et al. (2020)
Soy protein/wheat gluten	Extruded meat alternatives	Reduced hardness of sausage	Chiang et al. (2020)
Various plant proteins	Meat free sausage, and sausage with 20% and 60% meat	Minimized cooking loss and shrinkage; improved emulsion stability of meat sausage	Kamani et al. (2019)
Vicia ervilia	100% plant based burger	Improved water holding capacity and extended shelf life	Hatamikia et al. (2019)
Bakery products			
Oat flour and Pea protein	80% oat flour and 20% pea protein were used	Improved nutritional quality and reduced cracker hardness	(Morales-Polanco et al., 2017)
Rice/pea protein	Adding 5 and 10%	Less crispy texture of bread crust; higher water activity	Pico et al. (2019)
Succinylated pea protein	Adding 3.45% modified pea protein	Improved viscosity, cell count, and rise and softness of eggless cake	(Shah et al., 2019)
Cowpea protein	Cowpea protein mixed with wheat flour at 2, 4 and 6% in wheat bread and sponge cake	Improved water holding capacity; softer texture	(Campbell et al., 2016)

Quinoa protein	Adding 0-30% quinoa protein	Increased firmness of cupcake and water activity; decreased growth of microorganisms and extended shelf life	(López-Alarcón et al., 2019)
Soy protein	Adding 4% soy protein to rice flour	Lower gluten-free bread volume with inhomogeneous crumb structure	(Masure et al., 2019)
Soy protein hydrolysate	Adding 1% soy protein hydrolysate	Improved dough fermentation ability and acidification of steam bread	Liu et al. (2016)
Extruded soy protein	1000 g soy protein or extruded soy protein added with 400 g water	Improved volume and interior structure of Chinese steam bread	Du et al. (2016)
Sauces and dressings			
Lupin seed globulin protein	50:50 oil/water ratio, with protein concentration from 1.5-4%	Improved emulsion stability and rheological behavior of mayonnaise	Papalamprou et al. (2006)
Hydrolyzed canola protein	Prepared with 70% oil, and replaced 10-50% egg yolk with the hydrolyzed protein	Improved emulsion stability	Aluko and McIntosh (2005)
Faba bean protein	Prepared with 40% oil, 0-0.75% protein	Finest mean particle diameter and better texture of mayonnaise	Ouraji et al. (2020)
Broad bean protein/chickpea protein	Prepared with 75-76% oil, and 3% protein	Improved lightness of mayonnaise and antioxidant activity	Alu'datt et al. (2017)
Soy protein	Prepared with 37-43% oil, and 4-7% soy protein concentrate	Optimized sensory attributes	Garcia et al. (2009)
Soy protein-dextran conjugates	50:50 oil/water ratio, and 1% protein	Improved viscoelastic properties and creaming behavior of salad dressing emulsion	Diftis et al. (2005)
Beverages			
Pea protein	1:1 milk and pea protein solution	Viscous liquid behavior; more rapidly hydrolyzed under gastrointestinal conditions	Štreimikytė et al. (2020)

Pea protein with ultra-high temperature	Prepared by adding 3% pea protein isolate under ultra-high temperature	Reduced beany off-flavor of beverage	Trikusuma et al. (2020)
Pea, lentil, soy protein	Hydrolyzed protein fining agent in plastic-stoppered 100 mL cylinders, filled with 120 mL wine, minimize air	Effective wine clarification	(Granato et al., 2018, 2014; Marangon et al., 2019)
Rice protein	Adding glutelin (19 or 38 g), albumin (1.2 or 2.4 g)	Improved sensory properties of Chinese rice wine	Chen et al. (2020)
Soy protein with high hydrostatic pressure	Adding 25 g soy protein in 1 L peach beverage	Improved physical stability of peach juice	Manassero et al. (2019)
Protein-carbohydrate extracts from soybean meal	Prepared by adding 0.6% whey powder and soybean meal extracts obtained through different electro-activation conditions	Better solubility, foaming properties, and water holding capacity	Gerliani et al. (2019)
Soy protein-dextran conjugates	5% modified protein added to the drink	Increased solubility, wettability, and dispersibility	Boostani et al. (2017)
Dairy products or analogues			
Soy protein	Low fat yogurt fortified with 0-5% soy protein concentrate	Higher viscosity; darker color of yogurt	Drake et al. (2000)
Soy protein	Yogurt enriched with 10 mg/mL soy protein isolate	Increased water holding capacity of yogurt	Soleymanpuori et al. (2014)
Soy protein	Yogurt was prepared by adding 4% soy protein isolate	Increased biologically activity of yogurt	Pham & Shah (2009)
Soy protein	Prepared by 4 g oil, and 8 g total solids/100 g soy protein nanoparticles emulsion	Improved emulsion stability	Sengupta et al. (2019)
Oat protein	Prepared by adding 150 g oat protein concentrate in 1 L distilled water	Increased gel properties of yogurt	Brückner-Gühmann et al. (2019)

Encapsulating wall materials			
Soy protein	Prepared by ionic gelation method. Soy protein ratio was from 0-100%	Increased hygroscopicity of carrot powder beads	Kamaldeen et al. (2020)
Pumpkin protein	Prepared by spray drying method. Protein suspended in beet root juice in 1:20 core-wall ratio	Higher encapsulation efficiency in freeze-dried beetroot juice and higher antioxidant activity	Čakarević et al. (2020)
Acylated soy protein	Prepared by spray drying method. 8% protein solution/ core ratio is 2:1.	Reduced oil droplet size of microencapsulation	Nesterenko et al. (2014)
Soy protein-gum acacia conjugates	Protein: oleoresin from 2:1 to 6:1.	Improved water vapor permeability and emulsion stability	Li et al. (2015)
Cross-linked soy protein by transglutaminase	Prepared by inulin/soy protein isolate ratio from 0.5 to 1.5.	Improved oil holding capacity and thermal stability	Rios-Mera et al.(2019)
Pickering particles			
Pea protein	Protein dispersion at 0.25-3.0 g/100 mL. Oil at ø=0.2, pH 3	Improved emulsion stability	(Liang & Tang, 2014)
Pea protein	Protein dispersion at 6%. Oil at ø=0.2-0.6, pH 3.	Acted as release delivery system for beta-carotene	(Shao & Tang, 2016)
Pea protein microgel particles	Oil: pea protein microgel=20:80	Responsiveness to pH and ionic strength	(Zhang et al., 2020)
Soy protein-anthocyanin	6% soy protein solution with 0-0.15% anthocyanins	Improved emulsion stability, oxidative stability, and resistance to *in vitro* digestion	(Ju et al., 2020)
Soy protein-Jackfruit filum pectin (SPP)	SPP solution (2%, pH 7): oil=9:1.	Improved wettability, thermal stability, and freeze-thaw stability	(Jin et al., 2019)
Quinoa protein nanoparticles	Quinoa protein nanoparticle dispersions (0.25-6%), oil at ø=0.2-0.7	Improved wettability and emulsification efficiency	(Qin et al., 2018)

Flaxseed protein and mucilage nano-assemblies	Flaxseed protein:flaxseed mucilage=70 :30 or 50:50, total concentration=0.45%, pH 3	Improved emulsion stability	(Nikbakht Nasrabadi et al., 2019)
Rapeseed protein nanogel	Acylated rapeseed protein concentration=0.1-1%, oil at ø=0.1-0.5	Improved emulsion stability	(Wang et al., 2020)

Chapter 2 - Drying methods affect physicochemical and functional properties of quinoa protein isolate[1]

Abstract

Quinoa protein possesses a well-balanced amino acid profiles and can be a potential food ingredient with broad applications. The objective of this study was to investigate the effect of different drying methods, including freeze drying, spray drying, and vacuum drying on the functional and physicochemical properties of quinoa protein isolate, e.g., morphology, amino acid composition, SDS-PAGE profile, sulfhydryl/disulfide content, secondary structure, surface hydrophobicity, and thermal stability. The freeze-dried protein exhibited the highest emulsification capacity and stability and oil binding capacity, which was attributed to its higher surface hydrophobicity, while the spray-dried sample had the highest solubility and water binding capacity at pH 7. Gels (8%) prepared with the freeze-dried protein had higher elastic and viscous modulus than that from others. The freeze-dried protein had the highest maximal denaturation temperature but lowest enthalpy, which may be attributed to its higher amount of random coil but lower percent of regular α-helix and β-sheet structures. Overall, quinoa protein isolate from different processing methods demonstrated distinct functional properties. This information will be useful to optimize quinoa protein production and benefit its applications.

Key words: Plant protein, quinoa protein isolate, drying methods, physicochemical properties, functional properties

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2.1 Introduction

The demand for proteins in human diet has been steadily increasing in recent years due to the increased awareness of their nutritional value and functional properties. Compared with animal proteins, production of plant protein is more sustainable and requires much less water, land, and fossil energy resources. Currently, wheat gluten, soy protein, and pea protein are the most available plant proteins. In order to meet the future needs of more diverse, affordable, and superior plant protein ingredients, additional protein sources should be vigorously explored and investigated. Quinoa (*Chenopodium quinoa* Wild.), known as a pseudocereal and ancient grain, is consumed mostly by people in the Andean region (Abugoch et al., 2009). Quinoa possesses good resistance to drought, frost, soil salinity environmental conditions and can be tolerate to a wide range of soil pH (Abugoch et al., 2009; Steffolani et al., 2016). Because of the relatively high protein content as well as balanced amino acid compositions, quinoa is receiving increased popularity as a new food and protein source (Abugoch et al., 2009). Quinoa contains higher content of lysine (5.1-6.4%), methionine (0.4-1.0%), and cysteine than common cereal grains (Elsohaimy et al., 2015) and is also a good source of fiber, polyunsaturated fat, minerals, vitamins and phytochemicals such as polyphenols and flavonoids (Abugoch et al., 2009; Hager, Wolter, Jacob, Zannini, & Arendt, 2012). These functional nutrients could help lower the risk of chronic disease and potentially promote human health.

The major proteins in quinoa are 11S globulin and 2S albumin, which account for about 37% and 35% of the total seed protein, respectively (Abugoch, 2009; Kaspchak et al., 2017). Quinoa proteins possess good functional properties, for example, emulsification, foaming, gelation, water and oil binding properties, and solubility (Abugoch, 2009; Abugoch et al., 2008; López et al., 2018; Steffolani et al., 2016). Kaspchak et al. (2017) indicated that quinoa protein formed

strong and stable gels at pH 3.5 with heating to 70-90 °C, and the gel formation potential was affected by pH through altering the secondary structure as well as protein solubility. Steffolani et al. (2016) found that quinoa protein had better water and oil binding capacity compared with some legume proteins, although the properties differed among different quinoa varieties. The functional properties of proteins are dependent on many factors, such as hydrophilic/hydrophobic ratio, water activity, ionic force, pH, temperature, charge density and changes in environment (Abugoch, 2009).

Protein functional properties are dependent on processing conditions. A few studies have been conducted on the functionality of quinoa proteins with different extraction or processing methods. Lilian et al. (2008) indicated that quinoa protein extracted at pH 9 had higher solubility at isoelectric point than that extracted at pH 11, although the proteins from both extractions had similar amino acid composition and water holding capacity. Mir et al. (2019) and Vera et al. (2019) reported that ultrasound treatment at high intensity improved functional properties of quinoa protein, especially gelling behavior. Ruiz et al. (2016) investigated the effect of extraction pH of quinoa protein on heat-induced aggregation and gelation properties. They found that with extraction at pH 8 or 9, protein aggregation was enhanced in the formation of a semi-solid gel, while at pH 10 or 11, the protein had less aggregation, lost particle arrangements, and could not form gels. Therefore, protein processing methods and conditions are critical in determining their functionalities. Previous studies reported that different drying methods affected the functional properties of cowpea and bambara proteins (Mune & Sogi, 2016), peanut protein (Liu, Li, Jiang, Yang, & Zhang, 2019), and whitecheek shark protein hydrolysates (Alinejad, Motamedzadegan, Rezaei, & Regenstein, 2017). Freeze drying prevents most of protein deterioration and minimizes microbiological reaction, but it is a more expensive and time-consuming drying

process. Spray drying is more time efficient and one of the most popular processes used in the food industry, although it may cause some quality deterioration. Vacuum drying is considered as a simple and popular technique; however, it can be expensive for the large scale production and some degradation of heat sensitive products (Alinejad et al., 2017; Pratap Singh et al., 2020). To our knowledge, there is little information available about the effect of dehydration methods of quinoa proteins on their functional and physicochemical properties. Therefore, the objective of this study was to investigate the effect of different drying methods, namely freeze drying, spray drying, and vacuum drying on physicochemical and functional properties of quinoa protein isolates. Critical protein functional properties including water/oil absorption capacity, emulsification and foaming properties, solubility, and gel properties were analyzed. Protein physicochemical characteristics including amino acid composition, surface hydrophobicity, sulfhydryl/disulfide content, SDS-PAGE profile, secondary structure, thermal stability, and morphology were also evaluated. Selecting appropriate processing methods will benefit industry in optimizing protein production and functional properties for targeted food applications.

2.2 Materials and methods

2.2.1 Materials

Commercial white quinoa (*Chenopodium quinoa* Wild.) flour (11.4% moisture, 12.6% protein, 2.4% ash) was provided by Ardent Mills (Denver, CO, USA). Sodium dodecyl sulfate (SDS), 8-anilinonaphthalene-1-sulfonic acid (ANS), Tris-HCl, β-mercaptoethanol, urea, ethylenediaminetetraacetic acid (EDTA), sodium sulfite, and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Amino acid standards and analysis kit (EZ: faast kit) were purchased from Phenomenex (Torrance, CA, USA).

2.2.2 Quinoa protein extraction

Quinoa flour was defatted with hexane at a flour/ hexane ratio of 1: 4 (w/v) for 1 hour and 3 times prior to protein extraction. The defatted quinoa flour was placed in a fume hood for at least 24 hours to evaporate residue hexane. Quinoa protein isolate was extracted based on a literature method (Ruiz et al., 2016) with some modifications. Briefly, 100 g defatted quinoa flour was dispersed into 1000 mL deionized water (DI), and the suspension was stirred for 1 hour at room temperature with pH maintained at 10 using 1 M NaOH, followed by centrifugation at 8000 g for 20 min at 4 °C using Avanti J-E centrifuge (Beckman Coulter, Indianapolis, USA). The supernatant was collected, pH was adjusted to 4 with 1 M HCl, and the sample was stored at 4 °C for 2 hours to maximize protein precipitation, followed by another centrifugation to recover the protein precipitate. The precipitated protein was washed with DI water twice and then adjusted to neutral pH. The re-dispersed quinoa protein suspension (10%) was dried respectively by using a freeze-dryer (Freezone 4.5L, Labconco Corporation, Kansas City, MO, USA) (-40 °C, 3 days), vacuum dryer (Fisher Scientific, Hampton, NH, USA) (40 °C, 2 days at –28 inHg), and Buchi Mini spray dryer B-290 (BUCHI Corporation, New Castle, DE, USA) (inlet temperature 180 °C, outlet temperature 60 °C, 90% of aspirator, and 10% of pump speed). The protein suspension was further homogenized before feeding to the spray drier. Freeze- and vacuum-dried proteins were ground into powder and kept at 4 °C for further analysis. The quinoa protein content and moisture were measured following AACC Method 46-30.01 and 44-19.01, respectively.

2.2.3 Measurement of particle size

Particle size of quinoa protein samples was measured using a laser diffraction particle size distribution analyzer LA-910 (Horiba, Ltd., Kyoto, Japan). Each protein sample was dispersed in

DI water at 0.1% concentration, and the sample was transferred into the reservoir tank of the instrument filled with water. The sample was further mixed inside the instrument with a set of agitating blades and ultrasonic vibration to achieve uniform dispersion of the protein particles in the water, which was then analyzed based on the diffraction of laser scattered by the particles. Both particle size distribution and average particle size were collected from the software.

2.2.4 Color analysis

The color of quinoa proteins from different drying methods was measured using a Minolta Chroma Meter CR-300 colorimeter (Minolta Camera Co., Osaka, Japan). Three color components were collected from the measurement, including L* (- black to + white), a* (- green to + red), and b* (-blue to + yellow).

2.2.5 Atomic force microscopy (AFM)

Atomic force microscope (AFM) imaging of the quinoa proteins was analyzed following our previous method (Wang et al., 2020) and carried out using a Bruker Innova AFM instrument (Billerica, MA, USA) operated in a contact mode.

2.2.6 Amino acid analysis

Amino acids composition of the proteins was analyzed following the method of Steffolani (Steffolani et al., 2016) with some modifications. One hundred milligram protein was transferred to 8 mL 6M HCl, mixed well and vortexed for 5 seconds, with nitrogen gas purging for 5 min. The sample tube was sealed and transferred to an oven set at 110 °C for protein hydrolysis for 24 hours. Tryptophan was analyzed using 5 M NaOH alkaline hydrolysis under the same condition. Amino acid extraction and derivatization were carried out using EZ: faast kit from Phenomenex (Torrance, CA, USA). Two microliter derivatized sample was injected to a GC-MS QP2010-SE system (Kyoto, Kyoto Prefecture, Japan) equipped with a ZB-AAA capillary column (10m

×0.25mm i.d., Phenomenex, Torrance, CA, USA). The parameters were set as the following: injection temperature at 300 °C, flow rate of the carrier gas (helium) at 0.6 mL/min, oven temperature at 110°C with heating at 20 °C/ min to 320 °C, split ratio at 10, and the ion source at 240 °C. Aspartic acid, methionine, glutamic acid, and phenylalanine were separated using SIM mode based on their major ions. Each amino acid concentration was calculated based on the calibration curve established with respective amino acid standard. The amino acids were determined based on the standard solution chromatogram provided by Phenomenex (Torrance, CA) based on their elution time and mass spectra.

2.2.7 Functional properties

2.2.7.1 Solubility

Solubility of quinoa proteins in water was measured at pH 3 to 11 with 4% solid content. After pH was adjusted to the desired level using 1 M NaOH or HCl, the suspension was further stirred for 30 min at room temperature, followed by centrifugation at 4000 g for 30 min. Protein concentration in the solution was determined according to the Biuret method and analyzed using a double beam spectrophotometer (VWR UV-6300PC) at 540 nm absorbance (Elsohaimy et al., 2015).

2.2.7.2 Oil/water absorption capacity

Oil/ water absorption capacities were analyzed following the method of Elsohaimy (Elsohaimy et al., 2015) with small modifications. For oil absorption capacity (OAC), approximately 1 g protein was accurately weighted (O_0) and thoroughly mixed with 10 mL soybean oil in a 15 mL centrifuge tube (O_2), then allowed to stand for 30 min at room temperature, and followed by centrifugation at 3000 g for 30 min (Z 366 K centrifuge, Hermle Benchmark, USA). The supernatant was discarded, and the test tube was inverted for 2 min to

drain the excess oil and weighed (O_1). The OAC was calculated as: $OAC = \frac{O1-O2-O0}{O0}$. For water absorption capacity (WAC) test, protein was accurately weighted (W_0, approximately 0.6 g) and thoroughly mixed with 10 mL DI water in a 15 mL centrifuge tube (W_2), followed by centrifugation at 3000 g for 30 min. The supernatant was discarded, and the tube containing the protein was weighted (W_1). The WAC was calculated as: $WAC = \frac{W1-W2-W0}{W0}$, and determined at pH 5–8. The OAC and WAC results were expressed as g oil/ g protein and g H_2O/ g protein, respectively.

2.2.7.3 Emulsion capacity and stability

Emulsion capacity and stability were measured according to a literature method (Yasumatsu et al., 2011) with small modifications. Quinoa protein (1.75 g) was homogenized with 25 mL DI water for 30 seconds using a Waring blender. Soybean oil (25 mL) was then added to the suspension and homogenized for another 30 seconds. The emulsion was then centrifuged at 1100 g for 5 min. Emulsion capacity (EC) was calculated as: $EC = \frac{H1}{H0} \times 100$, where H_0 is the height of the total emulsion in the tube, and H_1 is the height of emulsified layer in the tube. For emulsion stability, the emulsion was heated at 80 °C for 30 min and then centrifuged similarly. The emulsion stability (ES) was calculated similarly as for EC. Both ES and EC were measured at pH 5, 6, 7 and 8, respectively.

2.2.7.4 Foaming capacity and stability

Foaming capacity and stability were measured according to a literature method (Kaushik et al., 2016) with some modification. Briefly, 0.5 g protein was dispersed into 50 mL DI water in a beaker. The suspension was homogenized with a high-performance disperser (Ingenieurbure CAT, Germany) for 2 min at 20,000 rpm, immediately transferred to a graduated cylinder, and

volume was recorded (V_1). Foam capacity (FC) was calculated as $FC = \frac{V1-V2}{V0} \times 100$, where V_0 is the total volume of protein suspension, and V_2 is the total volume of protein suspension solution after homogenization at 0 min. The total volume was recorded at 0, 15, 30, 45, 60, 75 and 90 min, respectively. Foam stability (FS) was calculated by the equation: $FS = \frac{vt}{v0} \times 100$, where Vt is the volume of foam at a certain time after homogenization. The FC and FS were measured at pH 5, 6, 7 and 8, respectively.

2.2.7.5 Gel rheology

Gel rheological properties were measured using a Bohlin CVOR 150 rheometer (Malvern Instruments, Southborough, MA, USA) with a PP 20 parallel plate with a gap size of 500 μm. Protein was dispersed in DI water with mixing to a final concentration of 8%, which was heated in boiling water for 1 hour, cooled, and kept in a refrigerator at 4 °C for 2 hours. Frequency sweep was conducted in the range of 0.1 to 10 Hz at 25 °C with 1% strain. Rheological properties in terms of shear storage modulus (G´) and loss modulus (G´´) were recorded.

2.2.8 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The protein sample was dispersed into 1% SDS/NaPhos buffer (pH 7.0, 5 mg/mL) and vigorously mixed overnight followed by centrifugation at 10,000 g for 5 min at room temperature (Chen et al., 2019) before running the gel. Twenty microliter supernatant was mixed with 10 μL Laemmli buffer (0.01 M Tris-HCl, 10% (w/v) SDS, , 0.1% (w/v) bromophenol blue, and 10% (v/v) glycerol) with (reducing condition) or without (non-reducing condition) 2% (v/v) β-mercaptoethanol. The sample was then boiled for 10 min, and 15 μL solution was loaded on the gel slab which consists of 12% resolving gel (pH 8.8) and 4% stacking gel (pH 6.8). The electrophoresis was run using a PowerPac 1000 (Bio-Rad, USA) with running buffer prepared by diluting 100 mL 10 X Tris/Glycine/SDS buffer with DI water at the constant voltage 220 V, and

the gel was stained with Coomassie Brilliant Blue for 1 hour, followed by de-staining with DI water overnight.

2.2.9 Sulfhydryl/ disulfide content

Free sulfhydryl groups were determined according to a previous method (Rombouts, Jansens, Lagrain, Delcour, & Zhu, 2014) with some modifications. Briefly, 30 mg protein was added to 3 mL reaction buffer A (8 M urea, 0.2 M Tris-HCl, 3 mM EDTA, 1% SDS, pH 8.0), and then vortexed for 30 seconds and mixed vigorously for 1 hour. After that, 0.3 mL of buffer B (10 mM DTNB in 0.2 M Tris-HCl, pH 8.0) was added, mixed vigorously for another 1 hour, followed by centrifugation at 13,600 g for 15 min at room temperature. Total sulfhydryl content was measured using our previous method (Chen et al., 2019). Ten milligram protein was added into 1 mL reaction buffer which included 3 mM EDTA, 1% SDS, 0.2 M Tris-HCl, 0.1 M sodium sulfite, at pH 9.5 and 0.5 mM 2-nitro-5-thiosulfobenzoate (NTSB), vortexed and mixed vigorously in dark for 1 hour, followed by centrifugation at 13,600 g for 15 min. The supernatant (0.3 mL) was diluted with 2.7 mL of the reaction buffer without NTSB. The absorbance was measured at 412 nm using a double beam spectrophotometer (VWR UV-6300PC, Radnor, PA, USA). The free or total SH content was calculated by the equation: $C(SH) = \frac{\mathrm{A}}{\varepsilon \mathrm{b}}$, where A is the absorbance, ε is the extinction coefficient of 13,600 $M^{-1}cm^{-1}$, b is the cell path length. The disulfide (SS) was calculated by the equation: $C(SS) = \frac{C_{tSH} - C_{fSH}}{2}$, where C_{tSH} is the total SH content, C_{fSH} is the free SH content.

2.2.10 FTIR and protein secondary structure

FTIR spectra of quinoa protein were collected with a PerkinElmer Spectrum 400 FT-IR/FT-NIR Spectrometer (PerkinElmer, Inc., Waltham, MA, USA) equipped with an attenuated total reflectance cell (ATR) according to our previous method (Chen et al., 2019). A total of 64 scans

was run for each sample at an interval of 4 cm^{-1} in the range of 400 - 4000 cm^{-1}, and protein secondary structures were determined from the amide I region (1600-1700 cm^{-1}). The data was quantified using OriginPro 2016 software (OriginLab, Inc., Northampton, MA, USA) to fit the peaks, and a second derivative method was used to find the anchor points. The curve was then auto baseline-subtracted and rescaled to smooth the derivative by using Savitzky-Golay method. The protein secondary structure was then determined based on the peak wavenumber and peak area.

2.2.11 Surface hydrophobicity

Surface hydrophobicity of the extracted proteins was measured according to a previous method (Timilsena, Adhikari, Barrow, & Adhikari, 2016) with some modifications. Protein solutions in DI water with concentrations of 0.0125-0.1% (w/v) were prepared. Each prepared sample (4 mL) was mixed with 20 μL of 8 mM 8-anilinno-1-napthalenesulfonic acid (ANS) in a 15 mL test tube, vortexed for 30 seconds, and incubated in dark for 15 min at room temperature. The mixture (250 μL) was then added to a 96-well microplate, and fluorescent intensity was measured using a microplate spectrophotometer (BioTek, Synergy H1 Hybrid, Highland Park, Winooski, VT) at 390 nm for excitation and 460 nm for emission. Fluorescent intensity of each diluted protein samples without ANS was also measured. Protein surface hydrophobicity was calculated based on the absorbance difference between the sample with and without ANS plotted against protein concentration, and the linear slope was considered as the relative surface hydrophobicity.

2.2.12 Differential scanning calorimetry (DSC)

Thermal properties of quinoa proteins were determined by differential scanning calorimeter (DSC) (Q200, TA instrument, Schaumburg, IL). Approximate 5 mg protein powder was

accurately weighed and sealed in a high-volume stainless-steel pan. The sample was heated from 20 to 250 °C at a heating rate of 10 °C /min in an inert environment using nitrogen with the gas flow at 50 mL/min. Protein denaturation temperature and enthalpy were calculated using Universal Analysis 2000 software.

2.2.13 Statistical analysis

All the tests were conducted in at least duplicate. Data were analyzed using SAS statistical software, version 9.4 (SAS Institute, Cary, NC, USA). Results were evaluated by one-way ANOVA. Tukey's post-hoc test was used to assess the significant differences among individual data set. The results were presented as means ± standard deviation (SD), and $p < 0.05$ was considered as the significant level.

2.3 Results and Discussions

2.3.1 Protein physical and compositional characteristics

Moisture content of the protein powders obtained through different drying methods was not significantly different (13 – 14%) (Table 1). Protein content ranged from 83.2 to 86.2%. The freeze- and vacuum-dried quinoa proteins had a slightly higher protein content than that from the spray drying, which was caused by the loss of some protein fractions during the later process. Spray-dried proteins had significantly smaller and finer particle sizes (10.43μm) than that from freeze-dried (44.24μm) and vacuum-dried proteins (38.25μm). The freeze- and vacuum-dried proteins required further grinding after drying to reduce the particle size, which determined the average powder particle size and size distribution (Yu, Ahmedna, & Goktepe, 2007). Protein particle size distribution is shown in Figure 1, and AFM images are presented in Figure S1 (Supplementary Document). The spray-dried protein powders exhibited narrower and more

uniform distribution, while the freeze-dried and vacuum-dried proteins showed similar distribution patterns (Figure 1).

Color characteristics of the protein powders were significantly different ($p< 0.05$) (Table 1). Freeze- and vacuum-dried protein powders showed brownish color, while spray-dried proteins showed creamy white color (Figure S2, Supplementary Document). Based on the lightness (L*) values, spray-dried protein was the lightest, while the vacuum-dried protein was the darkest. This result was in agreement with a previous study of chia seed protein isolates (Timilsena et al., 2016). The color properties were determined by the intrinsic characteristics of proteins, protein purity and pigment contamination, particle sizes, etc. Shaviklo et al. (2010) reported that high speed homogenization before spray drying may disrupted the protein tissue and can lead to removal of some pigments.

Aspartic acid, glutamic acid and leucine are the most abundant amino acids in quinoa proteins (Table S1, Supplementary Document). Compared with most cereal proteins, such as wheat, barley or sorghum, quinoa protein possesses higher amount of lysine. Amino acid compositions of the quinoa proteins from different drying methods were mostly similar except for alanine and glycine, which were higher in freeze-dried proteins compared with the other two proteins (Table S1). This could be attributed to the partial thermal degradation of these amino acids during the drying involving intensive heating (Abugoch, 2009). Feyzi et al. (2018) and Uribe et al. (2019) also found that amino acid profiles of green seaweed and fenugreek proteins were significantly different from different drying methods. The freeze- dried green seaweed had significantly higher glycine and alanine content than vacuum- and spray-dried samples, which indicated that the amino acids were more susceptible to drying technologies and could be lost, changed or destroyed during processing (Uribe et al., 2019). In addition, extraction methods

(Abugoch et al., 2008) and cultivate varieties (Steffolani et al., 2016) could also affect the amino acid compositions of proteins.

2.3.2 Functional properties

Solubility

Protein solubility depends on its hydrophilic-lipophilic balance and the thermodynamics of its interaction with water (Ghribi et al., 2015). As expected, minimal solubility was observed for quinoa proteins from all the drying methods at the isoelectric point around pH 4.5, and solubility increased when the pH was further increased or decreased beyond the isoelectric point (Figure 2A) (Tang, 2007; Zhao et al., 2013). The freeze- and spray-dried quinoa proteins possessed similar solubility trend from pH 3 to 11. The vacuum-dried protein showed much lower solubility when the pH was above 6, but higher solubility when the pH was below 6. The maximal solubility values (pH 11) of the proteins from freeze drying, spray drying, and vacuum drying were 93.7, 95.3 and 61.3%, respectively. The lower solubility of vacuum-dried protein could be due to more severe protein denaturation during the drying process (Ghribi et al., 2015). Vacuum drying may promote hydrophobic interchange reaction among the protein molecules and film formation on the solution surface, resulted in protein aggregation (Ghribi et al., 2015). Zidani et al. (2012) reported that the vacuum drying allows the water vaporization at low temperature (below 25 ºC) and heat transfer occurred by conduction and radiation. It is not advantageous for vacuum drying to work at higher temperature, since the solubility would be decreased. Higher solubility of freeze-dried protein was attributed to less protein denaturation during the process. Approximately 90% of water was removed as a vapor causing minimum salts or carbohydrates migration to the drying surface, thus the interactions were reduced between components and solubility was minimally affected. The spray drying process had less extent of

denaturation than vacuum-dried protein, since the outlet temperature (60 ºC) was lower than denaturation temperature (191.4 ºC), and the process of spray drying was fast and the residence time of protein inside the drying chamber was very short (Timilsena et al., 2016).

Oil/water absorption capacity

Oil absorption capacity (OAC) indicates the ability of protein to absorb and retain fat, and water absorption capacity (WAC) is a critical attribute in determining water retention functionality, swelling, solubility and gelation properties of proteins, both of which affect food texture and quality (Foegeding & Davis, 2011). The freeze-dried protein had significantly higher OAC than spray- and vacuum-dried proteins (Table 1). Oil absorption capacity is related to the amount of exposed hydrophobic amino acid residues in the protein and hydrophobic amino acid content. Freeze-dried protein possessed significantly higher surface hydrophobicity than the proteins from the other two drying methods (Table 2), which contributed to the highest oil absorption capacity of freeze-dried proteins.

The WAC of quinoa protein was dependent on protein drying methods and pH values (Table 1). Relatively lower WAC values were observed when the pH was close to the isoelectric point of the protein. When pH values increased from 5 to 7, the WAC gradually increased as well, which was related to the alteration of the electrical charge distribution and net charge values with the pH. At pH 8, freeze-dried quinoa proteins had the lowest WAC, while vacuum-dried protein had the highest WAC compared to that at other pH values. Overall, spray-dried protein exhibited relatively better WAC than the proteins from the other two methods, which may be attributed to its finer particle size and larger specific surface area (Ragab, Babiker, & Eltinay, 2004; Yu et al., 2007). Water absorption capacity is related to the swelling phenomenon of hydrated protein matrix. Thus, changes of protein conformation with increasing binding sites

under high temperature could lead to better WAC functionality (Zayas, 1997). Our result was in agreement with Yu et al. (2007), but different from Timilsena et al. (2016), who indicated that spray-dried chia seed protein isolate had no significant differences of WAC from vacuum-dried proteins. This is because the water absorption behavior of protein can be affected by protein sources and structures, testing pH, and other constituents in the materials, such as residue polysaccharides (Ragab et al., 2004; Steffolani et al., 2016).

Emulsifying activity and stability

Emulsifying properties of proteins, such as emulsifying capacity (EC) and emulsifying stability (ES), are useful functional properties and play a critical role in food applications. The EC is the ability to absorb oil and water at the interfacial area to form emulsion, which depends on the size, shape, charge, composition, and hydrophobicity of protein molecules (Ragab et al., 2004). The ES is related the stability of emulsion over a certain time under specific conditions, and it depends on the magnitude of these interactions (Karaca, Low, & Nickerson, 2011; Ma et al., 2011). The EC of quinoa proteins ranged from 14.3 to 61.0% (Table 1), and the vacuum-dried protein had significantly lower EC compared to the proteins from other drying methods. The oil-water interface is dominated by hydrophobic interactions, and exposure of the non-polar hydrophobic residues at the interface greatly affects the emulsifying properties. Relatively higher surface hydrophobicity can lead to stronger binding between emulsifier and oil droplet and better emulsifying properties of the protein (Gong et al., 2016). The lower emulsifying properties of vacuum-dried protein could also be attributed to the lower solubility. The EC significantly increased with pH for all the proteins. When the pH was close to the isoelectric point (pH=5), electrostatic repulsion among the molecules was the lowest, which resulted in protein aggregation, and lower solubility and emulsifying properties. When the pH value is higher than

the isoelectric point, the protein molecules had a net negative charge which greatly enhanced protein-water interaction and resulted higher solubility, therefore, the EC was increased. The ES ranged from 6.0 to 58.0% (Table 1). The freeze-dried quinoa protein had relatively higher ES than spray-dried protein, and the vacuum-dried protein had the lowest ES at all pH values. The better emulsifying properties of the freeze-dried protein may be attributed to its higher surface hydrophobicity and favorable dissociation at oil and water interfaces (Ghribi et al., 2015). Zhao et al. (2013) and Liu et al. (2019) reported that spray-dried rice dreg and peanut proteins had higher emulsion capacity than freeze-dried proteins due to the smaller particle size and higher solubility, but freeze-dried protein had higher emulsion stability than spray-dried protein.

Foaming capacity and stability

The foaming properties including foam capacity (FC) and foam stability (FS) are important functionality that is utilized for aeration and whipping purpose in food industry. Foam formation is dependent on the interfacial film that is formed by the proteins and its ability to keep the air bubble in the suspension and slow down the coalescence rate (Ghribi et al., 2015; Ma et al., 2011). The foaming properties of quinoa proteins made from different drying methods at pH 5-8 are shown in Figure 2B. As pH increasing, the FC (i.e., defined as the value at 0 min) increased for both freeze- and spray-dried proteins. In contrast, the vacuum-dried protein showed the highest FC at pH 6 and the lowest FC at pH 8. The spray- and freeze-dried proteins had similar foaming stability (20 to 90 min). The vacuum-dried protein had similar FS as freeze and spray dried proteins at pH 7-8, but much lower stability at pH 5. The lower FC observed around isoelectric point is attributed to the low protein solubility. The increased FC at pH value higher than 5 could be explained by the increased protein solubility due to the increase in the net charge of protein in the aqueous dispersion. The increased repulsive force among the charged molecules

decreased protein aggregation and reduced the coalescence of air bubbles (Timilsena et al., 2016). Protein with smaller particle size, such as that from spray drying, could be more rapidly absorbed during whipping to generate more foams (Zhao et al. (2013)). Protein needs to be adequately unfolded and molecularly flexible in order to form interfacial membranes around the air bubbles (Aluko & Monu, 2003). Aluko et al. (2003) reported that enzymatically hydrolyzed quinoa protein possessed better foaming capacity, because hydrolysis reduced the molecular size and increased the flexibility of the protein to form interfacial membranes.

Gel rheology

Shear storage modulus (G') and loss modulus (G") of heat-induced quinoa protein gels are shown in Figure 3. The freeze-dried quinoa protein exhibited much higher G' and G" compared with that from spray and vacuum drying. The G´ of the freeze-dried protein was higher than G", implying the formation of stronger gels with better resistance to stress-induced rupture. The spray- and vacuum-dried protein gels had similar range of G' and G", indicating weaker gelation properties of the proteins from these two drying methods. The freeze-dried protein was less denatured during processing compared with the other two proteins, and most of the intrinsic properties were retained with higher solubility, which favored protein interaction and aggregation during the heating process to form gel networks (Ruiz et al., 2016). On the other side, the vacuum-dried protein could hardly form gels, and the G' and G" values were low. This result was in agreement with Joshi et al. (2011), and they found that vacuum-dried lentil protein could not form gels as rapidly as freeze-dried lentil protein because of the poor protein solubility. Therefore, higher concentration or longer heating time were required for the vacuum-dried proteins to form a gel.

2.3.3 SDS-PAGE

SDS-PAGE profiles (non-reducing and reducing) of the quinoa proteins are shown in Figure 4. Quinoa proteins are comprised of albumin and globular chenopedin proteins, which exhibited a complex band profile. The globular chenopedin has a hexamer structure and consists of six pairs of basic and acidic polypeptides with molecular weight of 22-23 kDa and 32-39 kDa, respectively, linked by single disulfide bond (Dakhili, Abdolalizadeh, Hosseini, Shojaee-Aliabadi, & Mirmoghtadaie, 2019; Ruiz et al., 2016). Under non-reducing condition, more intensive bands were observed for the freeze-dried protein, while much weaker bands were noticed for the vacuum-dried protein, and band intensity for spray-dried protein was in between. This result was expected because the freeze drying is the mildest drying process with the lowest temperature among the three methods evaluated. The vacuum drying process requires much longer time, though at a lower temperature, than the spray drying, which still caused severe protein denaturation, aggregation, and crosslinking. This reduced protein solubility, leading to very weak bands in the SDS-PAGE profile. This observation was in consistent with the solubility results, which showed that the vacuum-dried quinoa protein had the lowest solubility than freeze- and spray-dried proteins. With breakdown of intramolecular disulfide bonds and unfolding of protein molecules by the reducing agent, the band of higher molecular weight disappeared and several new bands at lower molecular weight range appeared in the reduced SDS-PAGE profile (Timilsena et al. 2016).

2.3.4 Sulfhydryl/ disulfide content

The content of free sulfhydryl group (SH) as well as disulfide bond (SS) of the quinoa proteins are presented in Table 2. The free SH content of freeze- and spray-dried proteins was significantly higher than that of vacuum-dried protein, indicating more intensive oxidation of

free SH to form disulfide bonds during vacuum drying (Visschers & Jongh, 2005). The total SH and SS were not significantly different among all the drying methods, ranging from 40.1 to 49.0 nmol/mg and 15.8 to 18.4 nmol/mg, respectively. Zhao et al. (2013) reported that spray drying led to higher protein denaturation than freeze drying, although there was no significant differences of free SH content for the freeze- and spray-dried rice dreg protein isolate. Gong et al. (2016) indicated that the free SH content of freeze- and spray-dried peanut protein was not significantly different, but the freeze-dried peanut protein had relatively higher SS content. This is in accordance with changes in protein hydrophobicity, which suggested that the higher amount of exposed hydrophobic groups would increase the formation of disulfide bond from adjacent free SH groups, and this interchange reaction may lead to the different extent of aggregation (Gong et al., 2016).

2.3.5 Secondary structure

The secondary structure composition of quinoa proteins derived from the amide I band (1600-1700 cm^{-1}) is summarized in Table 2. The quinoa proteins prepared from different dry methods exhibited significant differences in secondary structures. The freeze-dried protein possessed higher amount of random coil than β-sheet and β-turn, while no random coil structure was observed in the spray-dried protein. The spray drying process altered the protein secondary structure and promoted protein assembly into more regular β-sheet and α-helix structures. Zhao et al. (2013) reported that the spray-dried rice dreg protein had higher β-sheet than the freeze-dried protein, which is similar to our result. This is probably because more β-sheet structure could be formed in aggregated protein molecules. However, (Gong et al., 2016) reported that the freeze-dried peanut protein had higher β-sheet than the spray-dried protein, while the spray-dried peanut protein exhibited higher α-helix, and they explained that this was attributed to the

shrinkage of droplet during spray drying and related to the concentration of solute during freeze drying process (Gong et al., 2016). Overall, it seems that protein secondary structure composition is influenced by both protein types and drying methods.

2.3.6 Surface hydrophobicity

Protein surface hydrophobicity is related to the amount and type of hydrophobic amino acid residues exposed at the surface of the protein and affected by protein unfolding and denaturation (Timilsena et al., 2016). The surface hydrophobicity could also influence intermolecular protein-protein and protein-lipid interactions and determines protein surface activities that are important to functional properties such as solubility, emulsification, and foaming. The differences in surface hydrophobicity of all the quinoa proteins could be attributed to the degree of denaturation of the proteins during different drying processes. The freeze-dried protein exhibited the highest surface hydrophobicity, while that of the vacuum-dried protein was the lowest (Table 2). This could be attributed to a limited extent of denaturation occurred in the freeze-dried quinoa protein by exposing hydrophobic regions; while the vacuum-dried protein had more intensive denaturation due to the hydrophobic interchange reaction among the protein molecules, and it was also related to the film formation on the protein surface, and resulted in protein aggregation (Hu et al., 2010). This observation agreed with the higher oil absorption capacity for the freeze-dried protein compared to the spray- and vacuum-dried proteins (Table 1). Gong et al. (2016) and Mune & Sogi (2016) also found that freeze-dried peanut protein, cowpea and bambara bean proteins exhibited higher hydrophobicity than the spray- and vacuum-dried proteins. However, another study found that vacuum-dried fenugreek protein had the highest surface hydrophobicity, followed by spray-dried and freeze-dried proteins (Feyzi et al., 2018).

2.3.7 Thermal properties

DSC thermograms are presented in Figure S3, and denaturation temperatures (T_d), aggregation temperature (T_a), and phase transition enthalpy (ΔH_a and ΔH_d) are summarized in Table 2. During the first scan from 20 to 250 °C, one major endothermic peak was observed for all the three proteins with peak temperatures around 190 to 220 °C, which was attributed to protein denaturation. Freeze-dried protein exhibited two additional endothermic denaturation peaks at 131 and 183 °C. These multiple denaturation peaks were caused by the complex composition of quinoa albumin and globular chenopedin proteins (Dakhili et al., 2019; Ruiz et al., 2016). The two denaturation peaks at lower temperatures were not shown for spray- and vacuum-dried proteins, which is probably because the proteins were partially denatured during drying. However, there was a significant exothermic peak for the spray- and vacuum-dried proteins at 148 and 172 °C, respectively, and this is because of protein aggregation during heating (Goyal, Chaudhuri, & Kuwajima, 2014). Ruiz et al. (2016) reported that extraction methods could affect the denaturation temperature. They found that there was one endothermic peak for the protein extracted at pH 8 to 10; however, no endothermic peak was found when the protein was extracted at 11, due to protein denaturation during extraction. No endothermic or exothermic peaks were observed during the second DSC heating scan of all the three proteins (Figure S3), indicating that the protein denaturation and aggregation transitions are non-reversable.

2.4. Conclusion

In this study, quinoa proteins were prepared using freeze drying, spray drying and vacuum drying methods and systematically characterized side by side. The color, protein content, and particle size of freeze- and vacuum-dried proteins were similar, while the spray-dried protein had

significantly finer particles, lighter color, and lower protein content. The freeze-dried protein was less denatured during processing and exhibited better functional properties than the spray- and vacuum-dried proteins. The protein from freeze drying method had the highest emulsification capacity and stability as well as oil absorption capacity due to its higher surface hydrophobicity. Gels prepared from the freeze-dried protein had higher elastic and viscous modulus than that from spray- and vacuum-dried proteins. Conclusions from functional properties were well supported by protein structural features from SDS-PAGE, sulfhydryl and disulfide analysis, secondary structure, surface hydrophobicity, and thermal characterization. Overall, quinoa protein demonstrated good functional properties. This study provides useful guidance for the industry to optimize protein production and will benefit their applications as a new protein ingredient.

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Conflict of interest

None.

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Figures and tables:

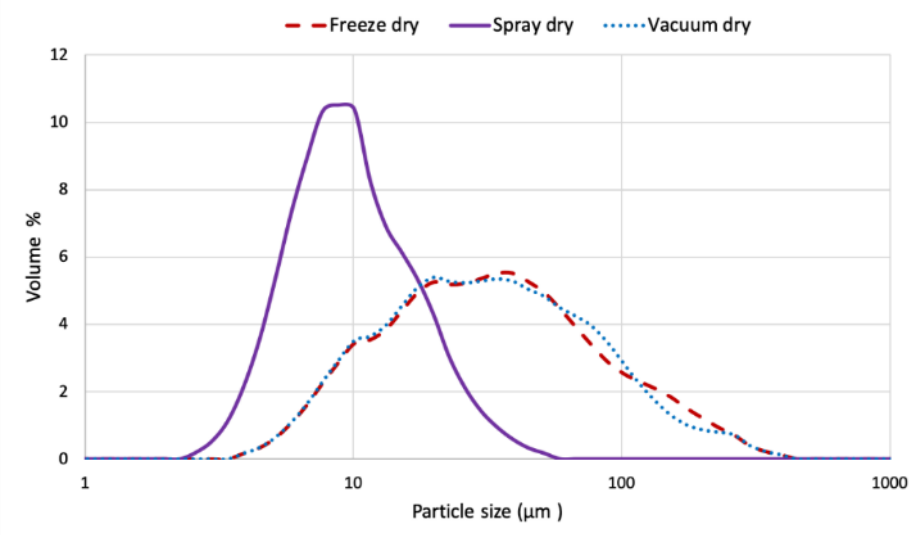

Figure 2.1 Particle size distribution of quinoa proteins from different drying methods.

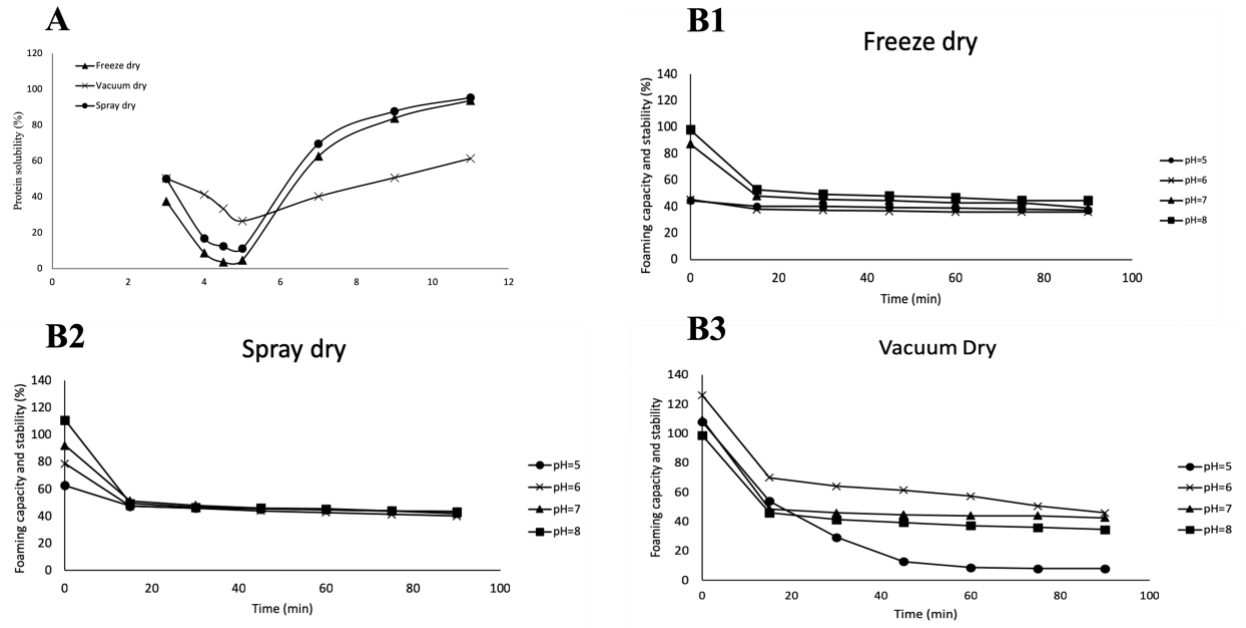

Figure 2.2 Solubility (A) and foaming properties (B1-B3) of quinoa proteins from different drying methods.

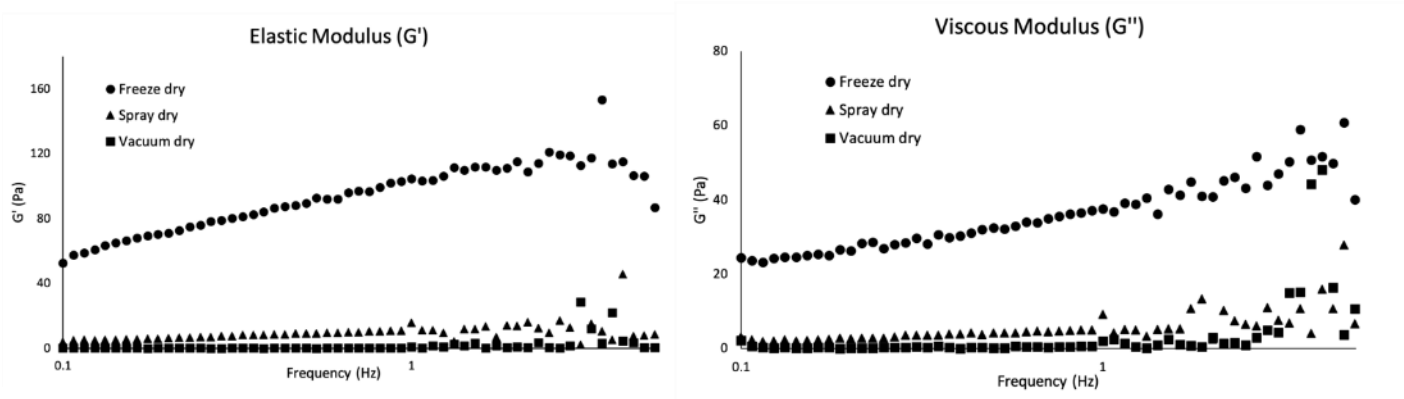

Figure 2.3 Rheological properties (G´ and G´´) of quinoa protein gels.

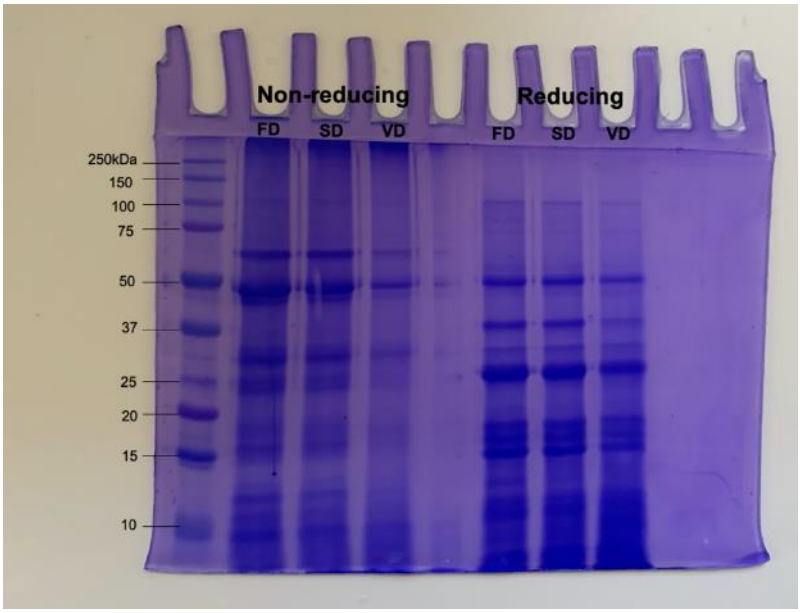

Figure 2.4 SDS-PAGE of quinoa proteins from different drying methods under non-reducing and reducing conditions.

Lane 1-molecular weight marker; FD-freeze dry, SD- spray dry, VD- vacuum dry.

Table 2-1 Physical and functional properties of quinoa proteins from different drying methods.

Physical property	**Freeze dry**	**Spray dry**	**Vacuum dry**
L*	52.44 ± 1.11^{b}	77.60 ± 0.98^{a}	48.86 ± 0.65^{c}
a*	2.70 ± 0.17^{a}	0.93 ± 0.09^{b}	2.78 ± 0.22^{a}
b*	11.72 ± 0.37^{a}	8.41 ± 0.18^{b}	12.20 ± 0.17^{a}
Moisture content (%)	13.32 ± 0.45^{a}	14.11 ± 0.54^{a}	13.58 ± 0.09^{a}
Protein content (%)	86.19 ± 0.18^{a}	83.22 ± 0.13^{b}	86.13 ± 0.44^{a}
Mean particle size (μm)	44.24 ± 3.64^{a}	10.43 ± 0.23^{b}	38.25 ± 6.00^{a}
Functional properties			
Oil absorption capacity (g oil/g protein) pH 7	3.19 ± 0.01^{a}	1.19 ± 0.05^{b}	0.94 ± 0.04^{c}
Water absorption capacity (g H_2O/g protein)			
pH 5	1.43 ± 0.13^{de}	1.43 ± 0.06^{de}	1.38 ± 0.11^{de}
pH 6	1.52 ± 0.21^{cde}	2.03 ± 0.25^{b}	1.29 ± 0.00^{e}
pH 7	1.84 ± 0.07^{bcd}	2.76 ± 0.12^{a}	1.46 ± 0.03^{cde}
pH 8	1.26 ± 0.41^{e}	1.94 ± 0.10^{bc}	1.55 ± 0.05^{bcde}
Emulsion capacity (%)			
pH 5	40.6 ± 1.2^{cd}	39.5 ± 1.0^{cd}	14.3 ± 1.4^{e}
pH 6	44.7 ± 1.0^{c}	40.7 ± 0.8^{cd}	35.0 ± 1.1^{d}
pH 7	56.6 ± 5.0^{ab}	51.6 ± 2.9^{b}	41.5 ± 1.4^{c}
pH 8	61.0 ± 4.4^{a}	59.3 ± 3.9^{a}	44.4 ± 1.5^{c}
Emulsion stability (%)			
pH 5	30.2 ± 3.3^{ef}	17.3 ± 1.0^{h}	6.0 ± 0.7^{i}
pH 6	42.0 ± 1.2^{cd}	37.7 ± 1.8^{de}	19.8 ± 2.2^{gh}
pH 7	51.9 ± 5.0^{ab}	48.0 ± 5.8^{bc}	25.4 ± 3.3^{fg}
pH 8	58.0 ± 3.5^{a}	55.4 ± 3.3^{ab}	24.4 ± 2.0^{fgh}

*Means with different letters within each attribute denote significant differences ($p< 0.05$).

Table 2-2 Structural and thermal properties of quinoa proteins from different drying methods.

Property	Freeze dry	Spray dry	Vacuum dry
Secondary structure			
β-sheet (%)	16.1±1.2^{b}	34.1±2.9^{a}	28.9±7.8^{a}
random coil (%)	51.3±3.2^{a}	0	31.1±3.2^{b}
α-helix (%)	16.0±1.2^{c}	52.5±2.9^{a}	26.2±5.2^{b}
β-turn (%)	12.3±3.1^{a}	13.5±0.3^{a}	11.9±5.3^{a}
Relative surface hydrophobicity (H_0)	360,937±11,426^{a}	293,106±3,721^{b}	32,915±1,538^{c}
Free SH (nmol/mg)	11.3±0.9^{a}	13.1±0.0^{a}	7.9±0.1^{b}
Total SH (nmol/mg)	49.0±7.4^{a}	44.5±0.3^{a}	40.1±2.5^{a}
S-S (nmol/mg)	18.4±3.1^{a}	15.8±0.0^{a}	16.1±1.3^{a}
T_d (°C)	131.2±0.1 183.2±0.3 220.4±0.0^{a}	/ / 191.4±2.6^{c}	/ / 208.3±0.2^{b}
ΔH_d (J/g)	3.4±0.1 1.1±0.1 8.5±0.5^{c}	/ / 38.8±1.3^{a}	/ / 25.1±0.1^{b}
T_a (°C)	/	147.7±1.9^{b}	172.0±1.2^{a}
ΔH_a (J/g)	/	17.9±0.2^{a}	18.7±1.5^{a}

*Means with different letters within each property denote significant differences ($p < 0.05$).

Chapter 3 - Acylation modification and/ or guar gum conjugation enhanced functional properties of pea protein isolate[2]

Abstract

There has been an increasing demand for diverse and more functional plant-based protein ingredients for food uses. This study aims to improve the functional properties of pea protein isolate through acylation or/and conjugation with guar gum and investigate the physicochemical characteristics of the modified proteins. Acylated pea proteins were prepared by reacting with acetic anhydride (AA) or succinic anhydride (SA) at 0.3 or 0.6 g of AA or SA per g protein, respectively. Guar gum-pea protein conjugates were prepared by incubating the mixture at a mass ratio of 1:20 and 1:40 at 60 °C for 24 hours, respectively. Acylated-guar gum-conjugated pea proteins were also prepared to investigate their synergistic effects. Both conjugated and acylated pea proteins showed significantly improved oil holding capacity of up to 2.20±0.05 and 2.09±0.03 g oil/g protein, respectively, compared to the unmodified protein (1.03±0.02 g oil/g). The acylated pea protein also had greater water holding capacity of up to 7.01±0.31 g water /g protein compared to the unmodified protein (3.57±0.05 g water/g). Emulsion capacity and stability were improved up to 96 - 100 % and 95 - 100 %, respectively, for the modified proteins (e.g., 1:20 conj., SA0.3/0.6, AA 0.3/0.6 conj., SA 0.3/0.6 conj.). The suspensions prepared with 9% acetylated pea protein formed firm gels. Sequential acylation and conjugation of pea proteins demonstrated more beneficial and synergistic effects on the water holding capacity and emulsifying properties. However, the *in vitro* gastrointestinal digestibility of the modified pea

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proteins decreased compared to that of the control pea protein. Overall, the acylated and conjugated pea proteins possessed superior functional properties that could be used as novel food ingredients in meat alternative or beverage applications.

Key words:

Pea protein isolate, protein modification, conjugation, acylation, functional properties, plant-based protein.

3.1 Introduction

There has been an increasing demand for plant-based proteins worldwide (Boye, Zare, & Pletch, 2010; Lin et al., 2017). Pea (*Pisum sativum L.*) is one of the most widely cultivated pulse legumes in the world, and has been utilized in human's diet for thousands of years. Pea protein has significant nutritional advantages such as providing essential amino acids and being associated with health benefits such as reduction of LDL (low density lipoprotein) cholesterol (Rigamonti et al., 2010), anti-inflammatory activity (Ndiaye, Vuong, Duarte, Aluko, & Matar, 2012), modulating intestinal bacterial activities (Światecka, Światecki, Kostyra, Marciniak-Darmochwa F, & Kostyra, 2010). Pea protein has been used to produce bioactive peptides with both antioxidant activity and angiotensin I-converting enzyme inhibitor activity (Roy, Boye, & Simpson, 2010). Additionally, pea protein hydrolysates showed beneficial effects on lowering blood pressure (Li et al., 2011). Pea protein has gained great attention in the food and beverages industries as a potentially alterative protein to animal protein for human foods.

Pea contains 20-25% protein, and pea protein contains many essential amino acids, especially that it is rich in lysine, approximately 6.3 g/100 g protein in raw pea (Khattab, Arntfield, & Nyachoti, 2009). Legumin (11S protein) and vicilin (7S protein) are the two major globulin proteins in pea (Burger & Zhang, 2019; Tamnak, Mirhosseini, Tan, Ghazali, & Muhammad, 2016). So far, the utilization of pea protein as a food ingredient is still very limited, partially due to their less-desirable functionalities. For example, pea protein contains high percentage of globulin fraction (49-81%) (salt soluble protein), which showed low solubility in aqueous food system (Tzitzikas, Vincken, De Groot, Gruppen, & Visser, 2006). Commercial pea protein is commonly subjected to harsh processing conditions, which may lead to protein denaturation and further reduce protein solubility (Tamnak et al., 2016). Other functionalities

that are associated with solubility may also be impaired, such as water holding capacity, foaming capacity/stability, and emulsifying capacity/stability.

To overcome these limitations, previous studies have been conducted to improve pea protein functional properties through chemical modifications. Conjugation between protein and polysaccharide is a popular modification approach, which builds chemical linkages between the protein and polysaccharide via the condensation of carbonyl and ε-amino group at the initial stage of Maillard reaction (Burger & Zhang, 2019). The conjugation reaction enables the protein to be covalently linked with hydrophilic polysaccharide, which enhances protein solubility and emulsifying properties (Guo, Su, Yuan, Mao, & Gao, 2019; Tamnak et al., 2016). Pea protein conjugated with gum Arabic showed improved solubility as well as emulsifying properties (Zha et al., 2019b). Additionally, the conjugation reaction mitigated the beany flavor of pea protein. Other studies also showed that pea protein conjugated with propylene glycol alginate (Guo et al., 2019) and pectin (Tamnak et al., 2016) had significantly improved functional properties.

Besides protein-polysaccharide conjugation, acylation is another chemical modification method that has been studied. Succinic anhydride and acetic anhydride are commonly used in the acylation modification of proteins. Acylation is a nucleophilic substitution reaction between acylating agents (e.g., succinic/acetic anhydride) with protein amino acid residues (particularly lysine), resulting in improved functional properties. A previous study demonstrated that acetylation and succinylation of pea protein improved emulsifying properties, foaming, and water holding capacity (Johnson & Brekke, 1983). Acylation modification has also been employed on other proteins, such as faba bean (Jens-Peter Krause, Ralf Mothes, & Schwenke, 1996), chickpea (Liu & Hung, 2008), and mung bean (El-Adawy, 2000).

Guar gum is derived from endosperm of *Cyamopsis tetragonoloba*, and it is a water soluble polysaccharide (Hamdani, Wani, Bhat, & Siddiqi, 2018). Guar gum is widely used in the food industry due to its excellent water absorption and stabilizing and thickening properties (Karaman, Kesler, Goksel, Dogan, & Kayacier, 2014). This study aims to improve pea protein functional properties in terms of water/oil holding capacity, foaming and emulsion properties, gelation, and solubility through acylation or/and conjugation with guar gum and understand the physicochemical characteristics and *in vitro* gastrointestinal (GI) digestibility of the modified proteins.

3.2 Materials and methods

3.2.1 Materials

Pea protein (83% protein content) was supplied by Roquette (Geneva, IL, USA). Guar gum (DeJa' GF Foods, Plain City, OH, USA) and soybean oil were purchased from Amazon. Acetic, succinic anhydrides, 8-anilinonaphthalene-1-sulfonic acid (ANS), β-mercaptoethanol, and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

3.2.2 Preparation of modified pea protein

Acylated pea proteins were prepared by reacting the protein with acetic anhydride (AA) or succinic anhydride (SA) at 0.3 or 0.6 g of AA or SA per g protein in distilled water at 10 wt% protein concentration, respectively. The protein slurry was adjusted to pH 8 using 5 M NaOH and mixed for 1 hour at room temperature to allow reaction. After that, the sample was transferred into a dialysis bag (3500 MW cut-off, Thermo Fisher Scientific, Waltham, MA, USA) for dialysis against distilled water at 4 °C for 48 hours to remove the residuals of acetic and succinic acids and salts. The distilled water used during the dialysis was changed every 10

hours. Then, the modified protein dispersion was lyophilized. All the dried protein powders were kept at 4 °C till further analysis.

The guar gum-pea conjugates were prepared through a wet heating Maillard reaction. Mixture of guar gum and pea protein (1:20 or 1:40 weight ratio) or acylated pea protein (1:20) was dispersed in distilled water at 10 wt% concentration, respectively. The mixture was mixed for 15 min at room temperature and then incubated in a water bath at 60 °C with continuous mixing for 24 hours. After that, the sample was lyophilized. All the dried protein powders were kept at 4 °C till further analysis.

3.2.3 Functional properties

Protein functional properties including solubility, water holding capacity, oil holding capacity, and foaming capacity and stability were measured following our previous methods (Shen et al., 2021) without modification. Emulsion capacity and stability were evaluated similarly to Shen et al. (2021), except that 1.0 g protein was dispersed in 50 mL 50:50 mixture of distilled water and soybean oil, instead of using 1.75 g protein.

The least gelation concentration (LGC) of pea proteins was evaluated following a previous method (Ogunwolu, Henshaw, Mock, Santros, & Awonorin, 2009) with minor modifications. The protein was added into 10 mL distilled water in 15 mL centrifuge tubes and thoroughly mixed to obtain a concentration from 2 to 20% (w/v). The protein suspension was heated at 100 °C for 1 hour, cooled under running cold tap water, and refrigerated at 4 °C for 2 hours. The LGC was considered as the concentration of protein dispersion that would not fall when the centrifuge tube was inverted.

3.2.4 Browning reaction during protein conjugation

The measurement of browning reaction was conducted following our previous method (Shen, Chen, & Li, 2018). UV absorbances at 304 and 420 nm are considered as an indicator of the Amadori compound (Wang & Ismail, 2012) and melanoidin (Martinez-Alvarenga et al., 2014) formation in protein-carbohydrate conjugates. The conjugated pea protein (50 mg) was dispersed in 4 mL distilled water in a centrifuge tube, which was vortexed for 10 seconds and further vigorously mixed for 30 min. After that, the dispersion was centrifuged at 10,000 xg for 10 min. The supernatant was obtained and analyzed using a double beam spectrophotometer (VWR UV-6300PC, VWR International, Radnor, PA, USA) at 304 and 420 nm.

3.2.5 Free amino group

Free amino group content of the modified pea proteins was measured following a previous method (Zha et al., 2019). One milliliter of protein sample solution (5 mg/mL) was added with 1 mL of 4% $NaHCO_3$ and 1 mL of 0.1% TNBS (2,4,6-trinitrobenzene sulfonic acid) in a centrifuge tube. The mixture was incubated in a water bath at 40 °C for 2 hours. After that, 1 mL of 10% (w/v) sodium dodecyl sulphate (SDS) was added to the mixture to solubilize the protein. Finally, the reaction was terminated by adding 0.5 mL 1 N HCl. The protein mixture was cooled at room temperature for 15 min, and absorbance at 340 nm was measured using the double beam spectrophotometer (VWR UV-6300PC). L-leucine was used as a standard to establish the calibration curve.

3.2.6 Surface hydrophobicity and Fourier transform infrared spectroscopy (FTIR)

Surface hydrophobicity information and FTIR spectra of the modified pea proteins were collected according to our previous method without modification (Shen et al., 2021).

3.2.7 Circular dichroism (CD) spectroscopy

Secondary structures of pea proteins were determined by using a Jasco J-815 circular dichroism spectrophotometer (Jasco Analytical Instruments, Easton, MD). The protein sample was dissolved in distilled water, which was further diluted to a certain concentration that could fit into the scanning regions. The protein solution was scanned from 190 to 250 nm. The following parameters were used: step interval 1nm, acquisition duration 50 nm/min, and bandwidth 0.5 μm. The data were recorded and corrected by subtracting the water blank. The data of protein secondary structure was estimated using BeStSel (Micsonai et al., 2018).

3.2.8 Sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) electrophoresis

SDS-PAGE of the modified proteins under reducing condition was performed according to our previous method (Chen et al., 2019), except that the protein sample (5 mg/mL) was extracted using 1% SDS/sodium phosphate buffer (pH 7.0) with 2% β-mercaptoethanol, instead of deionized water.

3.2.9 Free sulfhydryl (SH) content

The measurement of free SH groups was conducted following the method from a literature (Lagrain, Brijs, Veraverbeke, & Delcour, 2005). Protein solution (5 mg/mL) was prepared by dissolving the protein in 0.05 M sodium phosphate sample buffer (pH 6.5), which consisted of 2% SDS (v/v), 3.0 M urea, and 1.0 mM tetrasodium ethylenediamine tetraacetate. Five mL of the prepared solution was added with 500 μL of 0.1% (w/v) DTNB Ellman's reagent (5,5-dithio-bis-(2-nitrobenzoic acid), followed by mixing vigorously for 45 min, and centrifugation at 10,000 xg for 3 min. The absorbance was measured at 412 nm using the spectrophotometer (VWR UV-6300PC). Glutathione was used as a standard to establish the calibration curve.

3.2.10 *In vitro* gastrointestinal digestibility

In vitro digestibility of the proteins were determined following a simulated gastric and intestinal digestion method from literature (Wen, Li, Gu, Wang, & Wang, 2019; Wu, Taylor, Nebl, Ng, & Bennett, 2017) with some modifications. Briefly, 50 mg of protein was first dispersed in 20 mL of simulated gastric fluid solution, which contained 2.5 mM $CaCl_2$, 35 mM NaCl, and pepsin (182 U/ mg protein). The protein solution was acidified with HCl to pH 2, and digestion was continued at 37 °C for 1 hour in a water bath shaker. *In vitro* intestinal digestion was then carried out by adding 4 mL of simulated intestinal fluid containing 7.6 mM $CaCl_2$, 20.3 mM Tris, 7.4 mM bile salts, trypsin (40 U/mg protein), and chymotrypsin (0.5 U/mg protein) to the protein solution after the 1 hour gastric digestion. The pH of the protein solution was adjusted to 7 before incubating the sample in the water bath shaker for 2 hours. The digestion was stopped by heating the solution in boiling water for 5 min, cooled down, and centrifugated at 3780 xg for 5 min. The supernatant was diluted with 100 mM sodium bicarbonate (1: 200, v/v), which was further mixed with OPA reagent (100 mM sodium tetraborate, 0.01% SDS, 0.05 mg/mL OPA, and 0.05 mg/mL DTT) (1:50, v/v). Finally, 200 µL of the solution was added in a 96-well plate, and the fluorescence was determined using a plate reader (excitation at 340 nm, emission at 450 nm) (BioTek, Synergy H1 Hybrid, Highland Park, Winooski, VT, USA). L-Leucine was used to establish a calibration curve. The DH% (degree of hydrolysis) was calculated according to the literature (Wen, Li, Gu, Wang, & Wang, 2019) with h_{total} factor of 7.8 based on soy (Nelsen et al., 2001).

3.2.11 Statistical analysis

All the experiments were carried out in at least two replicates. Kruskal-Waillis non-parametric test and Conover-Iman procedure were used to analyze the specific sample pairs for

stochastic dominance ($p < 0.05$) among the treatments using Python 3.6 package scipy.stats (Python code and example are available in the Supplementary Document). The results are presented as mean ± standard deviation.

3.3 Results and discussion

3.3.1 Protein solubility

Protein solubility is considered as one of the most critical functionalities in food applications, because it is associated with many other functional properties, such as hydration, foaming, and emulsifying properties. Generally, all the modified pea proteins had greatly improved solubility compared with the unmodified pea protein above the isoelectric point (pI, around pH 5) (Figure 1). Guar gum-pea conjugates (1:20 and 1:40) also showed much higher solubility below the pI, while the solubility of the acylated pea proteins was much lower below pH 5, especially that the succinylated pea proteins were barely soluble. Thus, we can conclude that conjugation modification with polysaccharide is highly effective in improving protein solubility. This is because when protein is conjugated with hydrophilic polysaccharide at the early stage of Maillard reaction, protein hydration properties are improved, therefore, enhancing the solubility (Du et al., 2013).

The succinylated pea protein had relatively higher solubility than the acetylated pea protein when the pH was greater than 5, and it had lower solubility when the pH was less than 5. This could be explained by the fact that the succinylation process replaced the ammonium groups from lysine residues, which resulted in fewer hydrophilic cation groups to counterbalance the protein-protein hydrophobic interactions. Therefore, protein-protein interaction was stronger below the pI, which reduced its solubility. When the pH was above 5, the replacement of ε-amino group of lysine with negatively charged carboxyl groups enhanced the interaction between

protein-water, and promoted the intra- and intermolecular charge repulsion, thus, resulting in unfolding and dissociation of the quaternary structures and increased solubility (Anaya Castro et al., 2019; Arogundade et al., 2013; El-Adawy, 2000; Mirmoghtadaie, Kadivar, & Shahedi, 2009). Lower solubility of the acetylated pea proteins than the succinylated pea proteins above the pI was due to stronger aggregation between the unfolded protein via hydrophobic interactions (Yin, Tang, Wen, & Yang, 2009a). Our result was in agreement with other studies on the acylation of African yam bean protein (Arogundade et al., 2013), mung bean protein (El-Adawy, 2000), oat protein (Mirmoghtadaie et al., 2009), and rice protein (Du et al., 2013).

3.3.2 Water/ oil holding capacities

Water and oil holding capacities (WHC, OHC) determine the water/ oil retention of the proteins and protein-water/ oil interactions and affect texture and quality of food products. The WHC is also associated with other protein functional properties, such as solubility, emulsifying properties, and gelation. The physical mixture of guar gum-pea (1:20) had significantly higher WHC than the conjugated (1:20) and unmodified pea proteins (Table 1). Guar gum is a high molecular weight polysaccharide and strongly interacts with water, acting as a thickening agent (Karaman et al., 2014), and the higher WHC was achieved by its stronger water binding ability. Higher concentration of guar gum (1:20 vs. 1:40) resulted in higher WHC for the simple guar gum/protein mixture and the conjugated proteins, because more hydrophilic polysaccharides enhanced the affinity between protein and water molecules. However, the WHC of the conjugated protein was not obviously improved compared with the unmodified protein, which was probably related to the surface hydrophobicity of the proteins (Table 2). Conjugated proteins with decreased surface hydrophobicity showed stronger WHC (Amid, Mirhosseini, Poorazarang, & Mortazavi, 2013). Arogundade et al. (2013) and Lillard et al. (2009) also reported that protein-

polysaccharide conjugation did not increase the WHC of African yam bean and whey proteins; however, Wang et al. (2018) reported that conjugated rapeseed protein had significantly increased WHC. Overall, the WHC of conjugated protein depends on the conjugation conditions, degree of conjugation, types of polysaccharide, and its surface hydrophobicity.

The WHC of acetylated and succinylated pea proteins increased significantly compared with the unmodified and conjugated pea proteins (Table 1). Acylation modification unfolds the protein and alters protein electrical charge distribution, resulting in enhanced hydrophilic binding site of the protein molecules (El-Adawy, 2000). With increased concentration of acylation agents, there was no significant difference for the WHC of the succinylated pea proteins, but WHC of the acetylated protein decreased due to the conversion of protein net positive charge to neutral charge. Furthermore, AA-0.3 exhibited higher WHC than SA-0.3. The succinylated protein had higher solubility than the acetylated protein (Figure 1); therefore, more succinylated proteins were dissolved in water instead of absorbing and holding the water. In addition, sequential acylation and conjugation had synergistic effect on WHC, especially for SA-0.6 conjugate, which exhibited the highest WHC of 10.91 g water/g protein among all the modified proteins.

All the modified proteins (i.e., conjugation, acylation, and sequential modification) had significantly higher OHC compared with the unmodified pea protein (Table 1). The conjugation modification had a greater effect on increasing the OHC, because the heat treatment during the protein-polysaccharide conjugation altered and unfolded the protein structure and exposed more hydrophobic amino acid residues of the protein. Overall, the succinylated pea proteins exhibited higher OHC than the acetylated pea proteins, while there was no significant difference for OHC among the modified proteins with different levels of the same modifier. In addition, the protein

from sequential acylation and conjugation (SA 0.6 conj) showed the highest OHC among all the protein samples. Protein OHC could be affected by many factors, such as protein surface area, ratio of hydrophilicity/hydrophobicity, protein net charge, etc.

3.3.3 Emulsifying properties

Overall, most modified pea proteins exhibited significantly higher emulsion capacity (EC) and emulsion stability (ES) compared with the unmodified pea protein, except for AA 0.3/0.6 (Table 1). Generally, the guar gum-pea protein conjugates had higher EC and ES compared with the simple mixtures at the same gum concentration, indicating that the protein-polysaccharide interactions induced through Maillard reaction are crucial in improving the emulsifying activity of the protein. Higher gum concentration in the modified proteins (1:20 conj vs. 1:40 conj) resulted in greatly enhanced emulsion stability (94.7% vs. 60.7%), which was attributed to the hydrophilicity of the polysaccharide. Conjugation of guar gum and protein caused the formation of strong solvated layer at the oil-water interface, which favored the steric stabilization of the emulsion oil droplet (Keowmaneechai & McClements, 2002). The absorbed layer of conjugated protein has more effective steric stabilization of emulsion droplets than the unmodified protein (Du et al., 2013).

Acetylation and succinylation had distinct effects on the EC and ES of pea protein. The EC and ES of AA 0.3/0.6 were significantly decreased, while the EC and ES of SA 0.3/0.6 were significantly increased compared with the unmodified pea protein (Table 1). The addition of longer aliphatic groups by succinylation increased the protein-water interaction (El-Adawy, 2000; Johnson & Brekke, 1983), and exposed more hydrophobic residues of the protein; therefore, the emulsifying properties were significantly improved. The emulsifying properties were also positively related to protein solubility (Figure 1). The succinylated protein could form

more stable layers around the oil droplets to facilitate their interaction with aqueous phase because of higher solubility, and the emulsifying properties of the acetylated pea proteins were limited due to a lower solubility. Sequential acylation and conjugation modifications had exceptional synergistic effects on the emulsifying properties of the proteins, achieving nearly 100% EC and ES, except for AA 0.3 conjugate. The results showed that modification of protein structures by adding appropriate functional groups is highly effective in enhancing its functional properties (Du et al., 2013).

3.3.4 Foaming properties

Important characteristics of protein foaming properties include foaming capacity (FC) and foaming stability (FS). Foaming capacity is determined by the amount of interfacial area that can be created by the protein, and it is highly related to protein hydrophobicity, while foaming stability indicates its ability against stress during a certain period of time (Lam, Can Karaca, Tyler, & Nickerson, 2018). Foam formation is dependent on the interfacial film that is formed by the proteins and the ability to maintain the air bubble in the suspension and slow down the coalescence rate (Shen et al., 2021). In this study, most of the modified pea proteins showed decreased FC and FS compared with the unmodified pea protein, except for SA 0.3/0.6 (Figure 2). The conjugated proteins had much lower FC and FS than the acylated proteins. The higher FC of succinylated pea proteins may be attributed to their smaller molecular size and better solubility, so they could be more rapidly absorbed during the whipping process to generate more foams compared with the conjugated proteins with higher molecular weight and lower solubility (Aluko, McIntosh, & Reaney, 2001; Zhao et al., 2013).

When comparing different guar gum-pea protein conjugates, the 1: 40 conjugate exhibited better FC and FS than the 1:20 conjugate; however, the foaming properties of both conjugates

were weaker than that of the unmodified protein. The results implied that the addition of high molecular weight polysaccharide conjugated with the protein does not help in improving foaming properties. Other studies also found that some excessive modification of proteins could cause foam destabilization and poor stability due to the increase of net charge density, reduce the protein-protein interaction in the foam lamellae, and prevent the formation of elastic film in the air-water interface (Arogundade et al., 2013; Mirmoghtadaie et al., 2009).

3.3.5 Gelation property

Protein gelation is important in determining the texture, quality and sensory attributes of many foods (Foegeding & Davis, 2011). Overall, gelation properties of all the modified pea proteins were significantly enhanced with lower least gelation concentration (LGC) values compared with the unmodified pea protein (Table 1). The 1:20 protein conjugate had significantly decreased LGC compared with the simple protein-gum mixture (1:20), and both of them had better gelation properties than the 1:40 conjugate and mixture. This is because the addition of higher amount of hydrocolloid improved gel thickening function of the protein (Saha & Bhattacharya, 2010), and unfolding of the protein through conjugation enhanced protein hydrophobic interaction in the formation of more stable gel network, reducing the amount of proteins required for gel formation (O'Kane, Vereijken, Gruppen, & Van Boekel, 2005). Wang et al. (2018) reported that only moderate degree of conjugation of rapeseed protein with dextran could improve the gelation properties, while excessive conjugation decreased gelation properties, because additional static space was created between the conjugated protein molecules with polysaccharide coating, which inhibited protein hydrophobic interaction (Liu, Zhao, Zhao, Ren, & Yang, 2012). The acetylated pea proteins exhibited significantly lower LGC values, and thus better gelation properties, compared with the succinylated proteins. During the acetylation

process, the protein was unfolded and disulfide crosslinking was enhanced (Schmandke et al., 1981), improving the gelation properties. Furthermore, sequential acetylation and conjugation dramatically decreased the LGC, especially for the AA 0.6 conjugate, which formed stable gets at only 7% concentration. The result demonstrated that synergistic effect occurred when combining both modifications.

3.3.6 Browning reaction

The relative amount of browning compounds generated during the conjugation reaction in the modified proteins was measured based on the absorbance at 304 nm (early intermediate Amodari compounds) and 420 nm (final Maillard reaction products), respectively (Shen, Tebben, Chen, & Li, 2019). Generally, the conjugated proteins had significantly higher absorbance at 304 nm compared with the unmodified protein (Figure 3), but the absorbances at 420 nm were similar, which implied that majority of the protein-polysaccharide conjugates belongs to the early intermediates of Maillard reaction products. The 1:40 conjugate had relatively higher absorbance at 304 nm than the 1: 20 conjugate. This may be caused by the formation of more browning compounds with higher amount of proteins in the 1:40 conjugate during the Maillard reaction. Browning reaction depends on the conjugation conditions, such as reaction temperature, time, and the ratio of protein/polysaccharides (Zha et al., 2019). The simple guar gum-pea protein mixtures and unmodified protein had similar absorbance at 304 and 420 nm, because conjugation reaction was not expected for the mixtures as they were prepared at room conditions by simply mixing (Figure 3).

3.3.7 Free amino group content

The amount of available free amino group is another indicator of the degree of protein acylation and guar gum-protein conjugation. The acylated proteins had significantly lower

amount of free amino group compared with the unmodified pea proteins (Figure 4). This is because the acylation reaction mainly occurred between the acylating agent and free amino groups of the proteins, although reactions could also occur with other amino acid residues such as cysteine, tyrosine, serine and/or threonine (Lee, Groninger, & Spinelli, 1981). The succinylated proteins had a significantly higher amount of free amino group than the acetylated proteins with the same amount of acylation agent. When AA and SA were added at the same weight amount, more intensive reactions were expected for AA because of its higher molar ratios to protein and stronger reactivity. Although conjugation reaction occurred between carbonyl groups of polysaccharides and amino groups of protein, the amount of free amino group of the conjugated proteins was not reduced compared with that of the unmodified protein. This was caused by the interfered absorbance of guar gum molecules that was overlapped with the absorbance of the conjugated proteins during free amino measurement. In addition, we used a much lower amount of polysaccharide relative to the protein (1:20 and 1:40); therefore, relatively much less amount of free amino group was consumed during the conjugation modification.

3.3.8 Surface hydrophobicity

Surface hydrophobicity of protein is dominated by the hydrophobic amino acid group residues available at the surface of protein. The guar gum-pea protein conjugates had greatly larger ($p<0.05$) surface hydrophobicity compared to the unmodified pea protein (Table 2). This was because the inclusion of polysaccharide to the protein led to protein unfolding and exposure of more hydrophobic residues. However, the surface hydrophobicity of 1:20 conjugate was lower than that of the 1:40 conjugate, which may be attributed to the intrinsic hydrophilicity of the polysaccharide. Both the acetylated and succinylated pea proteins had significantly lower surface hydrophobicity than the unmodified pea protein, although higher level of modifier resulted in

slightly higher surface hydrophobicity (Table 2). Acylation modification of the protein introduced succinyl and acetyl groups onto the protein, which increased the electronegativity and enhanced the electronic repulsion, and this prevented ANS probe from binding to the protein hydrophobic area, thus showing decreased surface hydrophobicity. A similar trend was reported for acylated oat proteins (Zhao et al., 2017). Relatively higher surface hydrophobicity was observed for the succinylated protein compared with the acetylated protein with the same amount of modifier (Table 2), which is because of the more hydrophobic nature of the succinic group than the acetic group. Furthermore, the conjugated SA 0.3 and SA 0.6 had significantly higher surface hydrophobicity than the unmodified pea protein and succinylated proteins, which indicated that the conjugation had stronger effect in improving the hydrophobicity.

3.3.9 FTIR

Fourier transform infrared spectroscopy is useful in identifying protein functional groups and secondary structures after modification. The bands in the regions of 3700 - 3200 cm^{-1} and 1100 - 1000 cm^{-1} denote the hydroxyl group and C-O stretching vibration, respectively (Du et al., 2013). There were obvious differences when comparing the conjugated proteins with the unmodified protein (Figure 5). After protein conjugation with guar gum, it showed more intensive bands at 3700 - 3200 cm^{-1} than the unmodified pea protein and the sequential acylated and conjugated proteins (AA 0.6/ SA 0.6 conjugates) (Figure 5). A strong band at 1100 -1000 cm^{-1} was attributed to -OH bending vibration in the conjugated protein. Acylation modification greatly altered the protein secondary structures, which was related to the bands of amide I, II and III, attributed to 1635 cm^{-1}, 1546 cm^{-1} and 1450-1240 cm^{-1}, which defined the C=O stretching, N-H deformation, C-N stretching and N-H bending vibrations, respectively (Du et al., 2013; Pirestani, Nasirpour, Keramat, Desobry, & Jasniewski, 2018).

3.3.10 Circular dichroism (CD) spectroscopy

Secondary structures of the modified pea proteins including α-helix, β-sheet, β-turn, and random coil obtained from CD are summarized in Table 2. The unmodified pea protein consisted of 17.17% of α-helix, 23.97% of β-sheet, 1.17% of β-turn, and 57.67% of random coil, and random coil accounted for the majority of the secondary structures. The conjugated proteins (both 1:20 and 1:40 conjugates) had significantly higher amount of α-helix, but lower amounts of β-sheet and random coil compared with the unmodified pea protein. Du et al. (2013) reported a slight decrease in α-helix and β-sheet structures, but an increase in random coil in the rice protein conjugated with *k*-carrageenan. Liu et al. (2012) reported that the amount of both α-helix and random coil of peanut protein-dextran conjugates was decreased, while β-sheet structure was increased. The secondary structural differences could be attributed to the different protein types, reaction conditions, and the ratio of polysaccharide to protein. The acetylated pea protein had relatively lower amount of α-helix but much higher amount of β-turn structure. The succinylated pea protein possessed significantly higher amount of β-sheet structure but lower amount of random coil compared with the unmodified or conjugated pea proteins. Our results confirmed that conjugation and acylation can greatly alter protein secondary structures and further affect the functional properties.

3.3.11 SDS-PAGE

Globulins, including both legumin (11S) and vicilin (7S), are the major storage protein in pea. There was no obvious difference when comparing the SDS-PAGE bands of the gum-pea conjugates and the unmodified pea protein (Figure 6). This result was expected, because extremely small amount of polysaccharide relative to the protein was used for the conjugation modification, and changes of protein molecular size could not be observed from the SDS-PAGE

analysis. The succinylated proteins exhibited more intensive bands compared with the acetylated proteins. Although a strong solvent (i.e., SDS/sodium phosphate buffer) was used to dissolve the protein samples prior to the electrophoresis analysis, the acetylated protein still showed very low solubility due to the greatly reduced electronegativity by introducing acetic functionality, which is consistent with the solubility result (Figure 1). The 11S is a hexameric protein consisting of acidic (40 kDa) and basic (20 kDa) subunits, and the 7S is a glycosylated trimeric cluster consisting of three subunits, with molecular weight of 47.3, 33.3, and 28.7 kDa, respectively (Chéreau et al., 2016; Pirestani, Nasirpour, Keramat, & Desobry, 2017), all of which were observed on the SDS-PAGE under the reducing condition. The band at around 100 kDa was attributed to lipoxygenase (Barać et al., 2011) and may also indicate the formation of newly crosslinked protein structures during processing.

3.3.12 Free sulfhydryl (SH) group

The content of free sulfhydryl group in pea and modified pea proteins is summarized in Table 2. There was no significant difference for the free SH content between guar gum-pea protein conjugates and the unmodified pea protein, indicating that no or very minimal disulfide crosslinking occurred during the conjugation. Acetylated pea proteins (both AA 0.3/0.6 and AA 0.3/0.6 conjugates) had significantly lower free SH content compared with the unmodified protein, implying intensive disulfide crosslinking during acetylation modification. It was reported that conjugation reaction reduced the free sulfhydryl groups in pea, whey, and rapeseed proteins, respectively (Wang & Arntfield, 2016; Wang & Ismail, 2012; Wang et al., 2018), because heat treatment during the Maillard reaction promoted the formation of disulfide linkages. The different result from our study was attributed to the different conjugation conditions, such as reaction temperature, time, and ratio of polysaccharide to protein.

3.3.13 In vitro GI digestibility

The *in vitro* GI digestibility of pea and the modified pea proteins was indicated by the degree of hydrolysis, and the results are presented in Figure 7. Overall, the conjugated (1:20 conj and 1:40 conj) and acylated pea proteins (AA 0.6, SA 0.3, SA 0.6) showed decreased protein digestibility, while the digestibility of AA 0.3 was not significantly different compared with the control pea protein. The digestibility of the conjugated pea proteins was also decreased, because the conjugated protein had higher molecular weight, which became less accessible to the digestive enzymes. However, some papers (Siu & Thompson, 1982; Yin et al., 2009a; Yin, Tang, Wen, & Yang, 2009b) reported that the acylated proteins had increased digestibility compared with control protein, and this was attributed to their better solubility and unfolded molecular structures during modification.

3.4 Conclusions

In this study, modified pea proteins were prepared by acylation or/and conjugation through reacting with acetic anhydride (AA) or succinic anhydride (SA) and incubating the guar gum-pea protein mixtures to induce Maillard reaction, respectively. Both conjugated and acylated pea proteins demonstrated significantly improved OHC, and the acylated pea protein also had much greater WHC. The EC and ES of the modified proteins were improved by up to 112% and 140%, respectively, compared to the unmodified protein. Sequential acylation and conjugation of pea proteins demonstrated more beneficial and synergistic effects and further enhanced the WHC, OHC, emulsification and gelation properties, which could be used as novel plant protein ingredients for different applications. However, the *in vitro* GI digestibility of the modified pea protein was decreased compared to the control protein. Future research is necessary to conduct

safety evaluation of the chemically modified proteins and further understand protein nutritional changes during the modification.

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Conflict of interest

The authors declare that there is no known conflict of interest.

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Figures and tables

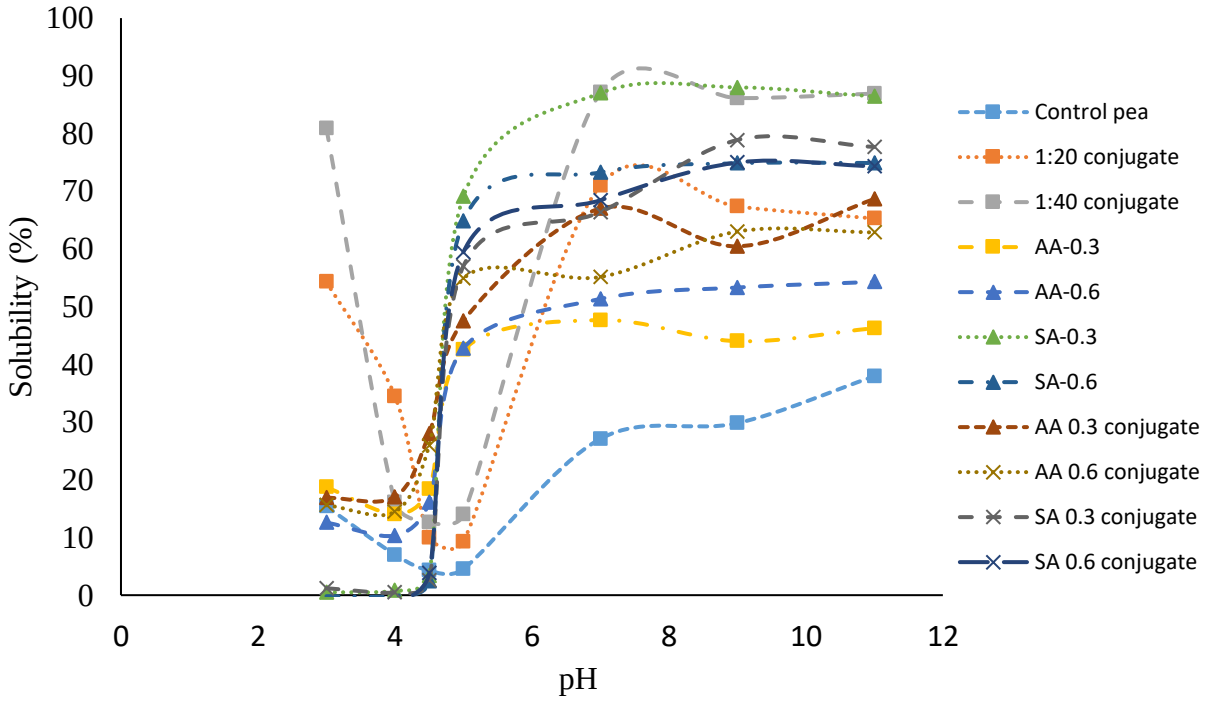

Figure 3.1 Solubility of pea and modified pea proteins.

*__Control Pea:__ control; __1:20 mix:__ 1:20 guar gum physical mix; __1:40 mix:__ 1:40 guar gum physical mix; __1:20 conj.:__ 1:20 guar gum conjugate; __1:40 conj.:__ 1:40 guar gum conjugate; __AA 0.3:__ acetylated pea protein at 0.3 g AA; __AA 0.3 conj.:__ acetylated pea protein at 0.3 g AA conjugated with guar gum (1:20); __AA 0.6:__ acetylated pea protein at 0.6 g AA; __AA 0.6 conj.:__ acetylated pea protein at 0.6 g AA conjugated with guar gum (1:20); __SA 0.3:__ succinylated pea protein at 0.3 g SA; __SA 0.3 conj.:__ succinylated pea protein at 0.3 g SA conjugated with guar gum (1:20); __SA 0.6:__ succinylated pea protein at 0.6 g SA; __SA 0.6 conj.:__ succinylated pea protein at 0.6 g SA conjugated with guar gum (1:20).

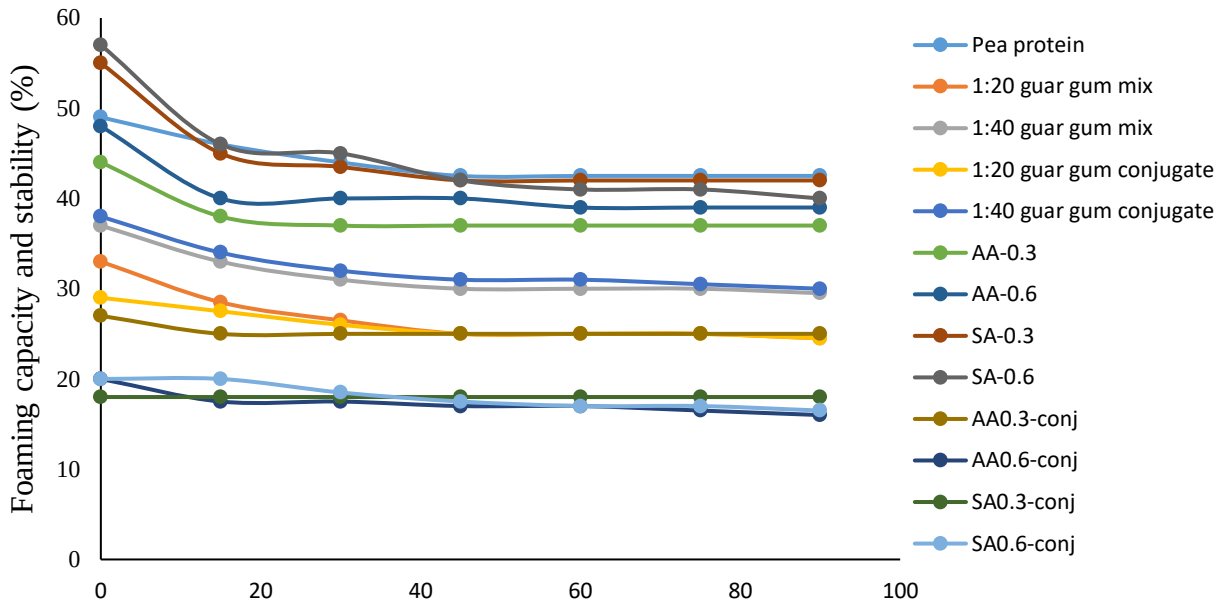

Figure 3.2 Foaming capacity and stability of pea and modified pea proteins.

*__Pea:__ control; __1:20 mix:__ 1:20 guar gum physical mix; __1:40 mix:__ 1:40 guar gum physical mix; __1:20 conj.:__ 1:20 guar gum conjugate; __1:40 conj.:__ 1:40 guar gum conjugate; __AA 0.3:__ acetylated pea protein at 0.3 g AA; __AA 0.3 conj.:__ acetylated pea protein at 0.3 g AA conjugated with guar gum (1:20); __AA 0.6:__ acetylated pea protein at 0.6 g AA; __AA 0.6 conj.:__ acetylated pea protein at 0.6 g AA conjugated with guar gum (1:20); __SA 0.3:__ succinylated pea protein at 0.3 g SA; __SA 0.3 conj.:__ succinylated pea protein at 0.3 g SA conjugated with guar gum (1:20); __SA 0.6:__ succinylated pea protein at 0.6 g SA; __SA 0.6 conj.:__ succinylated pea protein at 0.6 g SA conjugated with guar gum (1:20).

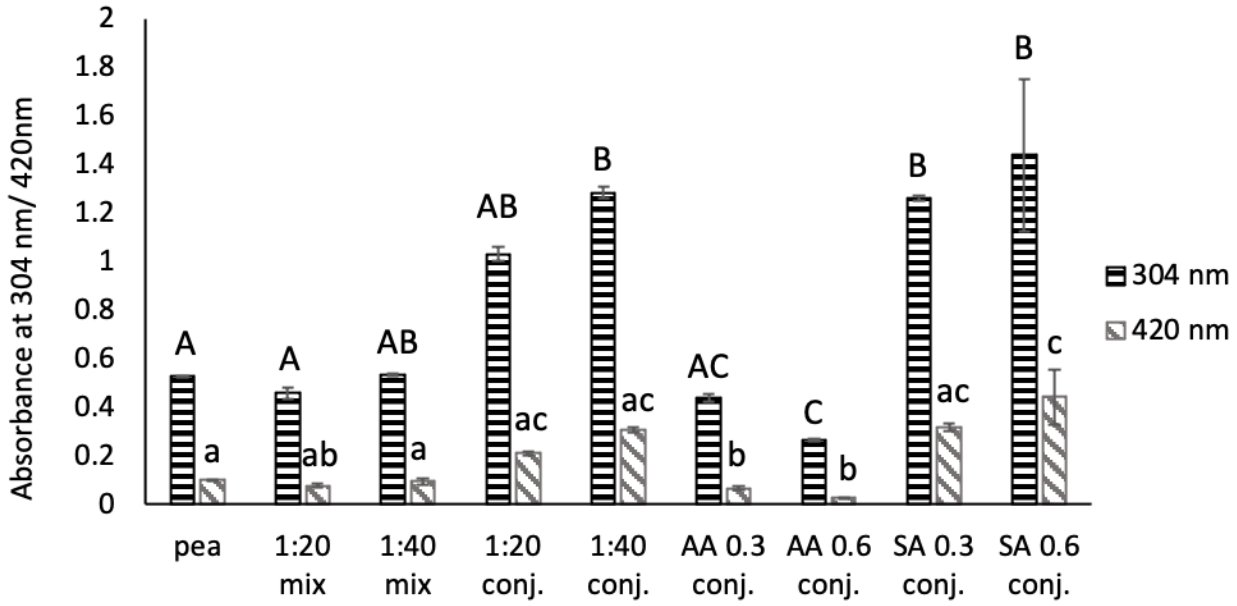

Figure 3.3 Browning reaction in modified pea proteins.

*__Pea:__ control; **1:20 mix:** 1:20 guar gum physical mix; **1:40 mix:** 1:40 guar gum physical mix; **1:20 conj.:** 1:20 guar gum conjugate; **1:40 conj.:** 1:40 guar gum conjugate; **AA 0.3 conj.:** acetylated pea protein at 0.3 g AA conjugated with guar gum (1:20); **AA 0.6 conj.:** acetylated pea protein at 0.6 g AA conjugated with guar gum (1:20); **SA 0.3 conj.:** succinylated pea protein at 0.3 g SA conjugated with guar gum (1:20); **SA 0.6 conj.:** succinylated pea protein at 0.6 g SA conjugated with guar gum (1:20).

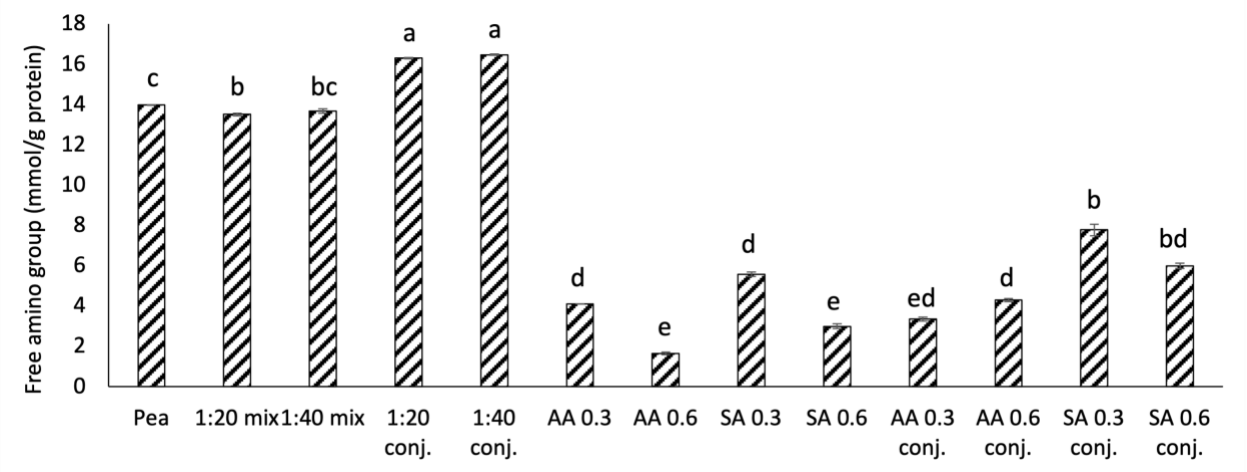

Figure 3.4 Free amino group content of pea and modified pea proteins.
*Means with different letters denote significant differences ($p < 0.05$).

Pea: control; **1:20 mix:** 1:20 guar gum physical mix; **1:40 mix:** 1:40 guar gum physical mix; **1:20 conj.:** 1:20 guar gum conjugate; **1:40 conj.:** 1:40 guar gum conjugate; **AA 0.3:** acetylated pea protein at 0.3 g AA; **AA 0.3 conj.:** acetylated pea protein at 0.3 g AA conjugated with guar gum (1:20); **AA 0.6:** acetylated pea protein at 0.6 g AA; **AA 0.6 conj.:** acetylated pea protein at 0.6 g AA conjugated with guar gum (1:20); **SA 0.3:** succinylated pea protein at 0.3 g SA; **SA 0.3 conj.:** succinylated pea protein at 0.3 g SA conjugated with guar gum (1:20); **SA 0.6:** succinylated pea protein at 0.6 g SA; **SA 0.6 conj.:** succinylated pea protein at 0.6 g SA conjugated with guar gum (1:20).

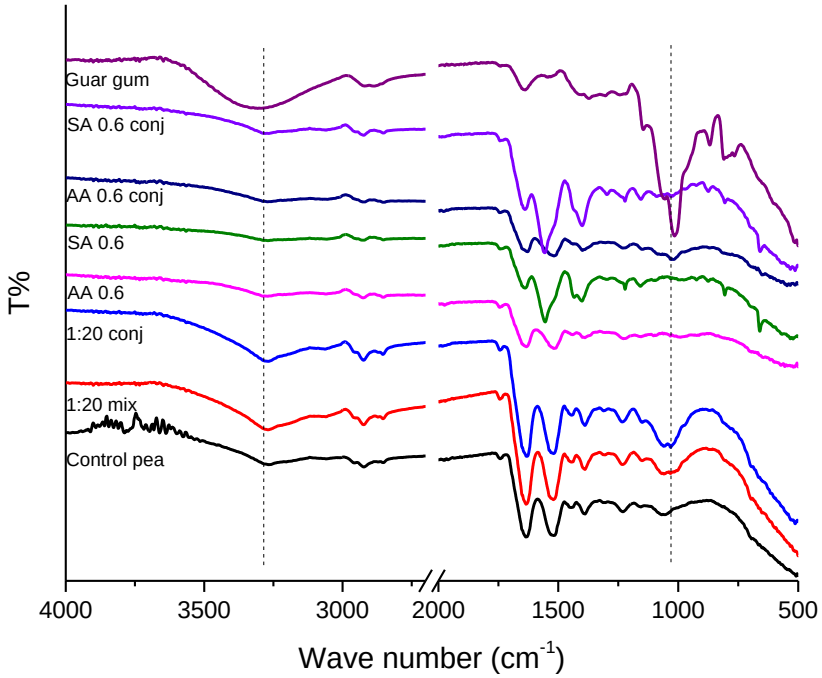

Figure 3.5 FTIR spectra of pea and selected modified pea proteins.

* **1:20 mix:** 1:20 guar gum physical mix; **1:20 conj.:** 1:20 guar gum conjugate; **AA 0.6:** acetylated pea protein at 0.6 g AA; **AA 0.6 conj.:** acetylated pea protein at 0.6 g AA conjugated with guar gum (1:20); **SA 0.6:** succinylated pea protein at 0.6 g SA; **SA 0.6 conj.:** succinylated pea protein at 0.6 g SA conjugated with guar gum (1:20).

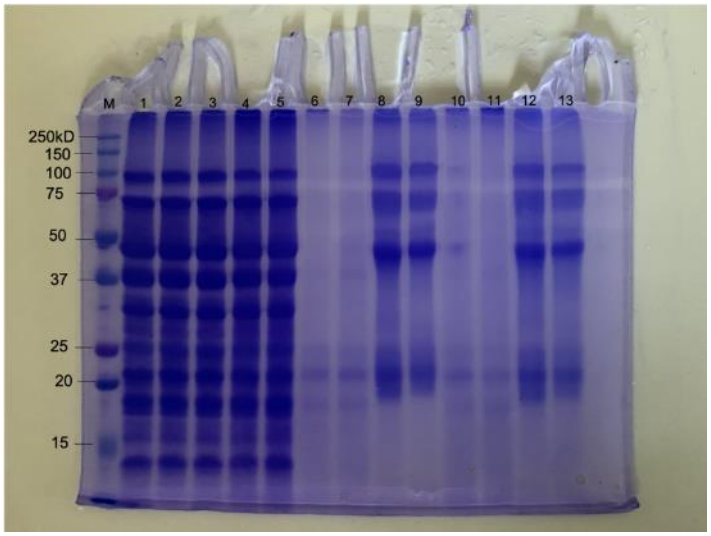

Figure 3.6 Electrophoretic patterns of pea and modified pea proteins under reducing condition. Lane M-molecular weight marker; Lane 1: pea; Lane 2: 1:20 guar gum mix; Lane 3: 1:40 guar gum mix; Lane 4: 1:20 guar gum conjugate; Lane 5: 1:40 guar gum conjugate; Lane 6: AA 0.3; Lane 7: AA 0.6; Lane 8: SA 0.3; Lane 9: SA 0.6; Lane 10: AA 0.3 conjugate; Lane 11: AA 0.6 conjugate; Lane 12: SA 0.3 conjugate; Lane 13: SA 0.6 conjugate.

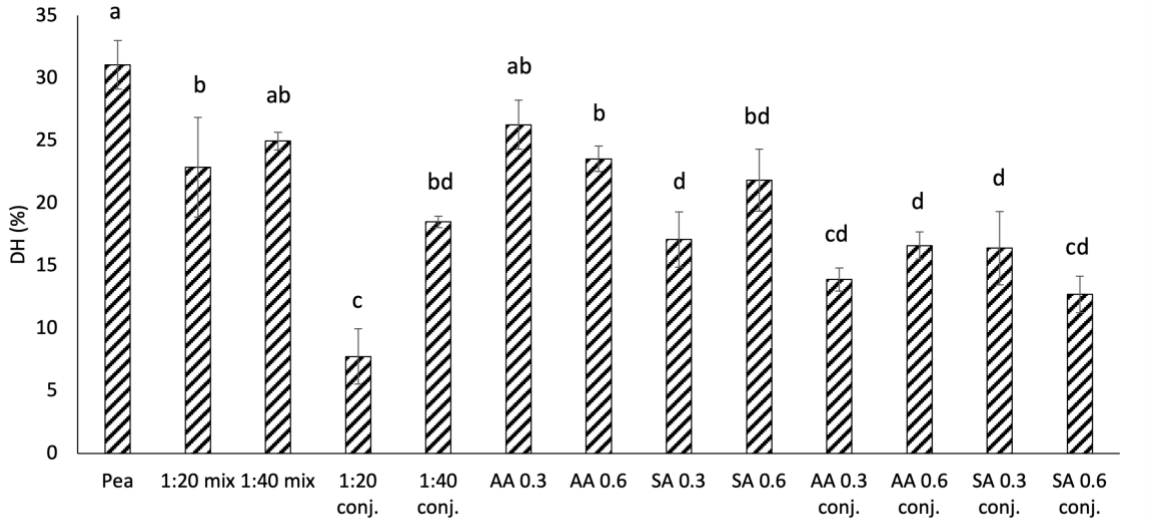

Figure 3.7 *In vitro* gastrointestinal digestibility in terms of degree of hydrolysis (DH) of pea and modified pea proteins.

*Means with different letters denote significant differences ($p < 0.05$).

****Pea:** control pea protein; **1:20 mix:** 1:20 guar gum physical mix; **1:40 mix:** 1:40 guar gum physical mix; **1:20 conj.:** 1:20 guar gum conjugate; **1:40 conj.:** 1:40 guar gum conjugate; **AA 0.3:** acetylated pea protein at 0.3 g AA; **AA 0.3 conj.:** acetylated pea protein at 0.3 g AA conjugated with guar gum (1:20); **AA 0.6:** acetylated pea protein at 0.6 g AA; **AA 0.6 conj.:** acetylated pea protein at 0.6 g AA conjugated with guar gum (1:20); **SA 0.3:** succinylated pea protein at 0.3 g SA; **SA 0.3 conj.:** succinylated pea protein at 0.3 g SA conjugated with guar gum (1:20); **SA 0.6:** succinylated pea protein at 0.6 g SA; **SA 0.6 conj.:** succinylated pea protein at 0.6 g SA conjugated with guar gum (1:20).

Table 3-1 Functional properties of pea proteins.

	WHC (g H_20/g protein)	OHC (g oil/g protein)	EC (%)	ES (%)	LGC (%)
Pea	3.6 ± 0.1^{f}	1.0 ± 0.0^{d}	45.1 ± 1.4^{dc}	39.7 ± 0.8^{d}	18^{a}
1:20 mix	5.2 ± 0.2^{cd}	1.1 ± 0.0^{d}	96.7 ± 1.0^{c}	67.9 ± 5.0^{c}	13^{d}
1:40 mix	4.1 ± 0.1^{de}	1.1 ± 0.0^{d}	67.5 ± 2.0^{c}	54.5 ± 1.0^{cd}	15^{b}
1:20 conj.	3.6 ± 0.1^{ef}	2.0 ± 0.1^{bc}	98.8 ± 0.6^{b}	94.7 ± 0.6^{bc}	11^{e}
1:40 conj.	2.7 ± 0.1^{f}	2.2 ± 0.2^{ab}	95.6 ± 0.6^{c}	60.7 ± 1.7^{c}	15^{b}
AA 0.3	7.0 ± 0.3^{ab}	1.7 ± 0.0^{cd}	41.6 ± 1.1^{d}	34.8 ± 3.6^{e}	9^{g}
AA 0.6	5.0 ± 0.1^{d}	1.6 ± 0.0^{d}	38.5 ± 1.9^{d}	33.7 ± 3.3^{e}	11^{e}
SA 0.3	5.7 ± 0.3^{c}	2.1 ± 0.0^{b}	99.0 ± 0.4^{b}	96.7 ± 0.6^{b}	14^{c}
SA 0.6	6.3 ± 0.7^{bc}	1.9 ± 0.1^{c}	99.1 ± 0.3^{b}	95.6 ± 0.7^{b}	14^{c}
AA 0.3 conj.	5.8 ± 0.2^{b}	1.8 ± 0.0^{cd}	100.0 ± 0.0^{a}	53.7 ± 1.2^{d}	9^{g}
AA 0.6 conj.	7.8 ± 0.2^{a}	1.9 ± 0.1^{c}	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}	7^{h}
SA 0.3 conj.	3.7 ± 0.2^{ef}	2.2 ± 0.1^{ab}	100.0 ± 0.0^{a}	99.1 ± 0.3^{a}	10^{f}
SA 0.6 conj.	10.9 ± 0.6^{a}	2.9 ± 0.1^{a}	100.0 ± 0.0^{a}	98.7 ± 0.6^{a}	10^{f}

Note: WHC: water holding capacity; OHC: oil holding capacity; EC: emulsion capacity; ES: emulsion stability; LGC: least gelation concentration. *Means with different letters for each functional attribute denote significant differences ($p< 0.05$).

***Pea:** control; **1:20 mix:** 1:20 guar gum physical mix; **1:40 mix:** 1:40 guar gum physical mix; **1:20 conj.:** 1:20 guar gum conjugate; **1:40 conj.:** 1:40 guar gum conjugate; **AA 0.3:** acetylated pea protein at 0.3 g AA; **AA 0.3 conj.:** acetylated pea protein at 0.3 g AA conjugated with guar gum (1:20); **AA 0.6:** acetylated pea protein at 0.6 g AA; **AA 0.6 conj.:** acetylated pea protein at 0.6 g AA conjugated with guar gum (1:20); **SA 0.3:** succinylated pea protein at 0.3 g SA; **SA 0.3 conj.:** succinylated pea protein at 0.3 g SA conjugated with guar gum (1:20); **SA 0.6:** succinylated pea protein at 0.6 g SA; **SA 0.6 conj.:** succinylated pea protein at 0.6 g SA conjugated with guar gum (1:20).

Table 3-2 Surface hydrophobicity, free S-H content, and protein secondary structures.

Protein samples	Surface hydrophobicity	Free S-H (μmol/g protein)	α-helix (%)	β-sheet(%)	β-turn (%)	random coil (%)
Pea	72,543±3,720^{a}	5.4±0.2^{a}	17.2±1.4^{a}	24.0±1.5^{a}	1.2±1.5^{a}	57.7±1.3^{a}
1:20 mix	116,861±2,343^{b}	4.6±0.5^{ab}	34.0±9.9^{b}	32.5±11.0^{ab}	/	33.5±20.8^{ab}
1:40 mix	160,597±5,462^{c}	5.1±0.1^{ab}	21.2±2.2^{ab}	25.6±3.0^{a}	/	53.2±4.8^{a}
1:20 conj.	152,126±7,239^{bc}	5.1±0.2^{ab}	42.9±6.0^{b}	14.0±7.7^{a}	/	43.2±13.6^{b}
1:40 conj.	178,954±6,750^{c}	5.5±0.0^{a}	53.5±9.6^{b}	15.6±4.2^{a}	/	30.9±10.8^{b}
AA 0.3	18,885±2,336^{d}	0.9±0.1^{b}	19.9±0.4^{a}	20.1±3.9^{a}	16.4±0.8^{ab}	43.6±5.0^{ab}
AA 0.6	35,482±2,255^{ae}	0.9±0.0^{b}	10.6±2.5^{a}	34.0±7.5^{b}	15.9±2.6^{ab}	41.1±2.6^{b}
SA 0.3	33,416±3,151^{e}	5.7±0.8^{a}	31.9±9.8^{b}	55.8±8.3^{b}	/	12.3±16.9^{b}
SA 0.6	52,467±3,024^{a}	4.2±0.7^{ab}	18.5±2.6^{ab}	52.0±6.5^{b}	/	29.5±7.9^{b}
AA 0.3 conj.	24,606±1,666^{ed}	0.8±0.0^{b}	18.4±2.8^{a}	26.1±3.5^{b}	15.4±4.3^{ab}	40.2±1.7^{b}
AA 0.6 conj.	21,801±1,685^{d}	0.8±0.1^{b}	10.6±4.5^{a}	31.5±2.1^{ab}	16.9±3.5^{b}	40.7±1.0^{ab}
SA 0.3 conj.	109,611±2,506^{b}	5.3±0.3^{a}	25.9±5.0^{b}	45.4±9.7^{b}	/	28.7±14.6^{b}
SA 0.6 conj.	94,011±3,939^{a}	3.6±0.4^{ab}	15.3±0.9^{a}	34.5±3.7^{ab}	/	50.2±4.5^{ab}

*Means with different letters in each column denote significant differences ($p < 0.05$).

__Pea:__ control; **1:20 mix: 1:20 guar gum physical mix; **1:40 mix:** 1:40 guar gum physical mix; **1:20 conj.:** 1:20 guar gum conjugate; **1:40 conj.:** 1:40 guar gum conjugate; **AA 0.3:** acetylated pea protein at 0.3 g AA; **AA 0.3 conj.:** acetylated pea protein at 0.3 g AA conjugated with guar gum (1:20); **AA 0.6:** acetylated pea protein at 0.6 g AA; **AA 0.6 conj.:** acetylated pea protein at 0.6 g AA conjugated with guar gum (1:20); **SA 0.3:** succinylated pea protein at 0.3 g SA; **SA 0.3 conj.:** succinylated pea protein at 0.3 g SA conjugated with guar gum (1:20); **SA 0.6:** succinylated pea protein at 0.6 g SA; **SA 0.6 conj.:** succinylated pea protein at 0.6 g SA conjugated with guar gum (1:20).

Chapter 4 - Improving functional properties of pea protein through "green" modifications using enzymes and polysaccharides

Abstract

Pea proteins, along with other plant proteins have gained significant attention due to the growing demands from environmental- and health-conscious consumers. The objective of this study was to enhance pea protein functional properties through enzymatic or/ conjugation modifications and understand the physicochemical properties of the modified proteins. Enzymatically modified pea proteins were prepared by mixing pea protein with 1% microbial transglutaminase or protein glutaminase at 40 °C and 55 °C for 3 hours, respectively. Conjugated pea proteins were prepared by incubating 10% pea protein slurry containing 5% guar gum or gum arabic at 60 °C for 24 hours. Sequential modifications through both enzymatic treatment and polysaccharide conjugation were also employed to investigate their synergistic benefits. Molecular changes of the proteins including free sulfhydryl and amino content, secondary structure, molecular size, and surface hydrophobicity were characterized. Protein functionality, *in intro* digestibility, and sensory properties were analyzed. The proteins crosslinked with transglutaminase showed significantly improved water holding capacity (5.2 - 5.6 g/g protein) compared with the control pea protein isolate (2.8 g /g). The pea proteins conjugated with guar gum showed exceptional emulsifying capacity (EC) and stability (ES) of up to 100 % compared with the control protein (EC of 58 % and ES of 48 %). Some sequentially modified pea proteins, such as transglutaminase crosslinking followed by guar gum conjugation had multiple functional enhancement (water holding, oil holding, emulsifying, and gelation). The functionally enhanced pea proteins had comparable sensory scores as the control protein. These green modification

approaches yield functionally enhanced protein ingredients that will have broader applications in various food products.

Key words: pea protein isolate, enzymatic modification, conjugation, functionalities, plant protein, green processes

4.1 Introduction

The demand for food proteins is continually increasing worldwide, due to the rapid growth of global population and need for healthy and nutritious diets. Proteins are the essential building blocks and dietary macronutrients for the human body. In addition to the nutritional value, protein ingredients deliver crucial techno-functional properties that contribute to food quality and sensory characteristics (Chen et al., 2021). In recent years, plant proteins have attracted more attention from consumers because of their lower cost, energy efficiency, and environmental sustainability compared with animal proteins (Li, 2020).

Pea protein is one of the most used plant proteins, behind only wheat gluten and soy proteins and contains high levels of lysine, threonine, and tryptophan and has good digestibility, is non-transgenicity, and has low allergenicity (Fang, Xiang, Sun-Waterhouse, Cui, & Lin, 2020; Xiong et al., 2018). However, the commercial utilization of pea protein is still relatively limited, owing to its less desirable functional characteristics in some applications and beany flavor (Tamnak, Mirhosseini, Tan, Tabatabaee Amid, et al., 2016; Zha, Dong, Rao, & Chen, 2019b), which may be improved through physical, chemical, or enzymatic modifications. When pea protein suspension with higher concentration was served, people could feel the gritty texture, and lumps could be stuck in throat during swallowing (Fang et al., 2020).

Enzymatic deamidation using protein glutaminase was reported to modify pea proteins, which converts some amide groups (glutamine or asparagine) to carboxyl groups (glutamic acid or aspartic acid) (Chen et al., 2021; Fang et al., 2020). The deamidation modification increased the concentration of negatively charged carboxyl group and exposed some hydrophobic side

chains of the protein, which shifted the isoelectric point to the acidic side (Fang et al., 2020; Jiang et al., 2015). Some protein functional properties, such as solubility, foaming capacity, and emulsifying stability were improved through the enzymatic deamidation under appropriate conditions (Kunarayakul, Thaiphanit, Anprung, & Suppavorasatit, 2018). Previous studies reported that the enzymatic deamidation enhanced protein solubility in wheat gluten (Yiehui Yong, Yamaguchi, & Matsumura, 2006), zein (Yong, Yamaguchi, Gu, Mori, & Matsumura, 2004), and oat proteins (Jiang et al., 2015). The sensory profiles were affected, such as enhanced umami and reduced bitter flavor in deamidated wheat gluten, and reduced beany taste and lumpiness in deamidated pea protein (Fang et al., 2020; B. Liu, Zhu, Guo, Peng, & Zhou, 2017). Transglutaminase is another enzyme commonly used to modify food proteins, and it catalyzes the covalent crosslinking between amino group on lysine residues and carboxyamide group on glutamine residues in protein (Marco, Pérez, Ribotta, & Rosell, 2007). This modification can convert some soluble proteins to insoluble higher molecular weight polymers through inter-and intra-molecular interactions (Sun & Arntfield, 2011). In addition, many studies reported that pea protein modified by transglutaminase had enhanced gelation property (Shand, Ya, Pietrasik, & Wanasundara, 2008; Sun & Arntfield, 2011, 2012).

Protein-polysaccharide conjugation is another green approach to modify the protein through glycosylation reaction between the carbonyl groups of polysaccharide and amine groups of protein. The conjugation modification enhances protein hydrophilicity and affects the balance of protein hydrophilicity and hydrophobicity. The modified protein may have more favored protein-water interaction, resulting in some improved functional properties, for example, emulsification property (Tamnak, Mirhosseini, Tan, Tabatabaee Amid, et al., 2016; Zha, Dong, Rao, & Chen,

2019a). Pea proteins conjugated with pectin, gum arabic, and soybean polysaccharide showed improved emulsifying, foaming properties, solubility, and thermal stability (Lan, Chen, & Rao, 2018; Tamnak, Mirhosseini, Tan, Ghazali, & Muhammad, 2016; Zha, Yang, Rao, & Chen, 2019).

Previously, we investigated the effect of acylation or/and conjugation on pea protein functionalities, and we found that the sequential acylation and conjugation modifications had exceptional synergistic and positive effects on protein emulsification, oil holding capacity, and gelation properties (Shen & Li, 2021). Because of the concerns of using synthetic chemicals such as acetic anhydride or succinic anhydride during acylation modification, the aim of this study was to develop greener approaches based on enzymes and natural polysaccharides for protein functional enhancement. Although some previous studies have reported the functional improvement of plant proteins through enzymatic or conjugation modification alone with different enzymes or polysaccharides, combining both modifications may deliver some synergistic effects and produce more functional protein ingredients. Therefore, the objective of this study was to enhance the functional properties of pea protein through sequential enzymatic modification and polysaccharides conjugation, in comparison with enzymatic modification or polysaccharide conjugation alone, and understand the physicochemical properties of the modified proteins. The new modification methods has many advantages, such as clean-label, mild reaction, safety, and efficiency. The newly modified and functionally enhanced pea proteins will further expand the uses of plant proteins in broader food applications and better meet the increasing protein demands.

4.2 Materials and methods

4.2.1 Materials

Yellow pea flour was provided by ADM (Chicago, IL, USA). Guar gum (Judee's, Plain City, OH, USA), gum arabic (Fisher Scientific, Hampton, NH, USA), protein-glutaminase (Amano Enzyme Inc, Nagoya, Japan), and transglutaminase (Modernist pantry, Eliot, ME, USA) were used as received. Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

4.2.2 Preparation of pea protein isolate

The yellow pea flour was first defatted with hexane. The defatted yellow pea flour was dispersed in distilled water at a 10% solid concentration. The pH was adjusted to 8.5 using 1.0 M NaOH, and the slurry was mixed at 500 rpm for 1 hour at room temperature. Then, the slurry was centrifuged at 8000 ×g for 20 min at 4 °C. The supernatant was collected, and pH was adjusted to 4.5 using 1.0 M HCl, which was then allowed to precipitate the protein at 4 °C for 2 hours. After that, the protein was recovered by centrifugation (8000 ×g, 20 min), washed twice using distilled water, and re-adjusted to pH 7.0. Finally, the protein suspension was lyophilized and stored at 4 °C for further study.

4.2.3 Preparation of modified pea proteins

Enzymatically modified pea proteins were prepared by reacting the protein (10% concentration in water) with 1% transglutaminase at 40 °C or 1% protein-glutaminase (pH 6.5) at 55 °C for 3 hours, respectively. At the end of the reaction, the protein slurry was heated to 100 °C to inactivate the enzyme. Conjugated pea proteins were prepared by incubating the protein (10% concentration in water) with 5% guar gum or gum arabic (protein basis) at 60 °C for 24 hours. Enzyme treated/polysaccharide conjugated proteins were also prepared to investigate their synergistic effects, where after the deactivation of the enzyme, the protein slurry was added with guar gum/

gum arabic (5%, protein basis) at 60 °C for 24 hours. The slurries of modified proteins were lyophilized and stored at 4 °C till further analysis.

4.2.4 Functional properties

Protein functional properties, including solubility, emulsifying properties, water and oil holding capacities, and least gelation capacity were determined using our previous methods without modification (Shen & Li, 2021).

4.2.5 Physicochemical properties and *in vitro* gastrointestinal digestibility

Protein physicochemical properties, including free sulfhydryl group content, free amino group content, protein secondary structures, surface hydrophobicity, and *in vitro* gastrointestinal digestibility were determined following previous methods without any modification (Shen & Li, 2021).

Size exclusion chromatography (SEC-HPLC) was conducted to estimate molecular size changes of pea proteins with different modifications. The protein sample (1 mg/mL) was dispersed in sodium phosphate buffer (pH 6.8). The suspension was vortexed and vigorously mixed for 1 hr to dissolve the protein, followed by centrifugation at 4000 ×g for 5 min. The supernatant was collected and filtered through a 0.45 μm filter (Biomed Scientific, Forest, VA, USA). The protein separation was achieved using a Phenomenex SEC-4000 column (7.8 ×300 mm) at 30 ºC with Agilent 1100 HPLC system (Santa Clara, CA, USA). The mobile phase included phase A (water with 0.1 % trifluoroacetic acid) and phase B (acetonitrile), with gradient elution of 20 % phase B at 0 – 20 min, 30% phase B at 20 – 25 min, 35 % phase B at 25 – 40 min, and 20% phase B again at 40 min to elute all the residues. Flow rate was set at 0.7 mL/min. Proteins were detected at 214 nm using a diode array detector (Agilent, Santa Clara, CA, USA).

4.2.6 Sensory analysis

Descriptive sensory analysis of pea and the modified pea proteins was conducted by six well-trained panelists to determine the flavor characteristics, including beany, starchy, grain, green, powdery mouthfeel, umami, sweet, astringent, bitter, and metallic flavors. The descriptive analysis was conducted using a universal intensity scale with 0.5 increments (0 = absence of sensation; 15 = extremely intense sensation). For each protein sample, 1.2 g protein was dispersed in 30 mL distilled water to obtain an aqueous dispersion of 4%. The protein dispersion was placed in a transparent cup with a lid labeled with a randomly selected three-digit code. Before being served, the panelists manually remixed the suspension to achieve a homogenous dispersion. Pure water, unsalted crackers, and mozzarella cheese were used for mouth rinsing between samples to avoid any carry-over effect. The references and definitions of flavor attributes used for this study were provided in the Supplementary Document. The sensory analysis was approved by the KSU Institutional Review Board committee, IRB-5930.

4.2.7 Statistical analysis

All the tests were conducted in at least duplicates, and the results were presented as mean± standard deviation (SD). All the results were evaluated by one-way ANOVA, and Tukey's post-hoc test was conducted using SAS University Edition software (SAS Institute, Cary, NC, USA) to assess the significant differences ($p < 0.05$) among different treatments.

4.3 Results and discussion

4.3.1 Free sulfhydryl group and free amino group

The free sulfhydryl (SH) content of the control and modified pea proteins is summarized in Table 1. The enzymatically modified or/ and conjugated pea proteins showed significantly reduced free SH content than the control pea protein (13.5 µmol/g). The pea protein deamidated

by PG, crosslinked by TG, and conjugated with guar gum or gum arabic all had decreased free SH group, which was attributed to the fact that the mechanical mixing in air condition during the modification processes favored the oxidation reaction by converting some free SH groups to disulfide bonds (Netto et al., 2007). The conjugated proteins exhibited significantly lower free SH group content than the enzymatically modified proteins, which was ascribed to the higher reaction temperature during the conjugation than the deamidation and crosslinking reactions; thus, more disulfide linkages were formed. The sequentially modified proteins exhibited even lower free SH group content than the proteins from deamidation or crosslinking reaction alone, which is because the former proteins underwent heat treatments twice during the combined modifications.

Free amino group content indicates the degree of enzymatic and conjugated modifications in the modified pea proteins, as the amino group was a major reaction site during the modifications. Overall, all the modified pea proteins showed significantly ($p < 0.05$) lower content of free amino group compared with the control protein (8.44 mmol/g) (Table 1). The pea protein crosslinked by transglutaminase or/ and conjugated with guar gum or gum arabic exhibited the lowest free amino group content, which was attributed to formation of ε-(γ-Glu)-Lys polymers with the free aminos (Sun & Arntfield, 2011). The decreased free amino group in deamidated proteins is because the conversion of amide groups to carboxyl groups in the presence of protein glutaminase, during which ammonia was formed, and free amino group content was reduced. Furthermore, the reduced free amino group in the proteins conjugated with gums was due to the Maillard reaction that consumed some amino groups (Zha, Dong, et al., 2019a).

4.3.2 Protein secondary structures

Protein secondary structure compositions of the control and modified pea proteins derived from FTIR spectra, including α-helix, β-sheet, β-turn, and random coil, are summarized in Table 1. The control pea protein consisted of 18.64% α-helix, 27.52% β-sheet, 11.48% β-turn, and 42.37% random coil. With different modifications, the secondary structure composition was greatly changed. For example, the proteins modified by PG, TG, guar gum, and gum arabic did not have any random coils, while the proteins modified by TG, guar gum, and gum arabic had greatly increased α-helix and β-sheet, and the protein modified by PG and TG had increased β-turn, compared with the control. However, the sequential enzymatic and conjugated modifications increased the random coil, reduced β-turn, and slightly reduced α-helix contents (in PG-Guar and PG-Arabic) compared with the enzymatic or conjugated protein alone. These results demonstrated that the enzymatic or conjugated modifications enabled the protein to be unfolded, and some random structures could be converted to more regular and ordered structures. Jiang et al. (2015) reported that α-helix content was increased in deamidated oat protein compared with the control because of increased flexibility protein molecules. Further, they observed that β-sheet was decreased with higher degree of protein deamidation. Mattice and Marangoni (2021) reported that both β-sheet and random coil were increased in TG crosslinked zein. Therefore, it can be concluded that secondary structure composition of modified proteins was affected by the nature of the modification, degree of modification, enzyme and protein types, and extent of non-covalent interactions.

4.3.3 Surface hydrophobicity

Protein surface hydrophobicity was measured to estimate the availability of nonpolar amino acid residues exposed to the surface of the protein (Vanessa Cabra, Roberto Arreguin,

Rafael Vazquez-Duhalt, & Amelia Farres, 2006). Overall, the enzyme modified or/ and conjugated pea proteins showed significantly decreased surface hydrophobicity compared with the control, except for the PG-Arabic (Figure 1). The decreased surface hydrophobicity for the protein deamidated by PG might be because the deamidation modification increased carboxylic acid residues and favored hydrophobic interactions of the protein (Chen et al., 2021). Our result agreed with that reported by Miwa et al. (2013), who showed that deamidated whey protein by protein glutaminase had decreased surface hydrophobicity. However, some other studies reported increased surface hydrophobicity for deamidated proteins, such as barley hordein (Zhao, Tian, & Chen, 2010), wheat gluten (Qiu, Sun, Cui, & Zhao, 2013), and zein (Cabra, Arreguin, Azquez-Duhalt, & Farres, 2007). Surface hydrophobicity of deamidated proteins are affected by many factors, such as protein type and original hydrophobicity/hydrophilicity, enzyme concentration, and other reaction parameters (Chen et al., 2021). The proteins crosslinked by transglutaminase (e.g., TG, TG-Guar, TG-Arabic) showed dramatically decreased surface hydrophobicity compared with the control and other modified proteins, which was attributed to the aggregated proteins formed during crosslinking and partial burial of the hydrophobic cavities in the protein core (Agyare & Damodaran, 2010), thus reduced protein surface hydrophobicity. Shen et al. (2021) indicated that freeze-dried quinoa protein had higher surface hydrophobicity than spray-dried protein, which was attributed to the extent of protein denaturation during the different drying processes.

4.3.4 SEC-HPLC

The size exclusion chromatograms of the pea and modified pea proteins are shown in Figure 2. Four proteins with known molecular sizes, including thyroglobulin bovine (670 kDa), γ-globulins from bovine blood (150 kDa), bovine serum albumin (60 kDa), and chicken egg grade

VI albumin (44 kDa), were separated with the same chromatography conditions and marked on the chromatogram as molecular weight references. With enzymatic modification and/or conjugation with polysaccharides, some proteins with larger molecular sizes were formed compared to those in the control pea protein, as indicated by the left shift of the first peak (670 kDa) on the chromatograms. The modified pea proteins from conjugation alone (e.g., Guar, Arabic) had similar peak patterns as the control, except that the peak size between 150-670 kDa was increased, while the peak around 670 kDa was relatively decreased, which was caused by the alteration of the sizes of medium molecule proteins during conjugation. For all the modified proteins involving enzymatic treatment, there was a dramatic decrease of peak sizes in the range of 60 to 150 kDa, which was caused by the formation of larger proteins (670 kDa) through various crosslinking mechanisms. The mechanical mixing during the enzymatic and conjugation modifications along with elevated temperature favored the oxidation reaction to induce protein crosslinking. The PG and TG protein samples underwent enzyme deactivation (i.e., boiling the protein slurries at 100 °C for 10 min) after protein deamidation and crosslinking reactions, which also favored protein crosslinking, besides the enzymatically induced crosslinking reactions. Furthermore, the sequential enzymatic and conjugated proteins exhibited even larger molecular size, especially for the TG-Guar and TG-Arabic samples. Several peaks disappeared, and some small peaks were merged into one prominent peak, similar to the sample TG. This SEC-HPLC result can be associated with the free sulfhydryl content (Table 1) and confirmed that the modified pea protein had exhibited a larger molecular size partially due to the protein crosslinking reaction.

4.3.5 Solubility

The control pea protein, which was extracted from pea flour in the lab and lyophilized, exhibited great solubility when the pH was away from the isoelectric point (PI, pH 4-5). The solubility was also much better than commercial pea protein (Shen and Li (2021), which implied that the commercial processing conditions of the proteins might cause more intensive structural denaturation that impaired the solubility (Zha, Dong, et al., 2019a). With the enzymatic or/ and conjugation modifications, most of the modified pea protein had similar or decreased solubility than the control pea protein when the pH was away from the PI, while the modified pea proteins had slightly increased solubility at the PI (Figure 3). Some of the pea proteins crosslinked with transglutaminase (e.g., TG, TG-Guar) were the least soluble at pH above the PI compared with the other modified protein samples. Pea protein contains high amount of lysine, and it favors the crosslinking reaction catalyzed by transglutaminase (Marco et al., 2007). This reaction enabled the formation of larger protein polymers, which became less soluble (Marco et al., 2007). However, it should be noticed that the protein sample treated with TG and gum arabic had much greater solubility at PI and pH 11 compared with the control and TG and TG-Guar proteins, which may be attributed to the synergistic effects of transglutaminase and gum arabic modifications. Zha, Dong, et al. (2019a) reported that commercial pea protein conjugated with gum arabic showed significantly improved solubility, because the less soluble 11S and 7S subunits of pea protein and hydrophilic gum arabic were involved in forming conjugates, which improved the overall solubility. Shen and Li (2021) also reported a similar finding showing improved solubility for commercial pea protein isolate conjugated with guar gum. Even for the lab extracted protein, our results showed that pea protein conjugated with gum arabic or treated with PG-Arabic had slightly increased solubility at pH 4.5-7 compared with the control and other

treatments. Previous studies reported that enzymatic deamidation improved the solubility of gluten proteins (Yiehui Yong et al., 2006) and zein proteins (Yong et al., 2004), because the induction of additional carboxyl groups to the protein molecules provided a newly balanced amphiphilicity that favored protein interaction with water. As for some of our modified pea proteins from deamidation or/ and conjugation, the solubility was not improved, which was because the native structure of the control pea protein was more favorable to solubility, compared to the denatured and modified structures.

4.3.6 Water and oil holding capacity

Water and oil holding capacities of the pea and modified pea proteins are summarized in Table 2. Overall, the proteins treated by transglutaminase, for example, TG, TG-Guar, and TG-Arabic, had significantly higher water holding capacities of 5.31, 5.62, and 5.21 g water /g protein, respectively, compared with the control pea protein (2.66 g/g). In addition, the PG-Guar also exhibited a significantly higher water holding capacity of 5.06 g/g. Transglutaminase catalyzed covalent crosslinking between lysine and glutamine residues in forming inter- or intra-molecular ε-(γ-Glu)-Lys polymers, which resulted in larger protein molecules and more intensive protein aggregation, favoring water holding capacity (Sun & Arntfield, 2011). Further, the newly formed crosslinking structures may enhance protein gel formation with better water holding capability due to the stronger hydrogen-bonded water shown in Raman bands (Kang et al. (2016)). The pea proteins modified by protein glutaminase or guar gum alone also had improved water holding capacity up to 3.62 g/g compared with the control. With sequential modification using both protein glutaminase and guar gum, the water holding capacity was further improved to 5.06 g/g, implying synergistic effects from multiple modification approaches.

The control pea protein had an oil holding capacity of 2.76 g oil/g protein, which was more than twice of that reported for commercial pea protein (1.03 g/g) (Shen & Li, 2021). Among all the modified pea proteins, the PG-Guar protein exhibited significantly higher oil holding capacity than the control and other treatments (Table 2). However, the oil holding capacity of the protein deamidated by protein glutaminase or conjugated with guar gum alone did not significantly differ from the control protein, which may be attributed to their lower surface hydrophobicity as compared to the control or PG-Guar (Figure1). The PG-Guar treatment showed synergistic effect benefiting oil holding capacity. The oil holding capacity of pea protein conjugated with guar gum was similar to the control protein in this study, all around 2.6 - 2.7 g/g. Shen and Li (2021) reported that the commercial pea protein conjugated with guar gum had significantly increased oil holding capacity (2.02 g/g) than the control protein (1.03 g/g). This was because the heat treatment during the conjugation had altered and unfolded protein structures, and more hydrophobic amino acid residues were exposed, resulting in improved oil holding capacity.

4.3.7 Emulsifying properties

The emulsifying characteristics of proteins, including emulsion capacity (EC) and emulsion stability (ES), are affected by the rate of protein adsorption and the ability to reorganize at the oil/water interface during emulsifying. The protein molecules act as barrier against the droplet coalescence and provide steric and electrostatic repulsions against flocculation in forming stable interfacial layer (Ma, Forssell, Partanen, Buchert, & Boer, 2011). As shown in Table 2, some of the modified pea proteins possessed greatly ($p < 0.05$) improved emulsifying properties than the control pea protein (EC: 58 %, ES: 48 %), especially for the treatments involving guar gum, such as Guar, PG-Guar, and TG-Guar with emulsion capacity of 97 -100%

and emulsion stability of 96 - 100%. On the other side, the pea proteins conjugated with gum arabic (i.e., Arabic, PG-Arabic, TG-Arabic) had similar emulsifying properties as the control. Gum arabic has a very different structure compared with guar gum, and it is a complex mixture of glycoproteins and polysaccharides predominantly consisting of arabinose and galactose. After conjugating with pea protein, the proteins with guar gum seem to have a more balanced hydrophilicity and hydrophobicity that favored their surface activities at oil/water interface compared to the proteins with gum arabic. Gum arabic had a relatively low hydration radius and effective volume (Bai, Huan, Li, & McClements, 2017), and it is less viscous than guar gum when applied at the same concentration in water. The conjugated proteins with gum arabic might be insufficient to span the surface of oil droplet when used at the same concentration as the protein conjugates with guar gum, resulting in the destabilization or flocculation of protein emulsions (Liu, Elmer, Low, & Nickerson, 2010).

The emulsifying properties of the protein deamidated by PG were not significantly different from the control, while the protein crosslinked by TG had significantly increased emulsion capacity and stability, although the stability was still much lower than those conjugated with guar gum. The interfacial film formed by the crosslinked protein by transglutaminase had higher resistance to destabilization, and relatively lower solubility of the crosslinked protein enabled a thicker interface with better steric stability, thus improved emulsion capacity (Nivala, Nordlund, Kruus, & Ercili-Cura, 2021). However, the absorption of the crosslinked proteins at the oil and water interface was not able to sustain the environmental stress (e.g., high temperature and shearing) during stability tests due to the larger molecular sizes and lack of molecule flexibility, which led to lower surface coverage and decreased emulsion stability

(Færgemand, Otte, & Qvist, 1998). The pea protein deamidated by protein glutaminase had no significant differences with the control protein, because the protein deamidation had increased carboxylic acid residues and improved electrostatic repulsion, but it might weaken the hydrophobic interaction and hydrogen bonds, which resulted in structures that were less surface active (Chen et al., 2021). In summary, the sequential enzymatic modification and conjugation (PG-Guar and TG-Guar) had synergistic effects on the emulsifying properties, implying that protein functionalities could be better enhanced by combining different modifications approaches.

4.3.8 Protein gelation property

Heat-induced gelation is one of the most important functional properties of protein, as it is associated with the texture, quality, and sensory of the foods. When pea protein slurry was heated above the denaturation temperature, the globulins were unfolded and rearranged to form soluble aggregates; while when the protein solution was cooled, the electrostatic repulsions were reduced between the aggregated proteins, and the proteins were assembled to form the structured get network entrapping water molecules (Jean-Luc Mession, Nicolas Sok, Ali Assifaoui, 2013). The control pea protein had a good gelation potential, with a least gelation concentration (LGC) of 11%, which was much lower than that of commercial pea protein (LGC of 18%). The modified pea proteins from guar gum conjugation (i.e., Guar) or transglutaminase crosslinking plus conjugation (i.e., TG-Guar, TG-Arabic) had further significantly improved gelation property with LGC of 9%, compared with the control protein (Table 2). The inclusion of guar gum during the protein conjugation can unfold the protein structure and enhance the hydrophobic interaction to create more stable and firm gel networks (Shen & Li, 2021). The addition of transglutaminase in the protein promoted the crosslinking among protein molecules and improved gelation ability

(Sun & Arntfield, 2011). On the other side, the proteins deamidated by glutaminase (i.e., PG, PG-Arabic, PG-Guar) had significantly decreased gelling property than the control, which might be partially attributed to the increased electrostatic repulsion between carboxylic acid groups (Miwa et al., 2013). The pea protein conjugated with gum arabic alone did not show gelation improvement, as contract to that with guar gum. This was probably related to the lower viscosity of gum arabic in water than guar gum (Saha & Bhattacharya, 2010). In addition, Alam et al. (2021) reported that the taro starch with guar gum had lower swelling power due to the fact that the tightening of starch granules restricted the exudation process, and improved gelation property. However, the gum arabic effectively facilitates the water penetration and eventually increases the swelling power due to the increased interactions between gelatinized starch granules; thus, the taro starch with gum arabic exhibited poorer gelation. Some of the polysaccharide properties may be carried over to the conjugated proteins and affect protein functional properties. The protein crosslinked by transglutaminase and followed by conjugation showed synergistic advantage in improving gelation property. These combined modification approaches could be used in many food applications that rely on protein gelation, such as condiments, meat patties, dairy, and cake batter products.

4.3.9 *In vitro* gastrointestinal digestibility

The digestibility of the pea and modified pea proteins was determined and presented as degree of hydrolysis of the proteins after the *in vitro* gastrointestinal digestion (Figure 4). Overall, the modified pea proteins showed significantly decreased digestibility ($p < 0.05$) compared with the control pea protein, except for the sample PG, which was also reduced but not significantly different from the control ($p > 0.05$). The conjugated proteins and the proteins modified by a combination of enzymatic crosslinking and conjugation had increased molecular

weight and were more potent to aggregate; thus, they became less accessible to the digestible enzymes as compared with the control. Gan et al. (2009) and Glusac et al. (2020) reported that soy and chickpea proteins crosslinked with transglutaminase also had decreased digestibility. The treatment of pea protein with protein glutaminase increased protein electrostatic repulsion, which may favor enzyme accessibility during digestion. Qiu et al. (2013) reported that the deamidated gluten had decreased pepsin digestibility, which was attributed to the acidic shift of the protein's isoelectric point after deamidation and resulted in more protein aggregates under pepsin digestion condition (pH =2). However, the digestibility of the deamidated gluten was increased during pancreatin digestion due to increased solubility and lose of protein structures.

4.3.10 Descriptive sensory analysis

The sensory scores from descriptive analysis are summarized in Table S1 (Supplementary Document), and the principal component analysis (PCA) describing the relationships of different attributes from different treatments is presented in Figure S1 (Supplementary Document), respectively. Overall, the modified pea proteins had comparable sensory scores for most attributes as the control pea protein, and all the modification treatments did not obviously decrease most sensory scores (Table S1). One interesting observation is that the proteins crosslinked with transglutaminase (e.g., TG, TG-Guar, TG-Arabic) had obviously increased pulpy mouthfeel (scores 3 – 5) compared with the control (score 0), which was attributed to the increased protein molecular sizes and aggregation because of crosslinking. The umami taste of several modified proteins (PG, PG-Guar, TG-Guar) was reduced to zero compared with the control (score 2). All the modified proteins had similar scores for beany related unpleasant attributes (beany, green, astringent, bitter, metallic) as the control. Principal component analysis was carried out to further understand the relationship between sensory

attributes and different modified proteins (Figure S1). Eigenvalues 1 and 2 from the biplot showed 56.9% of the variability, which indicated that some association of sensory characteristics and different modified pea proteins existed.

4.4 Conclusions

Enzymatic modification and/or conjugation with polysaccharides altered pea protein secondary structure compositions, molecular sizes, surface hydrophobicity, and contents of free sulfhydryl and amino groups, thus resulting in different functional characteristics. The pea proteins conjugated with guar gum (i.e., Guar, PG-Guar, TG-Guar) had greatly enhanced emulsifying properties compared with the control pea protein. The pea proteins crosslinked by transglutaminase (i.e., TG, TG-Guar, TG-Arabic) had water holding capacity twice of that of the control. Sequential modification of pea protein with transglutaminase and guar gum (TG-Guar) led to multiple functional enhancement of pea protein, including increased water holding capacity, oil holding capacity, emulsion capacity, emulsion stability, and gelation, and decreased protein solubility. The modified pea proteins had comparable sensory scores as the control pea protein, and these modifications overall did not negatively affect protein sensory properties. However, the modified pea proteins showed decreased *in vitro* gastrointestinal digestibility compared with the control protein. The newly developed pea proteins through green modifications may expand their uses in various food applications and better meet the increasing demand for more functional plant proteins.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Figures and tables

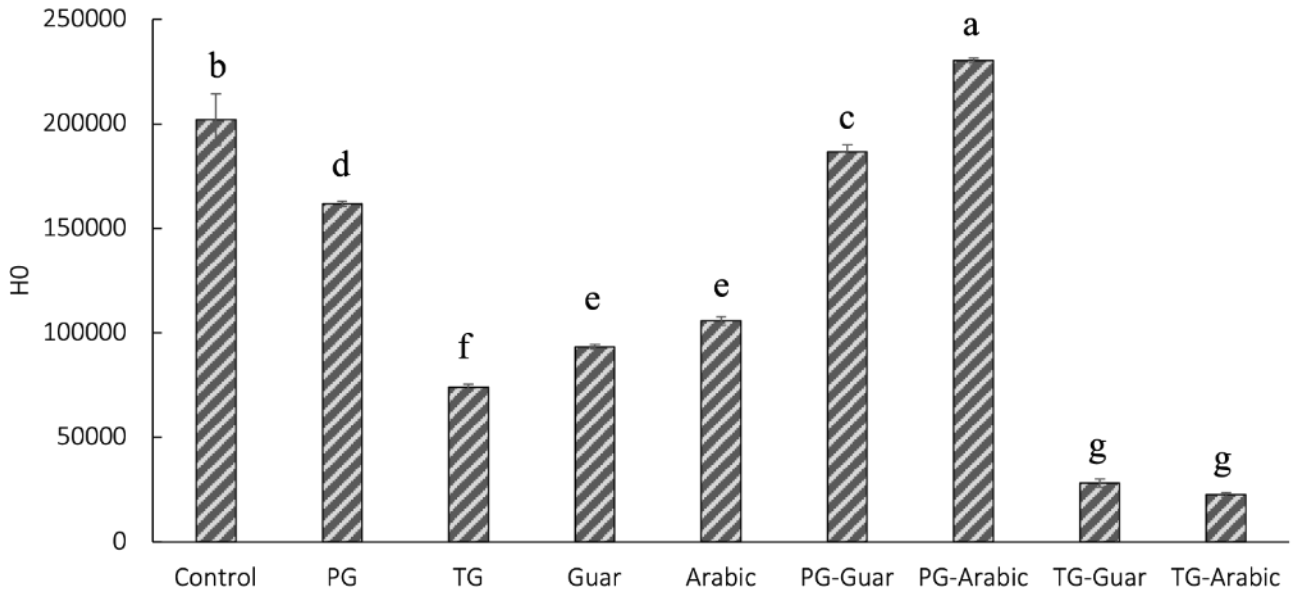

Figure 4.1 Surface hydrophobicity of pea and the modified pea proteins.
*Means with different letters indicate significant differences ($p < 0.05$).

Control: control pea protein; **PG:** pea protein deamidated with protein glutaminase; **TG:** pea protein crosslinked with transglutaminase; **Guar:** pea protein conjugated with guar gum; **Arabic:** pea protein conjugated with gum arabic; **PG-Guar:** deamidated pea protein conjugated with guar gum; **PG-Arabic:** deamidated pea protein conjugated with gum arabic; **TG-Guar:** crosslinked pea protein conjugated with guar gum; **TG-Arabic:** crosslinked pea protein conjugated with gum arabic.

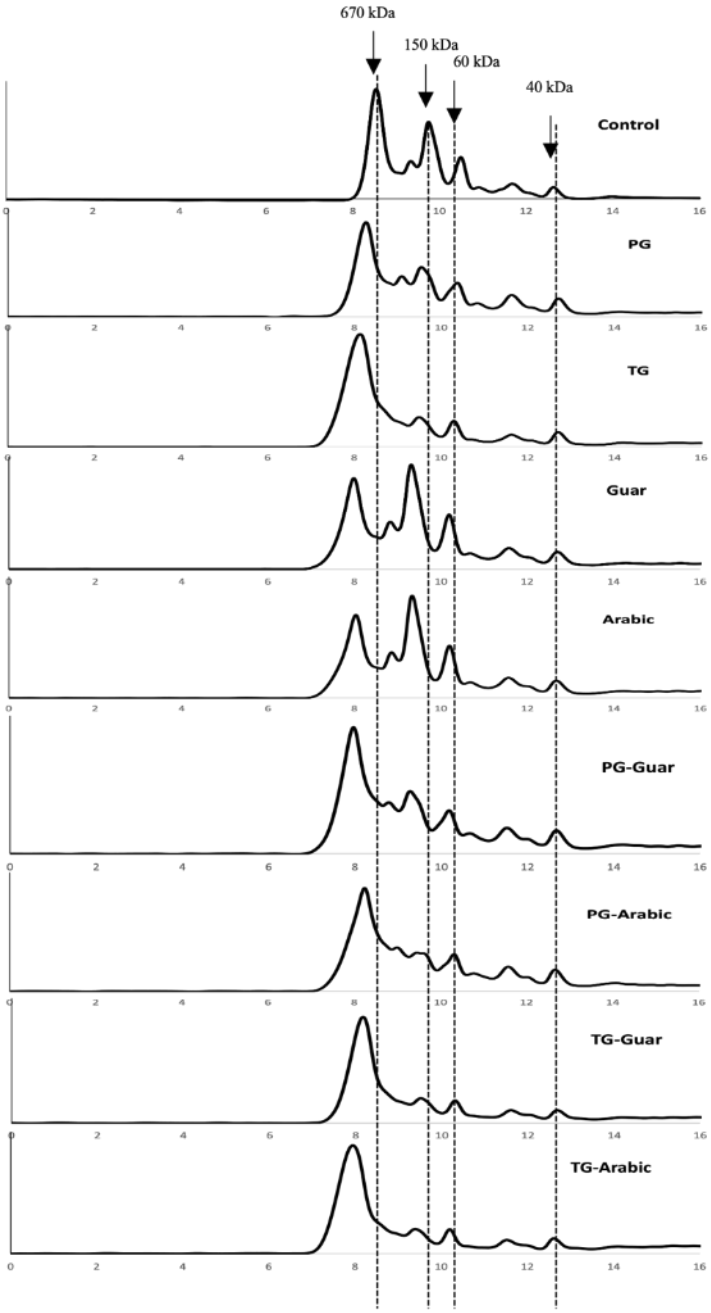

Figure 4.2 SEC-HPLC chromatograms of the control and modified pea proteins.

*__Control:__ control pea protein; **PG:** pea protein deamidated with protein glutaminase; **TG:** pea protein crosslinked with transglutaminase; **Guar:** pea protein conjugated with guar gum; **Arabic:** pea protein conjugated with gum arabic; **PG-Guar:** deamidated pea protein conjugated with guar gum; **PG-Arabic:** deamidated pea protein conjugated with gum arabic; **TG-Guar:** crosslinked pea protein conjugated with guar gum; **TG-Arabic:** crosslinked pea protein conjugated with gum arabic.

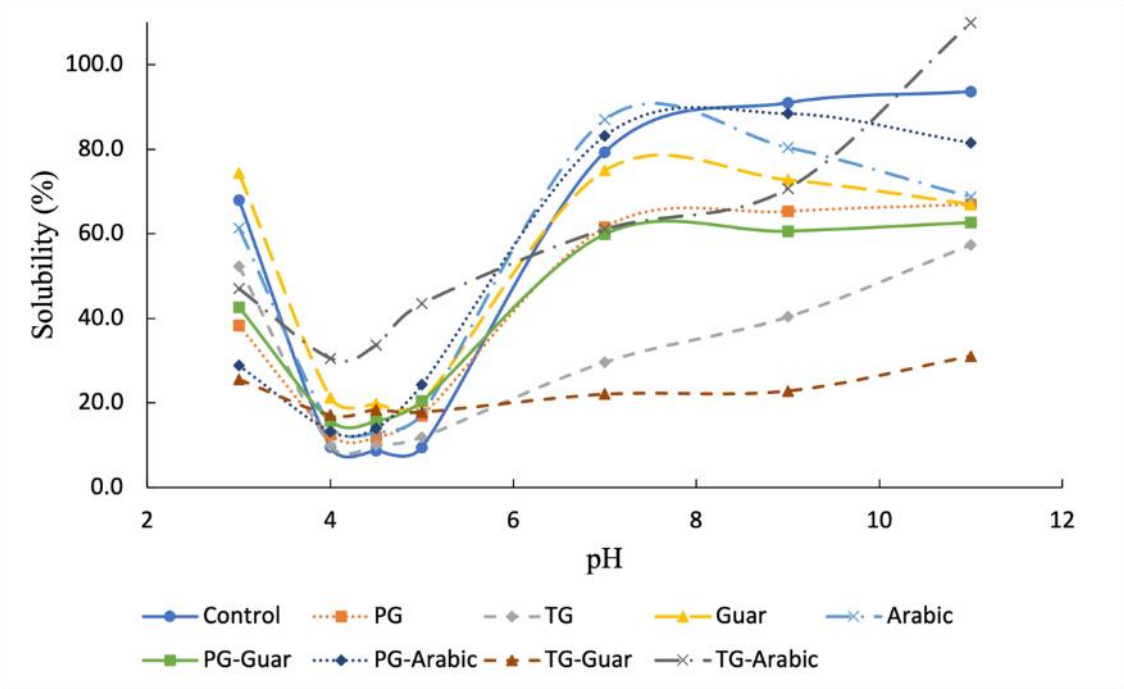

Figure 4.3 Solubility of pea and modified pea proteins.

*__Control:__ control pea protein; __PG:__ pea protein deamidated with protein glutaminase; __TG:__ pea protein crosslinked with transglutaminase; __Guar:__ pea protein conjugated with guar gum; __Arabic:__ pea protein conjugated with gum arabic; __PG-Guar:__ deamidated pea protein conjugated with guar gum; __PG-Arabic:__ deamidated pea protein conjugated with gum arabic; __TG-Guar:__ crosslinked pea protein conjugated with guar gum; __TG-Arabic:__ crosslinked pea protein conjugated with gum arabic.

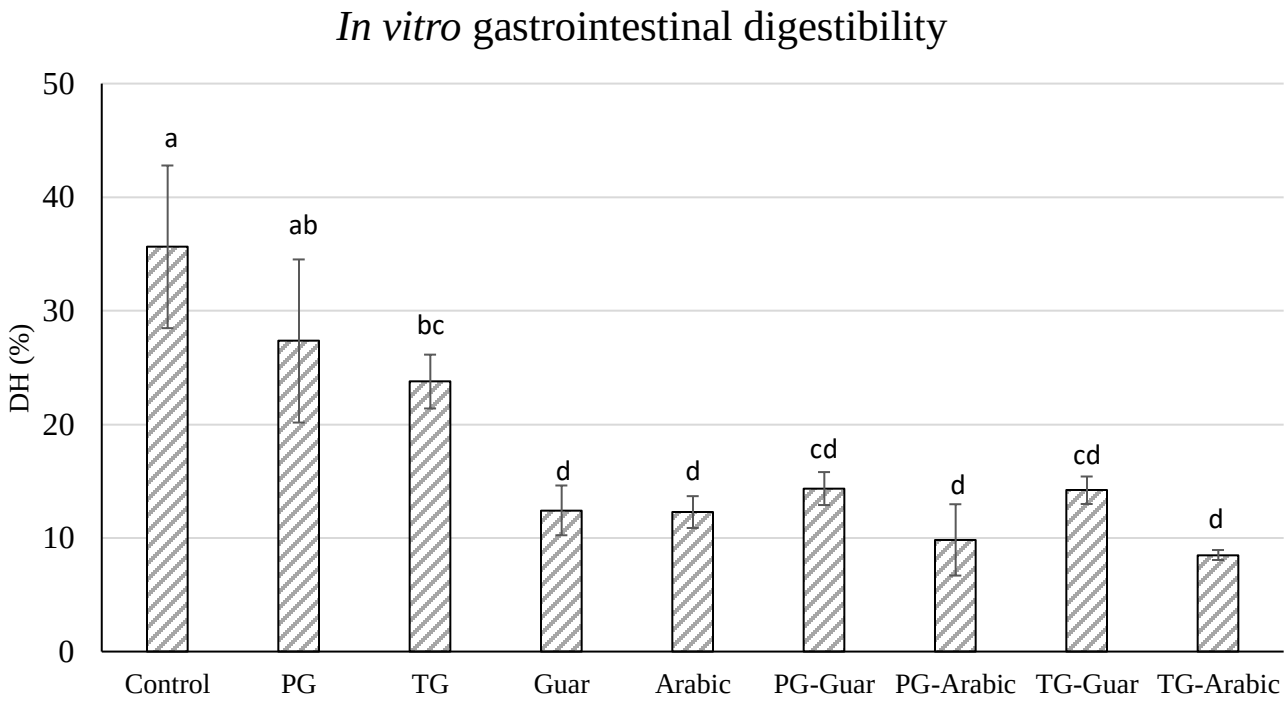

Figure 4.4 *In vitro* gastrointestinal digestibility (DH%) of pea and modified pea proteins.
*Means with different letters indicate significant differences ($p < 0.05$).

Control: control pea protein; **PG:** pea protein deamidated with protein glutaminase; **TG:** pea protein crosslinked with transglutaminase; **Guar:** pea protein conjugated with guar gum; **Arabic:** pea protein conjugated with gum arabic; **PG-Guar:** deamidated pea protein conjugated with guar gum; **PG-Arabic:** deamidated pea protein conjugated with gum arabic; **TG-Guar:** crosslinked pea protein conjugated with guar gum; **TG-Arabic:** crosslinked pea protein conjugated with gum arabic.

Table 4-1 Physicochemical properties including free sulfhydryl group content, free amino group content, secondary structures of pea and modified pea proteins.

Samples	Free SH (μmol/g)	Free NH_2 (mmol/g)	α-helix (%)	β-sheet(%)	β-turn (%)	random coil (%)
Control	13.5±0.1^{a}	8.4±0.1^{a}	18.6±0.1^{cd}	27.5±4.4^{bc}	11.5±2.3^{bc}	42.4±6.6^{a}
PG	9.9±0.1^{c}	7.5±0.1^{b}	53.7±0.5^{a}	26.7±3.0^{bc}	19.6±2.5^{a}	ND
TG	11.9±0.1^{b}	5.3±0.1^{c}	22.0±1.6^{c}	60.7±3.3^{a}	17.3±1.7^{ab}	ND
Guar	7.7±0.0^{d}	7.3±0.2^{b}	37.6±1.6^{b}	52.5±0.8^{a}	9.8±0.8^{cd}	ND
Arabic	6.5±0.0^{ef}	7.6±0.2^{b}	41.2±10.4^{ab}	48.1±9.8^{ab}	10.7±0.6^{cd}	ND
PG-Guar	5.6±0.0^{g}	7.3±0.3^{b}	9.7±0.4^{cd}	48.0±1.3^{ab}	6.9±0.1^{cd}	35.5±1.1^{a}
PG-Arabic	4.9±0.0^{h}	7.4±0.2^{b}	7.4±1.0^{d}	58.9±1.7^{a}	4.9±0.3^{d}	28.8±2.5^{a}
TG-Guar	6.7±0.0^{e}	5.4±0.1^{c}	10.3±2.1^{cd}	56.6±12.1^{a}	6.1±0.8^{cd}	27.1±15.1^{a}
TG-Arabic	6.3±0.2^{f}	5.2±0.1^{c}	20.9±0.5^{cd}	22.3±4.2^{c}	8.9±2.3^{cd}	47.9±6.0^{a}

*Means with different letters in each column indicate significant differences ($p < 0.05$). ** ND: not detected.

****Control:** control pea protein; **PG:** pea protein deamidated with protein glutaminase; **TG:** pea protein crosslinked with transglutaminase; **Guar:** pea protein conjugated with guar gum; **Arabic:** pea protein conjugated with gum arabic; **PG-Guar:** deamidated pea protein conjugated with guar gum; **PG-Arabic:** deamidated pea protein conjugated with gum arabic; **TG-Guar:** crosslinked pea protein conjugated with guar gum; **TG-Arabic:** crosslinked pea protein conjugated with gum arabic.

Table 4-2 Functional properties of pea and modified pea proteins.

Samples	WHC (g/g)	OHC (g/g)	EC (%)	ES (%)	LGC (%)
Control	2.7 ± 0.1^{f}	2.8 ± 0.1^{c}	58.6 ± 2.2^{c}	48.1 ± 1.8^{d}	11^{d}
PG	3.6 ± 0.0^{d}	2.7 ± 0.1^{c}	63.5 ± 5.0^{bc}	51.9 ± 1.0^{cd}	15^{a}
TG	5.3 ± 0.1^{b}	3.1 ± 0.0^{b}	94.5 ± 0.3^{a}	57.7 ± 1.4^{b}	11^{d}
Guar	3.6 ± 0.0^{d}	2.6 ± 0.0^{cd}	97.9 ± 0.3^{a}	96.3 ± 1.0^{a}	9^{e}
Arabic	2.7 ± 0.0^{f}	2.5 ± 0.1^{d}	57.8 ± 4.1^{c}	52.1 ± 2.8^{c}	13^{b}
PG-Guar	5.1 ± 0.0^{c}	3.4 ± 0.1^{a}	100.0 ± 0.0^{a}	97.7 ± 0.1^{a}	12^{c}
PG-Arabic	3.3 ± 0.0^{e}	2.8 ± 0.0^{c}	67.6 ± 1.5^{b}	56.7 ± 2.2^{b}	15^{a}
TG-Guar	5.6 ± 0.0^{a}	3.0 ± 0.1^{b}	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}	9^{e}
TG-Arabic	5.2 ± 0.1^{b}	2.7 ± 0.0^{c}	66.5 ± 4.7^{b}	54.6 ± 2.0^{bc}	9^{e}

*Water holding capacity (WHC), oil holding capacity (OHC), emulsion capacity (EC), emulsion stability (ES), and least gelation capacity (LGC).

**Means with different letters for each column indicate significant differences ($p< 0.05$).

***__Control:__ control pea protein; **PG:** pea protein deamidated with protein glutaminase; **TG:** pea protein crosslinked with transglutaminase; **Guar:** pea protein conjugated with guar gum; **Arabic:** pea protein conjugated with gum arabic; **PG-Guar:** deamidated pea protein conjugated with guar gum; **PG-Arabic:** deamidated pea protein conjugated with gum arabic; **TG-Guar:** crosslinked pea protein conjugated with guar gum; **TG-Arabic:** crosslinked pea protein conjugated with gum arabic.

Chapter 5 - Modulating molecular interactions in pea protein to improve its functional properties

Abstract

Proteins exist in numerous spatial arrangements and are stabilized by various inter- and intra-molecular forces. Different denaturants such as sodium sulfite, urea, sodium dodecyl sulfate (SDS), and trypsin can interfere with protein molecule interactions, particularly disulfide bond, hydrogen bond, hydrophobic interaction, and peptide bond, respectively, which further alters protein secondary and tertiary structures and functional properties. The objectives of this study were to investigate the functional properties of pea protein isolate in terms of water/oil holding capacity, emulsifying and foaming properties, solubility, and gelation by modulating protein covalent and non-covalent interactions and understand the physicochemical characteristics (e.g., free amino group, free sulfhydryl, surface hydrophobicity, SDS-PAGE profile, secondary structures) of the unfolded pea proteins that are responsible for the functional changes. All the denatured proteins possessed significantly increased solubility. Both urea and SDS unfolded proteins had significantly higher water holding capacity and oil holding capacity with up to 5.01 and 5.09 g H_2O /g, and 3.06 and 2.84 g oil /g compared with the control pea protein (4.12 and 1.29 g), respectively. The proteins unfolded with urea or SDS also showed improved emulsification properties. The trypsin hydrolyzed protein exhibited the highest foaming capacity and better gelation properties among all the treatments. Principal component analysis indicted strong associations between protein functional and physicochemical properties and molecular interactions.

Key words: pea protein, covalent/ non-covalent interactions, functional properties, protein unfolding

5.1 Introduction

Pea protein is attracting increasing interest due to its sustainability, nutritional value, availability, and low allergenicity, and it has been used in various food products such as bakery, meat analogues, and beverages. Water-soluble albumins (15-25 %) and salt-soluble globulins (65-80 %) are the two major protein classes in pea (Burger & Zhang, 2019). Pea globulins can be further classified into legumin (11S) and vicilin (7S) proteins. Legumins are hexameric proteins comprised of six pairs of subunits with molecular weight around 360 - 400 kDa, with the acidic-basic (α - β) subunits covalently linked through disulfide bonds (Burger & Zhang, 2019; Zhan, Shi, Wang, Li, & Chen, 2019). Glutamic acid residue dominates in the α-chain, and alanine, valine, and leucine residues are the primary amino acids in the β-chain (Lam, Can Karaca, Tyler, & Nickerson, 2018). Vicilin proteins are glycosylated trimeric clusters with molecular weight of 160 - 200 kDa consisting of three subunits (α, β, and γ, ~50 kDa each) (Burger & Zhang, 2019; Lam et al., 2018). Unlike legumin, the subunits of vicilin are non-covalently connected through hydrophobic interaction (Lam et al., 2018). The vicilin protein contains high levels of arginine, lysine, aspartic and glutamic acid residues but low levels of methionine, cysteine, and tryptophan residues. Both legumin and vicilin are dominated by β- sheet structure with low content of α-helical structure (Lam et al., 2018; Owusu‐Ansah & McCurdy, 1991).

Proteins exist in numerous spatial arrangements, and they are stabilized by covalent and non-covalent interactions, namely disulfide linkage, hydrogen bonding, hydrophobic interaction, and electrostatic forces (Li, Wang, Chen, Yu, & Feng, 2018; Schmid, Prinz, Stäbler, & Sängerlaub, 2017; Ustunol, 2014). The disulfide linkage is formed via sulfhydryl oxidation reaction by two cysteine residues, and it can be inter- or intramolecular interaction in stabilizing the structure of

folded proteins (Ustunol, 2014). Hydrophobic interaction is the major force involved in protein folding, and it allows non-polar amino acid side chains orient towards the interior of a protein molecule in aqueous systems. This is driven by thermodynamically unfavorable interactions of nonpolar molecular structures with water (Ustunol, 2014). Hydrogen bonding is dipole-dipole molecular attractions involving a hydrogen atom that is covalently bonded to more electronegative atoms, such as oxygen, nitrogen, sulfur, and another electronegative atom. It can be inter- or intramolecular interaction depending on the donor's property and acceptor atom that constitutes the bonds. Most of the hydrogen bonding is formed among N-H or C=O groups in protein and determines protein secondary structures, such as α-helix and β-sheet (Ustunol, 2014). Understanding the influence and contribution of protein structure and interactions on its functional properties will allow modulating protein structures by design to achieve more desirable functional properties and broaden their applications.

Proteins are folded into three-dimensional rigid and compact structures in their native state (Neurath, Greenstein, Putnam, & Erickson, 1944). Protein denaturation occurs along with the reactions to cleave disulfide bond or peptide bond or interfere with hydrogen bond or hydrophobic interaction. This can be achieved by using specific denaturing agents such as sodium sulfite, protease, urea, and sodium dodecyl sulfate, respectively. Sodium sulfite is a reducing agent that can reduce protein disulfide crosslinking, resulting in free sulfhydryl groups. This reaction can be reversible when an oxidizing agent becomes available (Schmid et al., 2017). Urea is a non-electrolyte polar chemical, and it denatures protein through dehydrating protein molecules and causes repulsion between proteins by hydrophobic interaction and competitive hydrogen bonding. Urea breaks down the hydrogen bonds more efficiently than affecting

hydrophobic interactions (Liu & Hsieh, 2008). Sodium dodecyl sulfate is a surfactant containing a hydrophobic tail and hydrophilic head, which dominantly interrupts hydrophobic interactions in protein (Schmid et al., 2017). Proteases hydrolyze protein peptide bonds, resulting in smaller peptides with better solubility (Ustunol, 2015). These modifications can change protein functional properties by altering protein hierarchical structures.

To our knowledge, there is very limited information on the effect of modulating protein intra- and inter-molecular interactions on its functional properties. Therefore, the objectives of this study were to investigate the functional properties of pea protein isolate in terms of water/oil holding capacity, emulsifying and foaming properties, solubility, and gelation by modulating protein covalent and non-covalent interactions and understand the physicochemical characteristics (e.g., free amino group, free sulfhydryl, surface hydrophobicity, SDS-PAGE profile, secondary structure, and thermal properties) of the modified pea proteins that are responsible for the functional changes.

5.2 Material and methods

5.2.1 Materials

Pea protein isolate (83 % protein content, 5 % ash) was obtained from a commercial source. Trypsin (bovine pancreas) was purchased from Alfa-Aesar (Tewksbury, MA, USA). Sodium sulfite, urea, sodium dodecyl sulfate, 8-Anilinonaphthalene-1-sulfonic acid (ANS), 2-mercaptoethanol, 2,4,6-trinitrobenzene sulfonic acid (TNBS) and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

5.2.2 Preparation of denatured pea proteins

Protein denaturation was carried out by reacting the protein dispersed in deionized (DI) water (10 % protein concentration) with different denaturants, including sodium sulfite (0.5 and 1%, w/w), urea (0.2 and 1 M), and sodium dodecyl sulfate (0.1 and 0.5%, w/w), for 2 hours at room temperature. The product was then transferred into dialysis bags to dialyze against DI water for 48 hours at 4 °C to remove the modifiers. Pea protein was also hydrolyzed using trypsin at a protein concentration of 6 % (protein base). Briefly, 24 g pea protein was dispersed into 400 mL of DI water and pH was adjusted to 8, and then 50 mg of trypsin (0.2% based on protein) was added. The reaction continued in a water bath shaker at 50 °C for 30 min. At the end of the hydrolysis, the protein suspension was adjusted to pH 6 and boiled at 100 °C for 10 min to inactive the enzyme, followed by cooling at room temperature. The proteins were then lyophilized and kept in a refrigerator until further analysis.

5.2.3 Analysis of protein physicochemical properties

Physicochemical properties of pea and the denatured pea proteins, including free amino group content, free sulfhydryl content, surface hydrophobicity, SDS-PAGE profile, and secondary structure were analyzed following our previously published methods (Shen & Li, 2021) without modification. Thermal properties were analyzed using a DSC following the method described in Shen et al. (2021).

5.2.4 Analysis of protein functional properties

Protein functional properties, including water/ oil holding capacity, solubility, emulsifying/ foaming capacity and stability, and least gelation concentration were analyzed according to the methods described in our previous paper (Shen & Li, 2021).

5.2.5 Statistical analysis

All the tests were conducted in at least duplicate. The data were analyzed using SAS University Edition 1 software (SAS Institute, Cary, NC, USA) and evaluated by one-way ANOVA, Tukey's post-hoc comparison test. Significant differences among the data set were considered as $p < 0.05$. The results were presented as mean ± standard deviation. Principal component analysis (PCA) was carried out using XLSTAT 2021 (Addinsoft, New York, NY, USA) to determine the relationship between protein functional and physicochemical properties from different denaturation approaches.

5.3 Results and discussion

5.3.1 Free amino and sulfhydryl groups

The increased concentration of free amino group indicates exposure of lysine side chain and amino terminus from protein unfolding and peptide bond hydrolysis. The trypsin hydrolyzed pea protein had the highest free amino content (51.74 mmol/g, $p < 0.05$) compared with the control (11.62 mmol/g) and other denatured proteins (Table 1). The proteins denatured with 0.2 M urea (16.72 mmol/g) and 0.5% SDS (16.64 mmol/g) also showed significantly higher free amino group content than the control. With 1% sodium sulfite, the free amino group content appeared to be decreased (9.52 mmol/g), not significantly ($p > 0.05$), compared to the control and the protein with 0.5% sodium sulfite. This might be related to amino oxidation and protein refolding during disulfide bond cleavage and thiol-disulfide exchange in the presence of the reducing agent. As shown from the solubility results (Figure 1), the trypsin hydrolyzed protein had a great reduction of insoluble protein fractions due to the hydrolysis reaction, which was also accompanied by a dramatically increased free amino group content (Achouri, Zhang, & Shiying, 1998). Specifically, trypsin cleaves peptide bonds at arginine and lysine residues from C-

terminal (Olsen, Ong, & Mann, 2004). Urea and SDS primarily interrupt hydrogen bonding and hydrophobic interactions in the protein, respectively, and the denaturation effect can be partially reversible to a certain extent. Therefore, the increased amino content with 0.2 M urea and 0.5% SDS can be mainly attributed to the exposure of free amino groups from lysine residue and protein N-terminus, because of protein unfolding and denaturation. However, it should be noticed that minor protein hydrolysis might occur in the presence of chaotropic agent or detergent.

On average, pea protein isolate contains approximately 0.2% cysteine (Gorissen et al., 2018) and 1% cystine residues (Banaszek et al., 2019). The relative amount of free sulfhydryl and thiol group can vary because of disulfide reducing, sulfhydryl crosslinking, and thiol-disulfide exchanges, which are influenced by protein processing and modification. With 1% sodium sulfite, free SH content significantly increased compared to the control and other denatured proteins. Sodium sulfite is a well-known reducing agent that is capable of unfolding protein through cleaving the inter- and intra-molecular disulfide linkages (Zhu, Wang, & Sun, 2016), resulting in increased concentration of free SH. However, with other denaturation conditions, such as 0.5% sodium sulfite, 0.1% SDS, or 1 M urea, free SH content decreased significantly compared to the control. This result could be attributed to the change of pH and ionic condition, as well as mechanical mixing during the processes that favored the oxidation reaction by converting some free SH groups to disulfide bonds (Netto et al., 2007). Moreover, urea addition in the protein solution destabilized both the hydrogen bonding and hydrophobic interaction and exposed SH groups that can be easily oxidized (Xiong & Kinsella, 1990). Free SH content also greatly decreased in the protein from trypsin hydrolysis compared to the control. This is because

the hydrolysis reaction was conducted at elevated temperature (i.e., 50 °C). The greater availability of exposed free sulfhydryl groups, the thermal energy, and mechanical mixing all favored disulfide crosslinking (Auvergne et al., 2007).

5.3.2 Protein surface hydrophobicity

As typical globular proteins in aqueous condition, pea proteins consist of hydrophobic cores formed by hydrophobic side chains, which are surrounded by hydrophilic external surface rich in hydrophilic side chains when interacts with water in its native sate (Zhu et al., 2016). Overall, 0.5% SDS denatured pea protein exhibited the highest surface hydrophobicity ($p < 0.05$) compared with the control and other denatured proteins, while the proteins denatured with 0.5 and 1% sodium sulfite, 0.1% SDS, and hydrolyzed with trypsin had significantly lower ($p < 0.05$) surface hydrophobicity compared with the control (Table 1). The hydrophobic tail of SDS vigorously interacts with the hydrophobic core of the protein in water, resulting in largely exposure of hydrophobic side chains, thus increasing protein surface hydrophobicity. With lower amount of SDS (0.1%) or sodium sulfite (both 0.5 and 1%), although the denaturation can lead to the exposure of hydrophobic groups, the exposure of additional hydrophilic groups during protein unfolding seems to dominate the process, resulting in an overall decrease of surface hydrophobicity. Protein hydrolysis with trypsin broke peptide bond and exposed large amount of hydrophilic amino and carboxyl groups and other polar side chains of amino acid residues, which contributed to the decreased surface hydrophobicity. The proteins modified with urea had similar surface hydrophobicity as the control, which implied a balanced effect on exposing hydrophobic and hydrophilic groups during the denaturation. This may be also related to the “molten globule” state of the protein, where the denaturant destroyed protein tertiary structure but stabilized the secondary structures (Zhu et al., 2016). Interestingly, Soy and camelina proteins modified with

sodium hydrogen sulfite and sodium bisulfite, respectively, had increased surface hydrophobicity compared with the control (Yue et al., 2019; Zhu et al., 2016). The different effects on protein surface hydrophobicity may be related to the reducing power of the agent, amount, and other reaction conditions.

5.3.3 SDS-PAGE profiles

The SDS-PAGE profiles of the control and modified pea proteins from both reducing and non-reducing conditions are shown in Figure 3. Under non-reducing condition, it is obvious that the proteins modified by sodium sulfite had overall lower molecular weight compared with others, evidenced by their weaker band intensity above 250 kDa, disappearance of band around 60 kDa, and higher band intensity at 37 kDa. This is because sodium sulfite cleaved the intermolecular disulfide bond, thus reducing the molecular weight of protein. The proteins denatured with urea or SDS did not show obvious differences in band distribution compared with the control, confirming that protein denaturation with urea or SDS dominantly interrupted the non-covalent interactions, while minor covalent side interactions, if any, could hardly be identified from SDS-PAGE profiles due to the limitation of the technique. Under the reducing condition, the high molecular weight bands (>250, 100-250, 50-75 kDa) for all the proteins mostly disappeared, while several lower molecular weight bands around 37 kDa and 20-25 kDa appeared. The modified proteins and the control had very similar bands profiles under the reducing conditions, further confirming that urea and SDS modifiers mainly affect non-covalent intra- and inter-molecular interactions in protein. As for trypsin hydrolyzed protein, no obvious protein bands were observed in the electrophoresis range of 10 to 250 kDa under both non-reducing and reducing conditions. This is because the molecular weight of peptides from trypsin hydrolysis was lower than 10 kDa, as also evidenced by their excellent solubility and

significantly increased free amino content. The small band above 250 kDa near the gel edge was possibly due to some protein aggregates that were resistant to hydrolysis.

5.3.4 Protein secondary structure

Secondary structure composition of the pea proteins, including β-sheet, random coil, α-helix, and β-turn derived from FTIR spectra, is summarized in Table 1. The control pea protein in the study was comprised of 19.3% β-sheet, 74.0% random coil, 3.7% α-helix, and 3.1% of β-turn, with random coil dominating the secondary conformation. The denatured proteins with sodium sulfite and urea had significantly increased β-sheet, α-helix, and β-turn structures compared with the control, while no random coil was identified in these proteins, implying that such protein unfolding approaches promoted the conversion of random structures to more regular and ordered structures (Matsuo, Sakurada, Yonehara, Kataoka, & Gekko, 2007). Roy and Bagchi (2014) reported that β-sheet conformation of proteins was unfolded preferentially in concentrated urea solution (8 M). The SDS denatured proteins also showed increased β-sheet, α-helix, and β-turn structures compared to the control, although there still remained a larger portion of random coils, which are different from the sodium sulfite and urea denatured proteins. Moreover, the composition of secondary structures in the modified proteins was also dependent on the concentration of denaturants. Trypsin hydrolyzed protein also had greatly decreased random coil and increased β-sheet compared to the control, but no α-helix structure was observed. It seemed that the hydrolyzed pea proteins tended to pack into β-sheet and β-turn structures driven by hydrogen bonding, instead of forming α-helix conformation (Zhao, Xiong, & McNear, 2013).

5.3.5 Thermal properties

The DSC thermograms are presented in Figure 4, and the onset temperature (°C), peak temperature (°C) and phase transition enthalpy (ΔH) of pea and denatured pea protein dry

powders are summarized in Table 3. All the proteins showed a smaller exothermic aggregation peak around 50 – 70 °C and a larger endothermic denaturation peak around 160 – 200 °C. Overall, the proteins denatured with sodium sulfite, urea, and SDS possessed higher peak denaturation temperatures (192.6 to 196. 3 °C) compared with the control (189.2 °C), while trypsin hydrolyzed protein had much lower denaturation temperature (180.3 °C). The shift to lower or higher denaturation temperatures was caused by either destabilization or stabilization of protein structures during modification. As discussed for the secondary structure (Table 1), all the denaturation approaches decreased random coil structures while increased the amount of more ordered structures of β-sheet, α-helix and β-turn, to different extent, compared with the control. Therefore, the increased denaturation temperature may be attributed to the formation of these new protein structures that required higher temperature to be re-unfolded. The pea protein denatured with 1 M urea had the highest denaturation temperature among all these proteins, implying that hydrogen bonding may play a dominant effect on protein's thermal stability. As for trypsin hydrolyzed pea protein, although it had more ordered structures than the control, the protein molecular size was also greatly reduced due to hydrolysis, which dominated the phase transition and resulted in the lowest denaturation temperature. The aggregation peak temperatures were not significant different among all the proteins (66 – 68 °C) except for trypsin hydrolyzed protein (63 °C), which was again attributed to its much lower molecular size that was prone to aggregation during heating. All the denatured proteins showed a larger aggregation enthalpy than the control, indicating a stronger aggregation tendency of these proteins, which may also partially explain the higher percentage of ordered secondary structures in these proteins. Goyal et al. (2014) reported that maltodextrin glucosidase protein showed an

aggregation peak at 65 °C, and they indicated that after completion of protein conformational unfolding, the protein aggregation at high temperature usually accompanies an exothermal effect.

5.3.6 Solubility

Solubility is one of the most critical functional attributes of protein for food uses, and it affects other properties, such as gelation, emulsification, and foaming properties. All the denatured proteins had greatly increased solubility at pH conditions away from the isoelectric point (pI, around pH 4.5) compared with the control pea protein (Figure 1). Further, trypsin hydrolyzed pea protein also had dramatically increased solubility (50%) at the isoelectric point compared with all the other proteins (lower than 20%). This can be explained by the more soluble nature of the smaller peptides formed through hydrolysis and the greatly unfolded protein-peptide chains (Latorres, Rios, Saggiomo, Wasielesky, & Prentice-Hernandez, 2018). Besides, solubility of protein hydrolysate is also dependent on the degree of hydrolysis (DH). Latorres et al. (2018) indicated that the hydrolysate with lower DH exhibited lower solubility compared to the higher DH hydrolysate, which was attributed to the rigid structure of larger peptides with stronger inter-and intramolecular interactions. All the other modified pea proteins showed similar solubility trend, i.e., less soluble at pI and more soluble at pH away from the pI. The increased solubility of the denatured proteins compared to the control was due to the disruption of molecular interactions that unfolded the protein and allowed them more accessible and interactive with aqueous solution during solubilization. At pH 7, the 0.1% SDS denatured pea protein showed the highest solubility among all the proteins, which might be related to its relatively lower surface hydrophobicity, while the 0.5% SDS modified protein had the highest surface hydrophobicity, thus slighted decreased solubility compared with the 0.1% SDS protein. Li et al. (2018) found that SDS and urea modified waxy rice flour had higher solubility than the

control and cysteine modified flour. Liu & Hsieh (2008) reported that protein unfolded with urea had higher solubility than that with reducing agent dithiothreitol. These results suggest that non-covalent interactions such as hydrogen bonding and hydrophobic interactions may play a more important role in protein solubility than disulfide bond crosslinking (Li et al., 2018).

5.3.7 Water/oil holding capacity

Water and oil holding capacities (WHC/OHC) indicate the ability of protein molecules to absorb and retain water and oil under specific condition, respectively. The WHC and OHC of natural and denatured pea proteins are shown in Table 2. Overall, pea proteins denatured by urea and SDS had significantly improved water holding capacity, and sodium sulfite denatured pea protein showed no significant differences compared with the control pea protein, while trypsin hydrolyzed pea protein had decreased WHC. SDS and urea denatured protein structure through affecting hydrophobic interaction and hydrogen bonding, which exposed some hydrophilic side chains, along with hydrophobic groups, and enhanced the ability to absorb water molecules (Bennion & Daggett, 2003; Li et al., 2018). The pea protein hydrolysate had the lowest WHC among all the protein samples, which was attributed to the greatly reduced molecular size of the peptides that were too small to trap and hold the water (Ustunol, 2015). As for the proteins denatured with sodium sulfite, although the cleavage of disulfide bonds can expose additional hydrophilic groups, it also reduced protein molecular size, thus resulting in a similar WHC as the control. The OHC values of all the modified pea proteins were significantly increased compared with the control pea protein. Exposure of hydrophobic core of the globular protein during denaturation allows stronger interaction with nonpolar lipids, thus resulting in enhanced oil absorption and holding properties.

5.3.8 Emulsifying capacity/stability

Some proteins are capable of forming and stabilizing oil-in-water emulsion where oil droplets are enveloped by surface-active protein molecules in water phase as interfacial films in preventing coalescence by decreasing interfacial tension (Kinsella, 1982). Emulsion capacity (EC) indicates the ability of a protein to emulsify oil and water at the interfacial area, and emulsion stability (ES) measures the stability of emulsion after treatment at higher temperature and centrifugation. The pea proteins denatured with urea and SDS had significantly improved EC and ES compared with the control pea protein (Table 2). Urea denaturation weakened hydrogen bonding and hydrophobic interaction of the protein molecules and unfolded the globular proteins that improved protein flexibility and enhanced film-forming potential. The increased EC and ES for SDS denatured pea protein is because the electrostatic interaction between negatively charged SDS molecules and positively charged groups on the protein surfaces reduced protein aggregation (Demetriades & Julian McClements, 2000). The 0.5% sodium sulfite unfolded protein and trypsin hydrolyzed protein had no significant differences in EC and ES compared with the control, while the EC was significantly decreased when sodium sulfite was increased to 1%. Lower amount of sodium sulfite cleaved protein disulfide bonds, while it had smaller effects on protein intramolecular interaction and quaternary structures. When the sodium sulfite concentration increased, the protein molecular size was further decreased, which may be responsible for the decreased emulsion capacity. Lee and Hirose (1992) reported that the emulsifying properties of bovine serum albumin with a reducing agent in the mild condition could be improved, and it was attributed to the increased flexibility of protein molecules with hydrophobic amino acid residues interact with oil phase, while hydrophilic amino acid residues interact with aqueous phase. They also reported that the emulsifying properties would decrease

when modifying with an extensive amount of reducing agent, because of the soluble aggregates formed with larger molecular size (Lee & Hirose, 1992). Trypsin hydrolysis resulted in small peptides and greatly altered protein's hierarchy structures. The peptide in the protein hydrolysates was surface-active, and it could form small droplets in the emulsion, but the emulsion can't be fully stabilized due to insufficient peptides (Padial-Domínguez, Espejo-Carpio, Pérez-Gálvez, Guadix, & Guadix, 2020). Therefore, the intramolecular interaction has dominant effect on protein unfolding and quaternary structures to improve the emulsifying properties.

5.3.9 Foaming capacity/stability

Protein could be spontaneously absorbed from the aqueous phase to the air-aqueous interface and stabilizes foams. The foam-forming potential is thermodynamically favorable because of the dehydration of the hydrophobic interface and hydrophobic protein residues (Foegeding, Luck, & Davis, 2006). Protein foaming potential increases when the surface tension of protein is decreased. Interfacial properties of protein foam mainly depend on protein types, co-solutes, and intermolecular interactions at the interface, including hydrophobic interactions, electrostatics, hydrogen bonding, and disulfide bond formations (Foegeding et al., 2006). The foaming capacity (FC) is measured by the number of interfacial areas that could be created when whipping the protein, and foaming stability (FS) is measured by the period of time when losing the air bubble volume of foam (Mauer, 2003). Overall, the trypsin hydrolyzed pea protein exhibited the highest foaming capacity than the control and other modified pea proteins (Figure 2). This is because the smaller peptides formed during protein hydrolysis could diffuse more rapidly to the air-aqueous interface and encapsulate air bubbles to form foams (Park and Yoon (2019). Further, extensively exposure of hydrophobic residues of polypeptides also allowed the formation of more foams.

Park and Yoon (2019) reported that protein foaming properties are influenced by pH condition, and foaming capacity decreased when the pH was near the isoelectric point (Park & Yoon, 2019). The pea proteins denatured by sodium sulfite, urea, and SDS had similar or even lower foaming capacity and stability than the control pea protein. This is probably because the denaturation of intermolecular interaction does not alter the protein molecular size, and the protein macromolecules are less capable of forming film interfaces and trapping air during whipping, compared with the hydrolyzed proteins.

5.3.10 Protein gelation

Protein gelation is the formation of a 3-D network from denatured proteins that entrap water through balanced protein-protein and protein-water interactions. Overall, the proteins hydrolyzed by trypsin or unfolded with SDS or 1 M urea had improved gelation properties (i.e., lower least gelation capacity value, 15 or 16%), while the protein denatured with sodium sulfite (i.e., LGC of 18%) had decreased gelation property compared with the control pea protein (i.e., LGC of 17%) (Table 2). Various covalent and non-covalent interactions, when balanced, can contribute to protein gelation, including chemical crosslinking (e.g., disulfide), salt bridging and other ionic interactions, hydrogen bonding, hydrophobic interaction, and van der Waals force attraction. The hydrolyzed protein with much higher free amino content favors hydrogen bonding and ionic interaction that may promote the formation of gels. The lower LGC of SDS denatured pea protein was possibly attributed to the fact that SDS disrupted protein hydrophobic and electrostatic interactions that reduced the energy barrier of protein-protein interaction in water and upon heating, enabling protein gel formation (Shan et al., 2015). Sodium sulfite cleaved protein inter- and intra-molecular disulfide bonds and decreased protein molecular size, resulting in reduced gel formation capacity. Utsumi and Kinsella (1985) reported that when the

concentration of 2-mercaptoethanol was increased, protein intramolecular interaction (hydrophobic interaction) would be destabilized. The lack of gel formation by adding reducing agent is caused by its dissociation of globulin protein into acidic and basic subunits or inhibition of thiol disulfide exchange reactions. A similar result was also reported by Sun and Arntfield (2012) when investigating the effect of dithiothreitol on pea protein gel formation. When the concentration of denaturant agents increased, there were no significant differences for sodium sulfite and SDS modified pea proteins. With 1 M urea, the protein gelation property was improved, while it remained the same as the control when the urea was at 0.2 M (Table 2). This is because higher amount of urea greatly interrupts protein hydrogen bonds and hydrophobic interactions that resulted in protein with an extensively unfolded state and altered protein secondary structure compositions that may favor gelation (Shan et al., 2015).

5.3.11 Principal component analysis

Principal component analysis (PCA) was conducted to further understand the relationships among protein functional and physicochemical properties and molecular interactions. The eigenvalues 1 and 2 interpreted 67.6% of the variability (Figure 5). The PCA results confirmed that different intra- and inter-molecular interactions strongly associate with protein functionality in terms of water/ oil holding capacity, emulsifying properties, solubility, foaming capacity and gelation property, and these functional properties are further associated with protein structural characteristics. Denaturing pea protein through interfering with the non-covalent interactions of hydrogen bonding and hydrophobic interaction is associated with improved water/ oil holding capacity, emulsifying properties and solubility (pH 7.0), which seems to be related to more β-sheet and β-turn structures and increased surface hydrophobicity of the unfolded proteins. Cleaving protein peptide bond through enzymatic hydrolysis is associated with enhanced

foaming capacity and increased solubility near protein isoelectric point (pH 4.5), and the hydrolyzed proteins are characterized by higher free amino group concentration. Reductive cleavage of the disulfide bonds in pea protein is associated with larger LGC values, implying poorer gelation capacity, and the proteins have higher free sulfhydryl group concentration and more α-helix structures.

5.4 Conclusions

In conclusion, we successfully manipulated pea protein inter- and intra-molecular interactions (e.g., disulfide bond, hydrogen bonding, hydrophobic interaction, peptide bond) with specific denaturation agents and achieved enhanced functional properties. Pea protein denaturation with sodium sulfite, urea, SDS, or trypsin all greatly improved protein solubility and oil holding capacity compared with the original protein. Trypsin hydrolyzed protein possessed excellent foam capacity and greater gelation property. The proteins unfolded with urea or SDS showed improved emulsification properties. Urea or SDS denatured proteins exhibited better water holding capacity and gelation property. The changes of these functional properties are well associated with molecular structural characteristics of the proteins. This study provides fundamental knowledge of protein functionalities related to protein covalent and non-covalent interactions and will contribute to the broader food applications of functionally enhanced pea proteins. Future research should focus on employing food-grade modifiers to modulate protein interactions and improve its functional characteristics in specific food applications.

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Conflict of interest

The authors declare that there is no known conflict of interest.

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Figures and tables

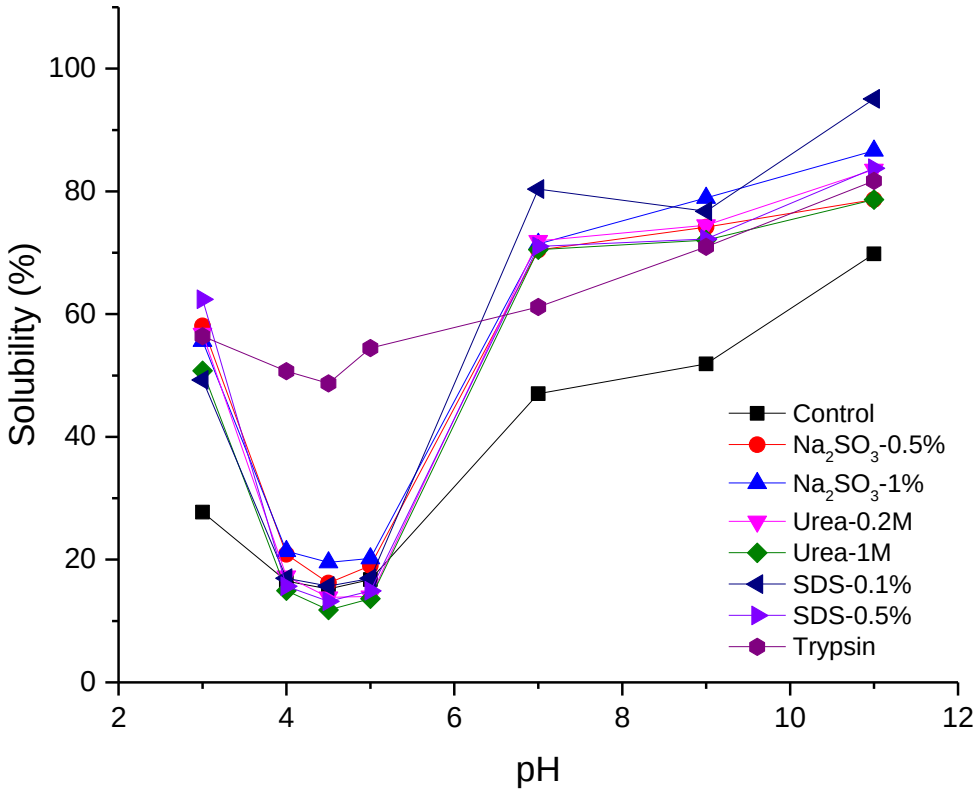

Figure 5.1 Solubility profiles of natural and denatured pea proteins.

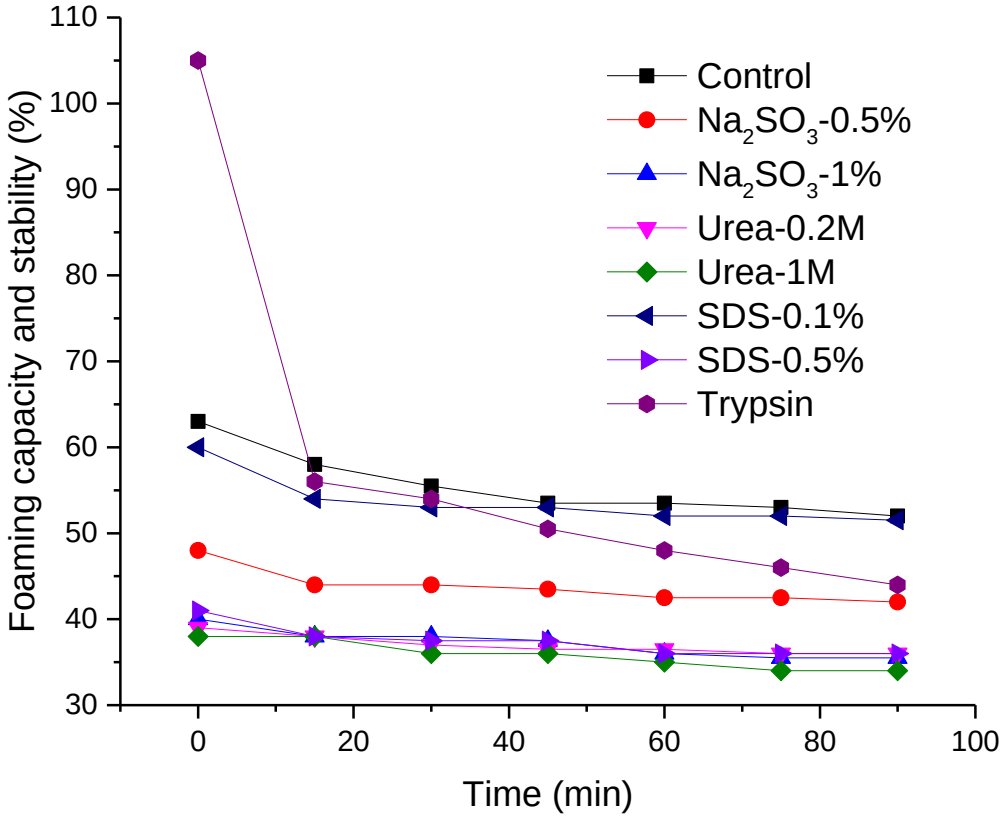

Figure 5.2 Foaming capacity and stability of natural and denatured pea proteins.

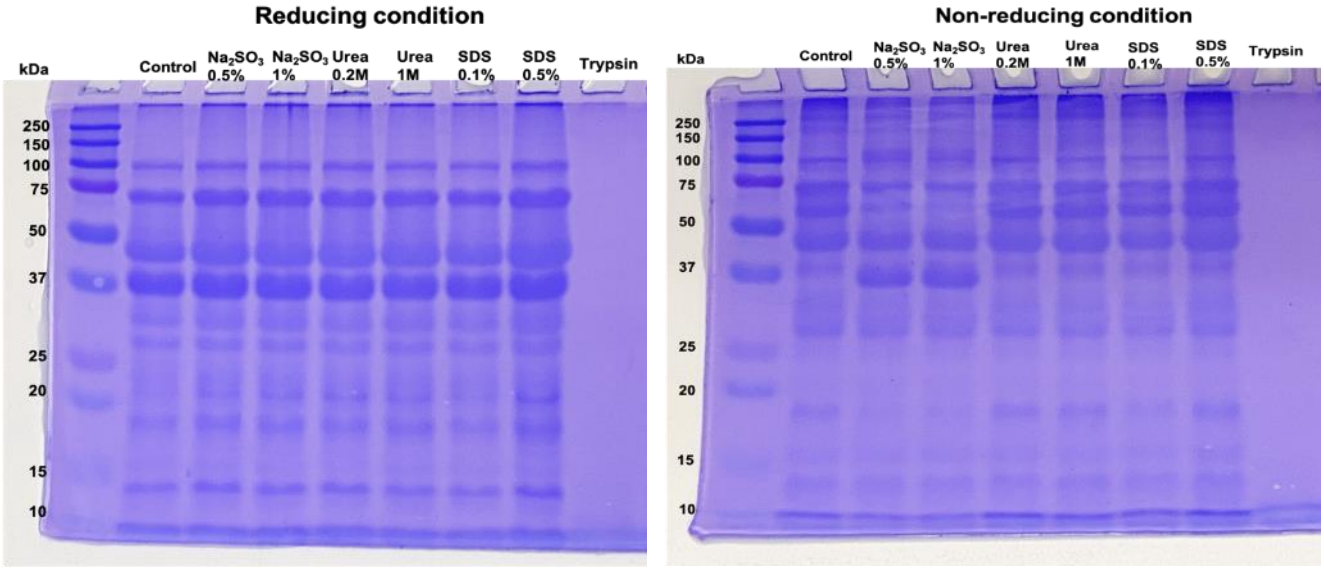

Figure 5.3 SDS-PAGE profiles of natural and denatured pea proteins under reducing and non-reducing conditions.

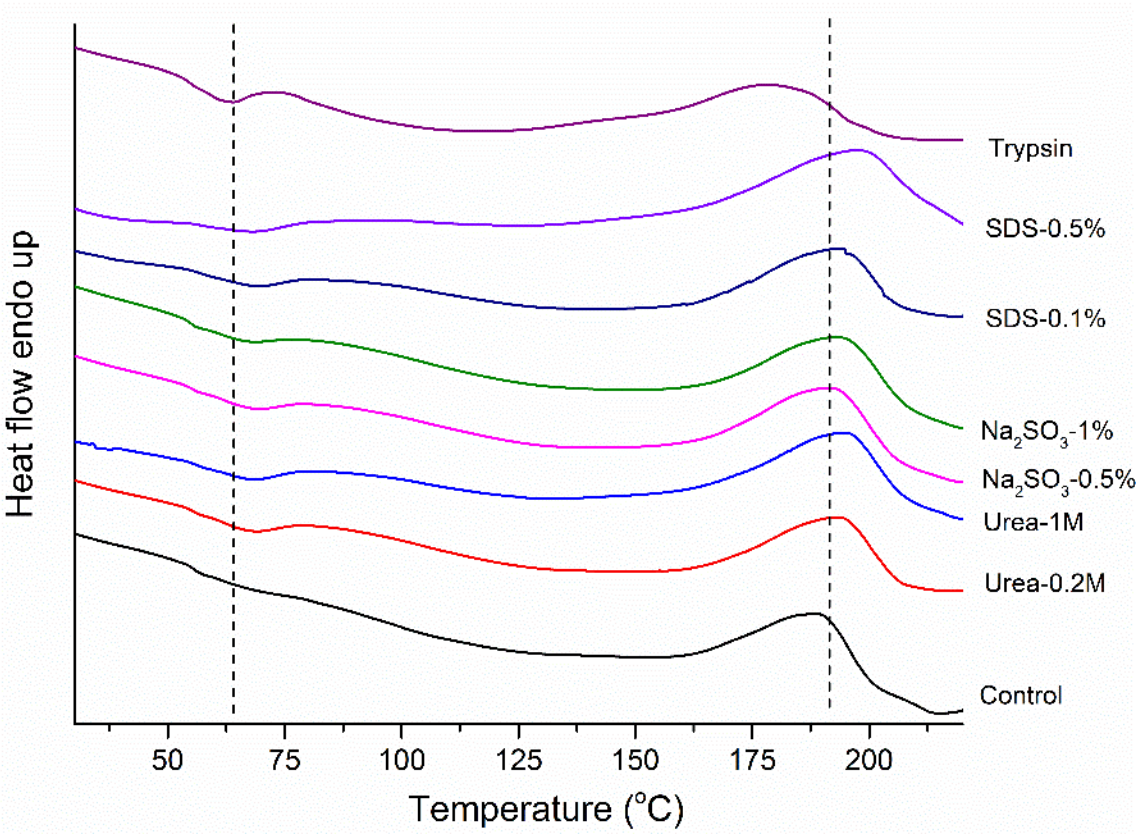

Figure 5.4 DSC thermograms of natural and denatured pea proteins.

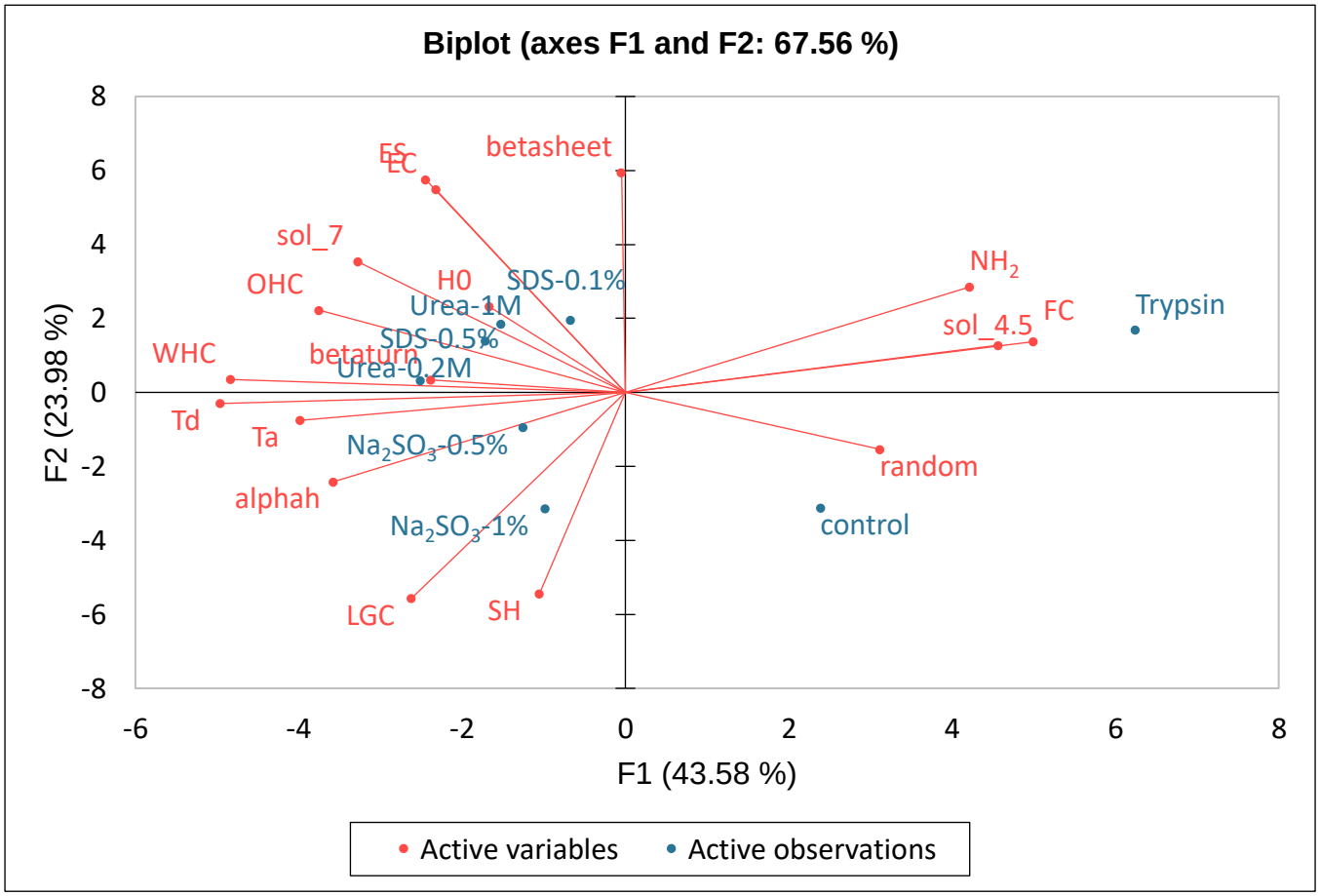

Figure 5.5 Principal component analysis (PCA) biplot describing relationships between protein functional and physicochemical properties with different denaturation agents.

*EC: emulsion capacity; ES: emulsion stability; sol_7: solubility at pH 7; sol_4.5: solubility at pH 4.5; H0: surface hydrophobicity; OHC: oil holding capacity; WHC: water holding capacity; Td: denaturation temperature; Ta: aggregation temperature; LGC: least gelation capacity; SH: free sulfhydryl concentration; betasheet: β-sheet; betaturn: β-turn; alphah: α-helix; random: random coil; NH_2: free amino concentration; FC: foaming capacity.

Table 5-1 Physicochemical properties of pea and denatured pea proteins.

Samples	Free amino group (mmol/g protein)	Free SH (μmol/g protein)	Surface hydrophobicity (H_0)	β-sheet (%)	random coil (%)	α-helix (%)	β-turn (%)
Control	11.6±0.1^{cd}	4.1±0.0^{b}	126,431±1,040^{b}	19.3±1.6^{d}	74.0±2.5^{a}	3.7±5.2^{de}	3.1±4.4^{c}
Na_2SO_3-0.5%	11.5±0.2^{cd}	3.7±0.0^{cd}	81,018±851^{cd}	51.4±1.1^{ab}	ND	33.9±2.8^{bc}	14.7±1.7^{a}
Na_2SO_3-1%	9.5±0.3^{d}	4.6±0.1^{a}	69,395±6,895^{d}	35.9±1.2^{c}	ND	53.7±1.3^{a}	10.4±0.2^{abc}
Urea-0.2M	16.7±0.3^{b}	4.2±0.1^{b}	139,753±11,725^{b}	41.3±4.2^{c}	ND	46.6±6.5^{ab}	12.1±2.3^{ab}
Urea-1M	13.3±0.7^{bc}	3.2±0.1^{f}	130,386±3,891^{b}	61.8±0.0^{a}	ND	30.2±0.0^{c}	7.9±0.0^{abc}
SDS-0.1%	11.3±0.3^{cd}	3.3±0.0^{ef}	96,788±3,263^{c}	62.0±1.9^{a}	24.6±2.0^{b}	7.9±0.2^{de}	5.6±0.0^{bc}
SDS-0.5%	16.6±1.2^{b}	3.9±0.1^{bc}	168,125±10,979^{a}	44.6±7.5^{bc}	28.9±4.4^{b}	15.5±2.2^{d}	11.1±0.8^{ab}
Trypsin	51.7±2.1^{a}	3.5±0.1^{de}	87,693±99^{c}	58.6±7.0^{abc}	33.1±8.0^{b}	ND	8.3±1.0^{abc}

*Means with different letters in each column indicate significant differences (p< 0.05), ND: not detected.

Table 5-2 Functional properties of pea and denatured pea proteins.

Samples	WHC (g/g)	OHC (g/g)	EC (%)	ES (%)	LGC (%)
Control	4.1 ± 0.0^{c}	1.3 ± 0.0^{f}	67.7 ± 2.4^{bc}	58.7 ± 3.8^{c}	17^{b}
Na_2SO_3-0.5%	4.2 ± 0.0^{c}	2.8 ± 0.0^{b}	68.2 ± 1.7^{b}	61.7 ± 1.8^{bc}	18^{a}
Na_2SO_3-1%	4.2 ± 0.0^{c}	2.8 ± 0.0^{bc}	64.0 ± 0.6^{c}	58.0 ± 2.0^{c}	18^{a}
Urea-0.2M	5.0 ± 0.0^{a}	3.1 ± 0.1^{a}	80.0 ± 3.0^{a}	65.6 ± 1.2^{ab}	17^{b}
Urea-1M	4.7 ± 0.0^{b}	2.5 ± 0.1^{d}	79.3 ± 1.6^{a}	65.2 ± 1.6^{ab}	16^{c}
SDS-0.1%	4.5 ± 0.2^{b}	2.7 ± 0.0^{c}	77.6 ± 1.0^{a}	65.3 ± 0.5^{ab}	16^{c}
SDS-0.5%	5.1 ± 0.1^{a}	2.8 ± 0.0^{b}	79.0 ± 0.3^{a}	67.7 ± 0.4^{a}	16^{c}
Trypsin	2.5 ± 0.0^{d}	2.1 ± 0.0^{e}	69.8 ± 1.7^{b}	61.3 ± 2.1^{bc}	15^{d}

*WHC: water holding capacity, OHC: oil holding capacity, EC: emulsion capacity, ES: emulsion stability, LGC: least gelation capacity.

**Means with different letters in each column indicate significant differences ($p< 0.05$).

Table 5-3 Thermal properties of pea and modified pea proteins.

Samples	Aggregation peak			Denaturation peak		
	Onset temp (°C)	Peak temp (°C)	Enthalpy (ΔH, J/g)	Onset temp (°C)	Peak temp (°C)	Enthalpy (ΔH, J/g)
Control	55.1±0.3^{a}	67.8±0.1^{a}	1.0±0.1^{b}	160.5±1.4^{a}	189.2±0.4^{b}	21.0±2.3^{ab}
Na_2SO_3-0.5%	53.5±1.2^{a}	68.0±0.0^{a}	2.8±0.3^{a}	162.4±0.8^{a}	192.6±0.7^{ab}	25.4±1.2^{ab}
Na_2SO_3-1%	52.6±0.6^{a}	66.3±0.3^{a}	3.0±0.8^{a}	165.7±1.3^{a}	193.9±0.5^{a}	26.9±0.5^{ab}
Urea-0.2M	53.3±0.2^{a}	67.6±0.1^{a}	2.9±0.0^{a}	164.8±0.2^{a}	193.2±0.6^{ab}	18.1±1.1^{b}
Urea-1M	54.0±0.7^{a}	67.2±0.9^{a}	1.9±0.0^{ab}	165.6±1.5^{a}	196.3±1.5^{a}	22.1±3.4^{ab}
SDS-0.1%	54.3±0.7^{a}	68.9±0.3^{a}	2.0±0.0^{ab}	164.2±1.2^{a}	193.7±0.2^{a}	20.7±0.2^{ab}
SDS-0.5%	53.6±0.4^{a}	67.7±1.6^{a}	2.4±0.2^{a}	162.5±0.3^{a}	194.0±0.7^{a}	24.6±0.9^{ab}
Trypsin	52.7±0.4^{a}	63.0±0.2^{b}	2.5±0.1^{a}	141.0±7.6^{b}	180.3±2.4^{c}	29.0±5.9^{a}

*Means with different letters in each column indicate significant differences ($p < 0.05$).

Chapter 6 - Effect of adding modified pea proteins as functional extender on the physical and sensory properties of beef patties[3]

Abstract

Plant-based ingredients are used as extenders in meat products to enhance nutrition, improve quality, and reduce cost. Pea protein modified via sequential deamidation and conjugation (PGG) exhibited greatly enhanced functionalities compared to the original pea protein (PPI). The objective of this study was to understand the effect of adding 2.5 and 5% PPI or PGG on the cookability, physical and texture properties, and sensory attributes of beef patties in comparison with regular patty. The beef patties containing PGG (especially at 5%) showed significantly decreased cooking loss (20%) and increased moisture and fat retentions compared with the control patty (33% cooking loss). In general, PPI patties exhibited harder texture while PGG patties showed much softer texture than the control. Sensory results indicated that the control patty had higher scores of juiciness and beef flavor intensity and less off flavor than the extended patties, while the PGG patties were tenderer and softer than the control and PPI patties. The patties containing PGG demonstrated some advantageous features in terms of higher fat/water retention, cooking yield, and tender texture, which may be preferred by the elderly or some other consumers. This study presents a novel approach to tailor meat texture using plant proteins.

Key Words: Beef patty, pea protein functionality, meat extender, texture, descriptive sensory analysis

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6.1 Introduction

There has been increasing demand for high-quality meat products with excellent eating quality, nutritional benefits, and lower cost (Balestra & Petracci, 2019). Beef products account for one quarter of total meat consumption in the U.S., with annual beef consumption of 58 pounds per person in 2021 (USDA ERS - Market Outlook). Non-meat ingredients are commonly added into meat products to reduce cost, enhance nutritional quality, and improve some quality attributes. Various types of functional ingredients such as starch, protein, fiber, and hydrocolloid, are used as extenders, fillers, or binders in meat products to increase cooking yield and water/oil retention capacity, optimize meat texture, bind among meat pieces, and stabilize water and fat components in meat emulsion during food preparation and cooking (Petracci, Bianchi, Mudalal, & Cavani, 2013).

Plant proteins are popular binders and extenders in meat products. They may enhance the emulsification of fat in comminute meat and bind fat and meat pieces in coarse ground meat products, which can deliver more structural integrity and functionality for meats (Petracci et al., 2013). The extension of meat systems with plant proteins results in a complex heterogeneous structure and alters the physical and textural characteristics of the meat product (Kassama, Ngadi, & Raghavan, 2003). Soy protein with good gelling and emulsifying properties has been used in meatball, sausages, and burgers for cost reduction and textural improvement (Balestra & Petracci, 2019). Akesowan (2010) found that pork burger with the addition of 2% soy protein isolate had significantly improved textural properties, such as cohesiveness, springiness, and chewiness. A similar finding was also reported by Hidayat, Wea, & Andriati (2017), which showed that beef sausage containing texturized protein had increased cooking yield and

decreased hardness; in addition, the sensory attributes were not affected with up to 30% substitution with the protein. Yi et al. (2012) reported that when glutinous rice flour was added to beef patties, there were decreased cooking loss, increased fat and moisture retention, and improved patty juiciness and tenderness compared with the regular patty.

Pea (*Pisum sativum* L.) is attracting increasing interest as a promising protein crop due to its many agronomic and food functional advantages (Khattab, Arntfield, & Nyachoti, 2009). However, commercial utilization of pea protein products is still relatively limited, partially due to their less desirable functional and sensory properties (Zha, Dong, Rao, & Chen, 2019). To overcome these limitations, protein modifications can be a useful strategy to improve the functionalities, such as solubility, emulsifying properties, gelation, and water/ oil holding capacities. For example, glutaminase deamidation of coconut protein and wheat protein increased the negative charge of proteins by converting amide groups in glutamine and asparagine residues to carboxyl groups, resulting in improved functional characteristics (Kunarayakul, Thaiphanit, Anprung, & Suppavorasatit, 2018; Wang, Gan, Zhou, Cheng, & Nirasawa, 2017). Pea protein and guar gum conjugation through Maillard reaction enabled the protein to be covalently linked with hydrophilic polysaccharide, which significantly improved protein solubility and emulsifying properties (Shen & Li, 2021). We recently developed a modified pea protein through sequential enzymatic modification of pea protein isolate (PPI) with protein glutaminase and conjugation with guar gum, namely PGG. This “green” modification approach exhibited synergistic advantages, and the modified pea protein PGG possessed excellent emulsification capacity, gelation property, and oil holding capacity. The new pea protein ingredient may have a better potential as functional extender in processed meat products.

Therefore, this study aimed to understand the effect of adding original (i.e., PPI) or functionally enhanced pea protein (PGG) on the cookability, physical and texture properties, and sensory attributes of beef patties in comparison with regular beef patty (i.e., no plant protein addition). This study will benefit researchers and food professionals interested in developing and utilizing novel plant protein ingredients.

6.2 Materials and methods

6.2.1 Materials

Ground beef (80% lean/ 20% fat) was purchased from a local grocery store. Pea protein isolate (PPI, 83% protein content) was obtained from a commercial source. Guar gum was purchased from Judee's (Plain City, OH, USA). Protein glutaminase was provided by Amano Enzyme Inc (Nagoya, Japan). Other chemicals and reagents of analytical grade were purchased from Sigma-Aldrich (St. Louis, MO, USA).

6.2.2 Preparation of functionally enhanced pea protein

The functionally enhanced pea protein (PGG) was prepared through a sequential modification of PPI with protein glutaminase and guar gum. Briefly, the PPI dispersion (10% protein concentration) was continuously mixed with 1% protein glutaminase (enzyme activity: 644 U/g) (PPI basis) at pH 6.5 in a water-bath shaker at 55 °C for 3 hours to allow deamidation reaction. The slurry was then boiled for 10 min to deactivate the enzyme and cooled down. After that, 5% guar gum (PPI basis) was added for conjugation reaction at 60 °C for 24 hours with continuous mixing. At the end, the protein slurry was lyophilized, and the dried protein sample was ground and kept at 4 °C for further analysis and usage.

6.2.3 Analysis of protein functional properties

Protein functional properties of PPI and PGG, including water/ oil holding capacity, emulsion capacity and stability, and solubility were measured following our previous methods (Shen, Tang, & Li, 2021) without any modification. The least gelation concentration was measured according to the method of Shen and Li (2021).

6.2.4 Preparation of beef patties containing pea proteins

Five patty treatments were designed for this study, including control patty (without pea protein) and patties with 2.5 or 5% PPI and PGG. Raw beef patties were prepared by hand mixing the ground beef with protein and then mounding to a round shape, with approximately 30 g per patty. The raw patties were cooked on a grill until reaching internal temperature of 71 °C. A total of 13 replicate patties were prepared for each treatment and used for the following tests: patty 1-10 for color measurement, patty 1-11 for cooking loss, patty 1-4 for TPA analysis, patty 5-6 for shear force test, patty 7-8 for pressed juiciness test, and patty 9-13 for moisture and fat retention measurements.

6.2.5 Color measurement

Color parameters of raw beef patties were measured using a digital precise colorimeter (CIELAB, XITIAN machine equipment Co., Ltd, Huizhou, China) to obtain the L*, a*, and b* values. Each beef patty was scanned twice at different locations on the surface, and each patty treatment was tested in ten replicates.

6.2.6 Measurement of cooking loss, moisture retention, and fat retention

Cooking loss was measured based on weight differences between a raw patty and the cooked patty according to the equation below:

$$\text{Cooking loss (\%)} = \frac{(\text{ raw weight}) - (\text{cooked weight})}{(\text{raw weight})} \times 100$$

Moisture content of beef patty (both raw and cooked) was measured according to AOAC 950.46 (AOAC, 2019), and the patty sample was dried at 135 °C for 2 hours. Fat content of beef patty (both raw and cooked) was measured according to AOAC 960.39 with small modifications. Briefly, beef patty was lyophilized, and the fat in the patty was extracted with ethyl ether for two times. The ether extract was combined and allowed to evaporate the solvent in a fume hood overnight. Moisture and fat retentions were calculated according to the following equations:

$$\text{Moisture retention (\%)} = \frac{(\text{cooked weight}) \times (\text{moisture \% in the cooked patty})}{(\text{raw weight}) \times (\text{moisture \% in the raw patty})} \times 100$$

$$\text{Fat retention (\%)} = \frac{(\text{cooked weight}) \times (\text{fat \% in the cooked patty})}{(\text{raw weight}) \times (\text{fat \% in the raw patty})} \times 100$$

6.2.7 Texture profile analysis (TPA)

Texture profiles of the cooked beef patty were measured using a TA-XT Plus texture analyzer (Stable Micro System, Godalming, Surrey, UK) with a cylinder probe with two-inch diameter. The measuring parameters were set as: 1.0 mm/s pre-test speed, 5.0 mm/s post-test speed, 1.0 mm/s test speed, and 50% strain compression with 20 g trigger force. Each patty treatment was conducted in four replicates. Patty textural parameters including hardness, resilience, cohesiveness, springiness, and chewiness were recorded by the equipped software and collected.

6.2.8 Shear force measurement

For shear force test, 2-cm wide strips were cut from cooked patties, and the strip was sheared perpendicularly to the patty surface using a Warner-Bratzler blade set attached to the Texture Analyzer (Stable Micro System, Godalming, Surrey, UK) with test speed at 5 mm/sec. The value

of shear force was collected as the maximal force during shearing. Each patty treatment was analyzed in four replicates.

6.2.9 Compressed juiciness

Juiciness value indicates the weight loss of cooked patty after a compression test. The test was measured following previously published method (Lucherk et al., 2017) with small modifications. Cooked patty was first cut into 1 cm^2 sample pieces, which was then covered with filter papers and pressed with a TA-4 probe (1-1/2 in. diameter acrylic cylinder, 20 mm tall) for 30 seconds at 8 kg force using the Texture Analyzer (Stable Micro System, Godalming, Surrey, UK). The trigger force was set at 5 g, and the test speed was set at 0.5 mm/sec. Each patty treatment was tested in four replicates The percentage of juiciness was calculated according to the following equation:

$$\text{Compressed juiciness (\%)} = \frac{\text{original sample weight} - \text{sample weight after compression}}{\text{original sample weight}} \times 100$$

6.2.10 Descriptive sensory analysis

Beef patties for sensory analysis were prepared and served at the Kansas State University Meat Science Lab (IRB# 7440, approved by the KSU Institutional Review Board committee). Ten different tubes of ground beef were purchased from a local grocery store, in order to prepare 10 replication samples for each treatment. Beef patties were prepared by mixing ground beef (80% lean/20% fat) with pea proteins by hand and pressing into 113.4 g patties using a patty maker, and the patties were then frozen, vacuum packed, and kept in a freezer until further sensory analysis. The patties were thawed 12 - 24 hours before cooking, and the patties were grilled on a clamshell-style grill until reaching the internal temperature of 71 °C. Fifteen panelists were further trained with the same testing samples during three training sessions before

the formal sensory evaluations. During formal analysis, six panelists were randomly selected from the pool based on their availability for each session, and the tests were conducted in 10 sessions arranged in each morning and afternoon for one week. Each cooked patty was cut into six equally sized wedges, and each panelist was fed six samples (1 wedge/sample) in random order including the warm-up ones. Each patty treatment was evaluated in ten replicates (i.e., ten different testing sessions). Sensory attributes including juiciness, tenderness, beef flavor, beef flavor intensity, texture, and off-flavor were scored on a continuous 100-point line with a midpoint of 50 (Figure S2, Supplementary Document).

6.2.11 Statistical analysis

All the data were analyzed using Python 3.6 package scipy. stats based on Kruskal-Waillis non-parametric test and Conover-Iman tests, and $p< 0.05$ was considered as a significant difference among the data sets. The data were presented as mean ± standard deviation. Principal Component Analysis (PCA) was conducted using XLSTAT 2021 (Addinsoft, New York, NY, USA) to determine associations among the different beef patty characteristics.

6.3 Results and discussion

6.3.1 Functional properties of pea proteins

The modified pea protein, i.e., PGG, showed significantly improved functional characteristics compared with the original pea protein isolate (PPI) (Table 1). The water and oil holding capacity (WHC, OHC) of PGG were 4.84 and 2.16 g/g, respectively, significantly higher ($p < 0.05$) than that of PPI (WHC of 4.09 g/g and OHC of 1.35 g/g). The emulsion capacity and stability of PGG were greatly increased to 99.4 and 98.2%, respectively, compared with PPI (88.7 and 66.7%, respectively). The PGG also exhibited much better gelation capacity, with a least gelation concentration (LGC) of 12%, while the LGC of the PPI was 17%. The solubility of

PGG was also twice that of PPI at pH 7 (50.5 vs. 22.9%). After deamidation of pea protein with protein glutaminase, some of the glutamine residues were converted to glutamate residues, resulting in improved functional properties (Fang, Xiang, Sun-Waterhouse, Cui, & Lin, 2020). Further inclusion of guar gum onto the protein structure through conjugation increased protein hydrophilicity, and the altered hydrophilicity/hydrophobicity balance favored protein-water interactions and improved protein dispersion stability (Baniel, Caer, Colas, & Gueguen, 1992). In addition, the inter-and intra-molecular interactions were partially disrupted and altered during the modifications, resulting in protein unfolding and structural rearrangement. These molecular changes favored many protein functional properties, leading to functionally enhanced pea protein ingredient, namely PGG in this study.

6.3.2 Physical properties of beef patties containing pea proteins

6.3.2.1 Color

The pictures and color parameters, including L* (– black to + white), a* (– green to + red), and b* (– blue to + yellow), of raw beef patties are shown in Figure 1 and Table 2, respectively. The patties containing PGG had significantly lower a* and b* values compared with the control and PPI patties, and a* value decreased with increased PGG addition (Table 2). This indicates that adding PGG decreased the redness of beef patties, while the effect of PPI on the redness was less obvious, which was attributed to the original color differences of PGG and PPI (Table S1, Figure S1). The patty with 5% PGG had the lowest a* value, because the concentration of myoglobin pigment was the most diluted (Wi, Bae, Kim, Cho, & Choi, 2020). Youssef and Barbut (2010) reported that the L* value decreased from 61.44 to 58.16 when protein content was increased in the cooked meat batters; however, the L* value was also dependent on other factors, such as protein content/types and oil types.

6.3.2.2 Cooking loss

Cooking of meat causes protein denaturation and shrinkage of myofibrillar and collagen proteins. The loss during cooking includes liquid drippings and volatile losses. Cooking loss determines cooking yield, and it is highly related to the sensory properties of meat products, in particular juiciness, tenderness, and other important quality attributes (Cao et al., 2016). The beef patties containing 5% PPI, 2.5% and 5% PGG had significantly decreased cooking loss compared with the control (32.8%) and 2.5% PPI patty (30.5%) (Table 2). As protein addition increased from 2.5 to 5%, the cooking loss was significantly decreased ($p < 0.05$) for both PPI and PGG based patties. This is because the plant proteins with good water and oil holding capacities and surface activity can form a cohesive gel matrix in the patties and can better stabilize the meat emulsions when at a higher concentration. The proteins may also act as fat-encapsulating agent to prevent oil dripping during cooking (Kamani, Meera, Bhaskar, & Modi, 2019). However, Youssef and Barbut (2011) reported that when the amount of meat proteins in beef batter emulsions with canola oil increased from 8 to 15%, the cooking loss was increased. This might be because the proteins formed a denser and aggregated network, which led to coalesce and migration of fat globules out of the protein matrix. Therefore, the amount and type of protein added to meat systems are important factors affecting cooking loss and final textural properties. The meat patties containing PGG had significantly decreased cooking loss (26.7% at 2.5% protein and 20.1% at 5% protein) when compared with PPI patties (30.5% at 2.5% protein and 25.8% at 5% protein) at the same protein addition level. The result implied that the functionally enhanced pea protein (PGG) with greater functional properties (e.g., water/ oil holding capacity, emulsifying properties, and gelation) can improve the cooking yield of meat patties compared to the original pea protein.

6.3.2.3 Moisture and fat retention

Moisture and fat retentions indicate the capacity of beef patty in holding the original water and fat after cooking. They are related to cooking loss and textural and sensory attributes of cooked patties, such as juiciness. The addition of PPI and PGG increased moisture retention of patties, though the values were not significantly ($p > 0.05$) different compared with the control (91.57%), while the beef patty with 5% PGG had significantly higher ($p < 0.05$) fat retention (89.15%) compared with the control (83.05%) and other patty treatments (Table 2). The increased water retention is because the added pea proteins can better absorb and hold water by forming a gel matrix during heating, and the plant protein may also interact with meat proteins in forming complex three-dimensional gel network that can better trap the water (Argel, Ranalli, Califano, & Andrés, 2020), resulting in firmer and more compact structures (Yi et al., 2012). In addition, due to the higher oil holding capacity of PGG compared with PPI, the beef patty with 5% PGG had significantly increased fat retention. The largest fat and moisture retention values of 5% PGG patty may also partially explain its lowest cooking loss among all the treatments.

6.3.2.4 Texture profile analysis

With the addition of PPI or PGG, the beef patties showed different texture profiles such as hardness, resilience, springiness, and chewiness (Table 3). For example, adding PPI significantly increased patty hardness (up to 7359 g with 5% PPI), while adding PGG significantly ($p< 0.05$) decreased patty hardness (as low as 3984 g with 5% PGG), compared with the control patty (5643 g). When the concentration of PGG increased from 2.5 to 5%, the cohesiveness and springiness also decreased. During cooking of the patties, heat-induced gelation of myofibrillar proteins is critical to deliver product integrity and needed texture and sensory properties (Sun &

Holley, 2011). The increased hardness of patties with the original PPI may be caused by the alteration of binding blocks among meat pieces and gel formation in the system from interactions among the meat and non-meat proteins (Youssef & Barbut, 2011). Similar results were found in beef patties with pea protein and emulsified meat batters with soy protein, as reported by Sephora, Joseph , Paul, & Ruth (2016) and Youssef & Barbut (2011), respectively. Akesowan (2010) reported that pork burger with 3% soy protein isolate (SPI) had significantly decreased hardness compared with the control (no SPI addition), but this may be attributed to the softer texture of hydrated SPI since water addition was increased in the patties based on different concentrations of SPI. On the other side, adding PGG greatly decreased the hardness of patties. As discussed previously, PGG possessed stronger water and oil holding capacities and gelation and emulsifying properties than PPI (Table 1), and the resultant patties also showed higher moisture and fat retention values (Table 2), which may partially contribute to the softer texture. In addition, modification (sequential deamidation and conjugation) of PPI in producing PGG changed protein secondary conformation and surface hydrophobicity (data not shown), which might weaken the binding and interactions among meat pieces compared to the original PPI. The raw PGG patties were much softer compared with the PPI and original beef patties. Further, the higher emulsifying potential of PGG may lead to more stable emulsions in the patties. Youssef and Barbut (2011) found that the hardness of meat was associated with the destabilization of emulsion, which can be caused by the separation of fat and water. Guar gum also has a softening effect when it is added to meat product (Sarteshnizi, Hosseini, Khaneghah, & Karimi, 2015). Ulu (2006) reported that low-fat meatballs showed decreased hardness and cohesiveness when guar gum was added at 0.5 and 1%. For the PGG patties, although a very low amount of guar gum was used during conjugation (i.e., PGG was prepared with 5% guar gum based on PPI,

corresponding to 0.025% gum addition in patties containing 5% PGG), it may still partially contribute to the softer texture of the patties.

6.3.2.5 Shear force

The Warner-Bratzler shear force indicates the maximum force as a knife cutting through meat sample, and it is useful for assessing meat tenderness (Novaković & Tomašević, 2017). With 2.5% PPI or PGG, the patties showed similar shear force as the control. When PPI addition was increased to 5%, the shear force of the patty was greatly increased to 1909 g; while the patty with a higher amount of PGG (5%) had significantly decreased shear force of 832 g, compared with the control patty (1429 g) (Table 3). Sungho et al. (2017) indicated that non-meat protein can be an alternative gelling agent, which enhances the binding of meat pieces, thus resulting in increased shear force of the patties with a higher amount of PPI. The decreased shear force of the patty with 5% PGG may be attributed to the better water and oil holding capacities of PGG protein, which can retain more moisture and fat in the cooked patties (Table 1 and 2). The shear force of patties had a similar trend as the hardness values. Chatterjee et al. (2019) and Huang et al. (1999) reported that an addition of tapioca starch and sorghum flour decreased shear force of chicken breast meat patties and beef patties, respectively. However, other studies (Sephora et al., 2016; Youssef & Barbut, 2011) also reported that the non-meat proteins increased hardness and shear force of meat products. It can be concluded that both protein concentration and functional properties of the added plant ingredients (e.g., flour, starch, and protein) influence the meat texture.

6.3.2.6 Compressed juiciness

Compressed juiciness values of the beef patties are summarized in Table 3. Overall, adding either PPI or PGG proteins decreased the values of compressed juiciness (ranging from 11.9 –

16.0%) compared with the control patty (19.1%). The beef patty with 5% PGG exhibited the lowest compressed juiciness value (11.9%). This is because the plant proteins (PPI or PGG) with good water and oil holding capacities and gelation properties can effectively bind water and oil in the beef patties and form gel matrix. Thus, the water and oil could not be easily extruded from the meaty matrix when the patty was compressed during testing, resulting in a higher amount of residue moisture and oil in the patties with added proteins compared with the control. Gujral et al. (2002) reported that adding texturized soy protein also decreased goat patty juiciness, and it was attributed to the better water absorption and holding capacity of the texturized proteins. They also showed that the juiciness was increased by increasing the content of liquid whole eggs in the patty formulation. Serdaroglu (2006) reported that the compressed juiciness of beef meatballs was not affected by adding whey protein of up to 4%, but decreased when the fat content was increased from 5 to 20%, which was related to the moisture and fat retention capacity of the meatballs. Overall, ingredient functionality, product formulation, and processing methods all determine the cookability and instrumental juiciness values of the product.

6.3.3 Descriptive sensory properties and principal component analysis (PCA)

Descriptive sensory characteristics of beef patties in terms of juiciness, tenderness, texture, beef flavor, beef flavor intensity, and off-flavor are presented in Figure 2 and summarized in Table S2 (Supplementary Documents). Overall, the beef patties containing PPI or PGG (both 2.5 and 5%) showed decreased juiciness, beef flavor, and beef flavor intensity, but increased off-flavor compared to the control. However, tenderness of the beef patties containing PGG significantly increased ($p < 0.05$) to around 72% for both 2.5 and 5% PGG patties, and the texture decreased to 51.5% for 2.5% PGG patty and 43.9% for 5% PGG patty, compared with the control patty (65.7% tenderness and 68.2% texture). These sensory results agreed with the

decreased hardness and chewiness of PGG patties from physical texture measurement (Table 3). Although juiciness was decreased for patties with the plant proteins compared with the control, PGG patties still showed significantly higher juiciness than PPI patties when protein was added at the same level, which was attributed to the better functional properties (water/oil holding capacity, emulsification, and gelation) of PGG than PPI. The results implied that some of the functional properties of plant protein ingredients can be carried over into end food products, such as in beef patties. The highest sensory juiciness score for the control patty was also in agreement with its largest compressed juiciness data from instrument measurement. However, the trend was somewhat different when comparing the compressed juiciness with sensory juiciness score for the patties containing added proteins. For example, the juiciness score of 5% PGG patty (44.2%) was much higher than that of 5% PPI patty (35.3%), but the former had a lower compressed juiciness value (11.9%) than the latter (16.0%). This is because sensory juiciness is mostly attributed to the available fats on the surface or crevice of patties perceived by the panelists during chewing, and it can also be associated to the tenderness and texture of patties, while compressed juiciness is determined by the liquid (oil/water) holding capacity of cooked patties. In addition, beef flavor and flavor intensity of the patties were greatly reduced, and off flavor was significantly increased even with only 2.5% plant protein addition. However, the beef patties containing PGG still demonstrated some advantages over the control patty, such as higher fat/water retention and cooking yield and softer and tender texture, which may be preferred by some elders. The flavor defect may be partially overcome by serving the patties with seasonings and dressings during meal service.

Principal component analysis was conducted to further determine the relationship between physical properties and sensory attributes of the different patty treatments (Figure 3). The eigenvalues 1 and 2 represented 87.85% of the variability. As shown on the biplot, the control beef patty without any plant protein addition was associated with strong beef flavor, high flavor score, large compressed juiciness value, and high cooking loss. The beef patties containing PPI (either 2.5 or 5%) were associated with high hardness, chewiness, and shear force value, while the beef patties with PGG were associated with better moisture and fat retention, lower cooking loss, softer texture, and tender sensory. All the beef patties containing the added proteins were also associated with off-flavor, which is common for many plant proteins.

6.4 Conclusions

Sequential modification of pea protein isolate (PPI) through deamidation and conjugation produced functionally enhanced protein, named PGG, with greater water and oil holding capacities, emulsifying properties, solubility, and gelation properties. Some of these functional properties in the protein ingredient can be carried over into end food products, such as in cooked patties. Extending beef patties with PPI or PGG reduced cooking loss, and thus increasing cooking yield, but also led to decreased juiciness and beef flavor scores and increased off-flavor score. The beef patties containing PGG also showed much softer and tender texture compared with the control patty, which would be advantageous features for some elder consumers with such sensory preference. Further research is needed to eliminate or reduce the off flavor in patties and other meat products extended with plant proteins. In addition to descriptive sensory analysis, consumer sensory evaluation can also be included to gain a more comprehensive understanding of the sensory properties of extended meat products.

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Conflict of interest

The authors declare that there is no known conflict of interest.

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Figures and tables

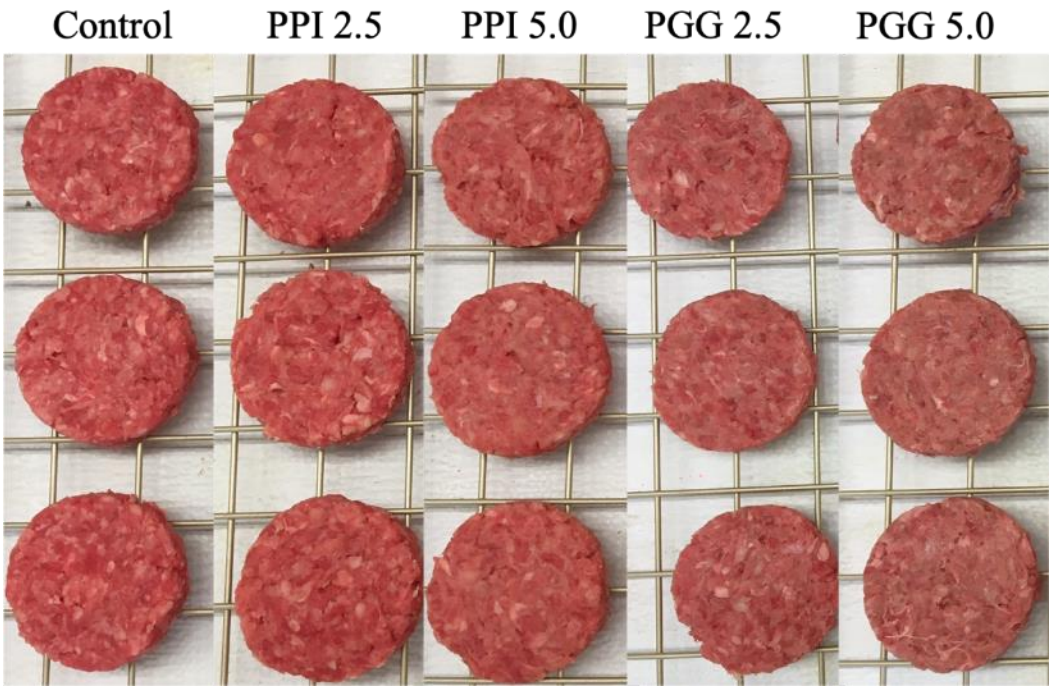

Figure 6.1 Pictures of raw beef patties extended with PPI or PGG.

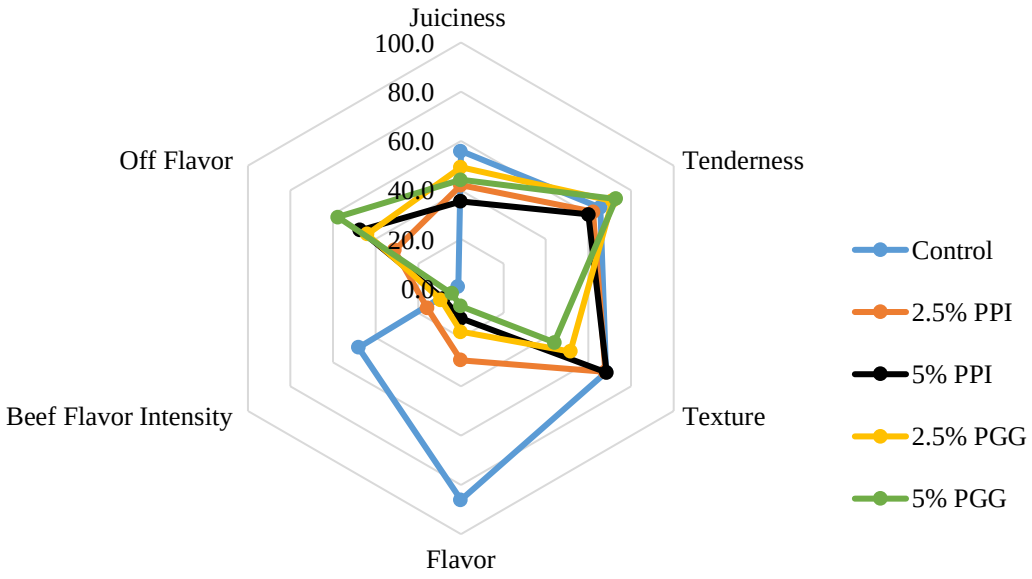

Figure 6.2 Descriptive sensory scores of different beef patties.

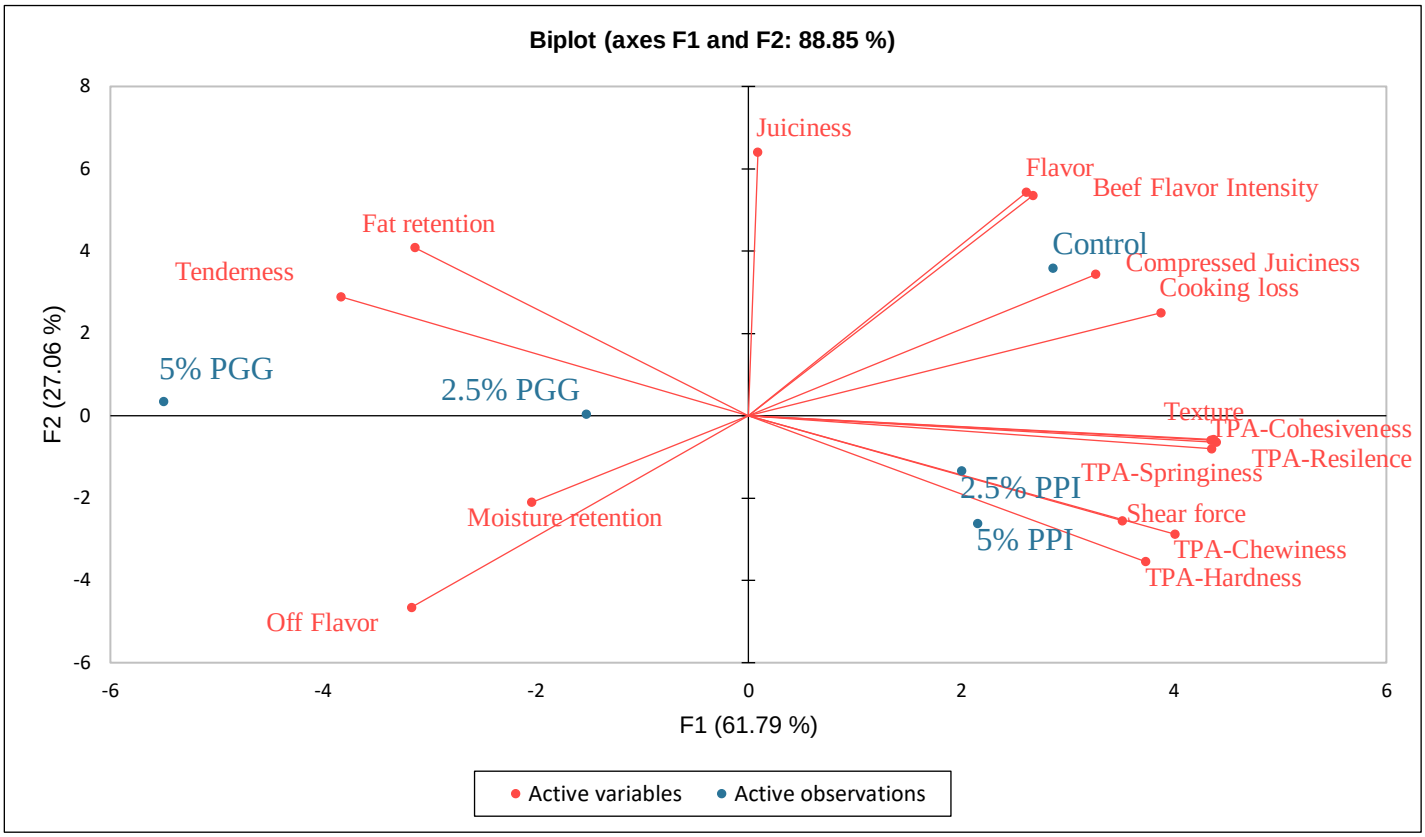

Figure 6.3 Principal component analysis (PCA) biplot describing the relationships between physical texture parameters and sensory attributes of different beef patties.

Table 6-1 Functional properties of pea protein isolate (PPI) and functionally enhanced pea protein (PGG).

Samples	WHC (g/g)	OHC (g/g)	EC (%)	ES (%)	LGC (%)	Solubility (pH 7) (%)
PPI	4.1±0.0[b]	1.4±0.0[b]	88.7±0.4[b]	66.7±0.6[b]	17%[b]	22.9±0.5[b]
PGG	4.8±0.0[a]	2.2±0.1[a]	99.4±0.1[a]	98.2±0.3[a]	12%[a]	50.5±0.5[a]

*Water holding capacity (WHC), oil holding capacity (OHC), emulsion capacity (EC), emulsion stability (ES), least gelation capacity (LGC), and solubility.

**Means with different letters in each column indicate significant differences ($p < 0.05$).

Table 6-2 Color, cooking loss, moisture and fat retention of beef patties.

*Means with different letters in each column indicate significant differences ($p < 0.05$).

	Color (raw patty)					
	L*	a*	b*	Cooking loss (%)	Moisture retention (%)	Fat retention (%)
Control	46.42±1.11[a]	17.09±0.74[a]	18.29±0.46[a]	32.82±1.69[a]	91.57±4.00[a]	83.05±3.26[a]
2.5% PPI	44.83±1.38[b]	17.13±0.69[a]	18.44±0.48[a]	30.54±1.93[a]	97.56±4.33[a]	74.58±1.64[a]
5% PPI	46.17±1.34[ab]	16.22±0.92[a]	18.28±0.56[a]	25.84±2.50[b]	97.23±1.81[a]	74.92±5.78[a]
2.5% PGG	47.33±0.61[a]	14.46±0.71[b]	16.79±0.81[b]	26.70±2.80[b]	98.74±3.27[a]	78.19±4.33[a]
5% PGG	47.31±1.27[a]	13.09±0.74[b]	16.42±0.78[b]	20.13±2.12[c]	100.15±0.36[a]	89.15±1.95[b]

Table 6-3 Physical attributes of beef patties from instrument analysis.

	Hardness (g)	Resilience (%)	Cohesiveness	Springiness (%)	Chewiness (g)	Shear force (g)	Compressed juiciness (%)
Control	5643.5±607.1^{a}	19.9±1.2^{a}	0.5±0.0^{a}	81.7±1.4^{a}	2365.5±428.0^{a}	1429.0±133.5^{a}	19.1±2.1^{a}
2.5% PPI	7061.8±425.0^{b}	20.5±0.7^{a}	0.5±0.0^{a}	81.6±1.0^{a}	3006.3±281.7^{a}	1344.7±190.8^{ab}	13.7±1.9^{b}
5% PPI	7359.2±323.0^{b}	19.6±1.0^{a}	0.5±0.0^{a}	81.8±1.7^{a}	3081.3±237.6^{a}	1906.9±192.1^{a}	16.0±0.8^{a}
2.5% PGG	4889.8±328.0^{ac}	18.2±1.2^{a}	0.5±0.0^{a}	78.3±3.6^{a}	1856.1±244.1^{b}	1379.8±360.3^{ab}	15.5±1.3^{ab}
5% PGG	3984.1±459.1^{c}	15.7±1.4^{b}	0.4±0.0^{b}	71.9±2.2^{b}	1197.4±155.1^{b}	831.7±142.1^{b}	11.9±0.6^{b}

*Means with different letters in each column indicate significant differences ($p< 0.05$).

Chapter 7 - Emulsifying properties and mayonnaise application of pea protein isolate conjugated with guar gum

Abstract

Plant proteins are receiving increasing interests in food and ingredient applications. Functionally improved plant protein may be used as a healthier emulsifier in food products. The objective of this study was to evaluate the emulsifying properties of functionally enhanced pea protein (i.e., pea protein conjugate with guar gum, G-PPI) and potential application in mayonnaise, compared with unmodified pea protein isolate. Emulsions containing G-PPI were prepared at different pH environments (3, 5, 7, 9), salt concentrations (0.01, 0.1, and 1 M NaCl), protein concentrations (1, 1.5, and 2%), and oil/water ratios (10/90, 30/70, 50/50, 70/30, 90/10). Mayonnaise samples were prepared using the pea proteins or egg yolk powder at different concentrations. Various characteristics of the emulsions, including particle size, zeta potential, apparent viscosity, viscoelasticity, and microstructure were analyzed. The emulsions with G-PPI had significantly increased stability of up to 89.37% and apparent viscosity of up to 48.62 mPa.s. The G-PPI emulsion had smaller droplet size of 934.40 nm at pH 7 compared with the PPI emulsion (stability: 62.66%, apparent viscosity: 22.80 mPa.s, droplet size: 1664.80 nm). The pH, NaCl concentration, protein concentration, and oil/water ratio greatly affected the emulsifying properties. The G-PPI mayonnaise at higher protein concentrations (6 or 8%) exhibited excellent emulsifying properties and viscoelasticity with G´ and G´´ of 1161.57 pa and 361.51 pa, respectively. The modified pea protein through the green process could be used as a safe and healthier emulsifier in different emulsified foods.

Keywords: plant protein, protein conjugate, emulsifier, mayonnaise, rheology

7.1 Introduction:

Plant proteins are receiving tremendous attention and interest in food and ingredient applications, due to their advantages such as lower cost and more sustainable nature compared with animal proteins (Zha, Dong, Rao, & Chen, 2019a). Pea proteins extracted from yellow pea (*Pisum sativum L.*) are among the most widely used plant proteins, only after soy proteins and wheat gluten. Pea protein consists of 15-25% water-soluble albumin and 65-80% of salt extractable globulin, and it contains high levels of essential amino acids such as lysine, threonine, and tryptophan (Burger & Zhang, 2019). The major globulin proteins in pea include legumin and vicilin, as well as small amount of convicilin (Tamnak, Mirhosseini, Tan, Ghazali, & Muhammad, 2016). Pea protein, as a promising plant protein, has great food application potentials due to its nutritional value, health benefits, less allergenicity, and diverse functional attributes (Boye, Zare, & Pletch, 2010). However, commercial pea proteins tend to have low solubility and less desirable functionalities (Zha et al., 2019a).

Polysaccharide gums are complex hydrophilic polymers with many functional properties, and they have been widely used in the food industry as thickeners, gelling agents, textural modifiers, etc. (Tamnak et al., 2016). Protein-polysaccharide conjugation is a chemical-free, mild, and safe modification method to improve protein functional properties such as emulsifying, foaming, and gelling. Conjugation reaction builds chemical linkages between the two polymers by condensing ε-amino group of the protein and carbonyls of the polysaccharide during early-stage Maillard reaction (Burger & Zhang, 2019). In the conjugation process, non-covalent electrostatic interactions can also contribute to the formation of new hybrid polymeric complex (Tabatabaee Amid & Mirhosseini, 2014). In the early stage, the Maillard reaction mainly induces the non-covalent electrostatic interactions between protein and polysaccharides in forming a new hybrid

polymer with much lighter color. The advanced Maillard reaction may accelerate the chemical reaction and form a less soluble polymer with a much darker color (Tabatabaee Amid & Mirhosseini, 2014). The covalent linkages of protein and polysaccharides may deliver better molecular integrity than non-covalent interactions (Dickinson & Galazka, 1991). The formation of protein and polysaccharide conjugate and complex can promote the structural and textural characteristics of food products via their aggregation and gelling behaviors (Neirynck, Van Der Meeren, Bayarri Gorbe, Dierckx, & Dewettinck, 2004). Several previous studies reported that pea protein conjugated with polysaccharides had significantly improved emulsifying properties (Qing Guo, Su, Yuan, Mao, & Gao, 2019; S. Liu, Elmer, Low, & Nickerson, 2010; Zha et al., 2019a; Zha, Dong, Rao, & Chen, 2019b). Zha et al. (2019a) indicated that pea protein conjugated with gum arabic possessed better emulsifying properties, and the resultant oil-in-water emulsions had smaller droplet size, higher surface charge, and stronger steric hindrance. Further, some studies reported that the pea protein conjugated with pectin showed good rheological behavior in oil-in-water emulsions (Gharsallaoui, Yamauchi, Chambin, Cases, & Saurel, 2010; Lan, Chen, & Rao, 2018; Tamnak et al., 2016).

Guar gum is a high molecular weight polysaccharide extracted from guar bean (*Cyamopsis tetragonolobus)*. The chemical structure of guar gum consists of a straight chain of D-mannose unit linked by β - (1-4) glycoside linkages with mannose to galactose ratio of 2: 1(Chityala, Khouryieh, Williams, & Conte, 2016). Recently, we developed a functionally enhanced pea protein through conjugation modification with guar gum based on wet Maillard reaction, namely G-PPI (Shen & Li, 2021). This novel ingredient exhibited excellent water and oil holding capacities, solubility, emulsifying, and gelling properties. Emulsions are widely used in food, cosmetic, and pharmaceutical applications. They are colloidal systems and consist of two

immiscible liquids (i.e., water and oil), which are thermodynamically unstable because of several physical mechanisms such as gravitational separation, coalescence, flocculation (McClements, 2016). Protein can reduce the interfacial tension between water and oil phases, and make the emulsion more stable through the formation of viscoelastic layers on the droplets and generating repulsive steric and electrostatic interactions between the droplets (McClements, 2004). On the other side, native pea protein is a less effective emulsifier compared with synthetic surfactants or emulsifiers. The modification approach is necessary for protein to possess better emulsifying properties and steric stabilization.

Mayonnaise is a semi-solid oil-in-water emulsion made from several major ingredients such as egg yolk, vinegar, oil, and water. The stability of mayonnaise depends on the amount of oil, water, egg yolk, viscosity, and production methods. Due to the health concern of cholesterol content in the egg yolk, replacement of egg yolk with plant proteins for mayonnaise preparation has attracted more interest. The objective of this study was to evaluate the emulsifying properties of the modified pea protein (G-PPI) at different pH, NaCl, protein concentrations, and oil/water ratio conditions, as well as application in mayonnaise, and characterize their properties in terms of particle size, zeta potential, apparent viscosity, viscoelasticity, and microstructure. This study will benefit the researchers interested in utilizing plant protein in various food applications.

7.2 Materials and methods

7.2.1 Materials

Pea protein isolate (83% protein content, 5% ash) was obtained from a commercial manufacturer. Guar gum was purchased from Judee's (Plain City, OH, USA). Soybean oil was purchased from Healthy Harvest Production, LLC (Berthoud, CO, USA).

7.2.2 Preparation of modified pea protein (G-PPI)

The modified pea protein (G-PPI) was prepared by mixing pea protein isolate (PPI) with 5% guar gum (based on PPI) in aqueous suspension (10% PPI concentration) through a wet heat Maillard reaction at 60 °C for 24 hours. The slurry was then lyophilized, and the dried conjugate powder was ground and kept at 4 °C for further analysis and emulsion and mayonnaise preparations.

7.2.3 Preparation of emulsions and mayonnaises

Emulsion preparation: For emulsions based on the unmodified pea protein, the protein (0.75 g) was added into deionized (DI) water (25 mL), which was then vortexed for 30 seconds to dissolve and disperse the protein particles. Soybean oil (25 mL) was then added to the protein slurry. The mixture was treated with a high-performance homogenizer (Fisher Scientific, Fair Lawn, NJ, USA) for 2 min at 20,000 rpm. Emulsions with different variables were prepared similarly except that the parameters were adjusted to the set conditions (pH, NaCl concentration, protein concentration, oil/water ratio). For the modified protein, we prepared emulsions by changing the pH condition (3, 5, 7, 9), salt concentrations (0.01, 0.1 and 1 M NaCl), protein concentrations (1, 1.5, and 2%), and the ratio of oil/water (10:90, 30:70, 50:50, 70:30, 90:10). Emulsions with the control pea protein at pH (3, 5, 7, 9), 0.1 M NaCl, 0.75 g protein concentration, and 50:50 oil/water ratio were also prepared similarly for the comparison.

Mayonnaise preparation: The formulation of low-fat mayonnaise was followed from a previous reference with small modifications (Liu et al., 2018). The basic mayonnaise formula included 25 mL soybean oil, 23.5 mL water, 1.5 mL vinegar, 0.75 g sugar, 0.35 g salt, and varied amounts of egg yolk powder (Modernist pantry, ME, USA), unmodified pea protein, or modified pea protein (2, 4, 6, or 8% based on total oil, water and vinegar weight). Briefly, all the

ingredients except for the oil were homogenized in a Waring blender for 30 seconds. The soybean oil was added into the suspension and homogenized for another 30 seconds. The mayonnaise sample was collected and stored in a glass jar at 4 ºC for further analysis within two days.

7.2.4 Functional properties of pea proteins

Protein functional properties, including water and oil holding capacities, solubility, least gelation concentration, and emulsion capacity and stability, were measured according to our previous methods without any modifications (Shen & Li, 2021).

7.2.5 Apparent viscosity

Apparent viscosity of the emulsions was measured using a rheometer (MCR-92 Anton Paar, Ashland, VA, USA) equipped with a CP50-mm diameter stainless cone with an angle of 1º and a 0.101-mm gap at 25 ºC. The apparent viscosity of the samples was measured at shear rate range of 0.1 -100 s^{-1}. The measurement was conducted in duplicates.

7.2.6 Particle size and zeta potential

The mean particle size and zeta potential of the proteins and emulsions were measured using a dynamic light scattering analyzer (DelsaMaz Assist, Beckman Coulter, Indianapolis, Indiana, USA). The protein samples were dissolved in the distilled water at 1%, and adjusted pH to 3, 4, 4.5, 5, 7, 9, and 11. The protein solutions were centrifuged at 10,000 ×g for 5 min, and the supernatants were collected. The supernatants were further diluted to 1/100 with buffers (citrate buffer for pH 3, 4, 4.5, and 5; phosphate buffer for pH 7; glycine-NaOH buffer for pH 9 and 11). The emulsion samples were prepared similarly as described above and diluted to 1/100 and then injected into the flow cell using a syringe at 20 ± 1 ºC to obtain the particle size and zeta potential (Singh & Amamcharla, 2021).

7.2.7 Viscoelastic properties

The viscoelastic properties of mayonnaise were measured using the same rheometer (MCR-92 Anton Paar, Ashland, VA, USA) equipped with PP25 mm parallel plate with a gap of 1 mm . The analyses were conducted with different testing modes, including strain sweep (strain range of 0.01-100% at a fixed frequency of 1 Hz), frequency sweep (frequency range of 0.1- 100 Hz, at 0.5% strain), and temperature sweep (from 30- 80 ºC) (Rathod & Amamcharla, 2021). Parameters including storage modulus (G') and loss modulus (G") were collected in duplicate.

7.2.8 Microstructure

Images of emulsions and mayonnaises were taken using an optical Microscope (Olympus America Inc., Melville, NY, USA). One tiny drop of the sample was transferred onto the microscope glass slide, then the sample was covered with a cover-slip and viewed with a 40 × objective lens. The images were collected with the Lightscreen software and processed with Image J.

7.2.9 Statistical analysis

All the data were analyzed using SAS University Edition software (SAS Institute, Cary, NC, USA), with one-way ANOVA and Tukey's post-hoc comparison test. Significant difference among all the data sets was considered as $p < 0.05$. The results were presented as mean ± standard deviation.

7.3 Results and discussions

7.3.1 Protein functional properties

The conjugated pea protein (G-PPI) possessed significantly better water and oil holding capacities, solubility (pH 7), and gelation property (Table 1) than the unmodified pea protein. The water and oil holding capacities of G-PPI reached 4.99 and 2.54 g/g compared with the

control protein (4.09 and 1.35 g/g), respectively. The solubility of G-PPI was greatly increased to 64.66%, compared with the control (22.89%). The G-PPI also exhibited better gelation property (with lease gelation concentration (LGC) of 12%) than the control (LGC of 17%). During the conjugation modification, the inclusion of guar gum to the protein molecules enhanced the hydrophilicity of the complex and altered the original balance of protein hydrophobicity and hydrophilicity, which resulted in improved protein dispersion, better solubility and emulsification properties. The more hydrophilic guar gum domains in the complex enhanced the affinity between water and the complex (Baniel, Caer, Colas, & Gueguen, 1992; Du et al., 2013); thus, the water holding capacity was also increased. Moreover, the heat treatment during the protein and polysaccharide conjugation unfolded protein globular structures and exposed more hydrophobic amino acid residues to the protein surface, which contributed to the improved oil holding capacity (Shen & Li, 2021). Guar gum had gel thickening properties, and the inclusion of guar gum in the protein conjugation enhanced protein hydrophobic interactions in forming more stable gel networks (O'Kane, Vereijken, Gruppen, & Van Boekel, 2005).

7.3.2 Zeta potential

Various repulsive and attractive forces are involved in the interactions that influence the stability of protein colloidal systems (Gerzhova, Mondor, Benali, & Aider, 2016). Zeta potential indicates the magnitude of electrostatic interaction between particles. When the particles carry some net charges, either positive or negative, the repulsive forces play a predominant effect, which prevents the protein suspensions from aggregation. In contrast, when the net charge is close to zero or neutral, the attractive forces become more essential, resulting in particles' aggregation and precipitation (Gerzhova et al., 2016). The stability and the surface charge of protein particles are mainly dependent on the pH of the medium. The zeta potential of PPI and

G-PPI at different pH conditions are shown in Figure 2. The PPI carried net negative charges in the alkaline medium with the maximum net charge at pH 7 – 9. The net charge was reduced when the pH decreased as it was near the isoelectric point (pH 3 – 4). Similarly, the maximum net charge of G-PPI was around pH 7, and the isoelectric point was around pH 4 – 5. However, when at pH 9, the net charge of G-PPI was decreased greatly compared to the PPI. This result may be attributed to the hydrophobic interactions between the unfolded protein and guar gum, and the G-PPI enhanced the gel formation during the conjugation process. Thus, the protein colloidal system became viscous and aggregated, and the net charge was decreased. Moreover, guar gum is a neutral polysaccharide, and the net charge was unaffected by the pH changes (Wang, Ellis, & Ross-Murphy, 2000).

7.3.3 Emulsifying properties of pea proteins

The emulsifying properties (e.g., emulsion capacity and stability) of oil-in-water emulsion and mayonnaise samples containing the pea proteins at different formulations and environmental conditions are summarized in Table 2 and Table 4, respectively. Overall, the G-PPI emulsions exhibited much better emulsion capacity and stability than the PPI emulsions. They showed higher resistance against the flocculation, coalescence, and phase separation than the PPI emulsions. This physical stability of emulsion against the gravitational separation can be improved by decreasing the droplet size, and the G-PPI protein could be absorbed faster at the oil/water interface and resulted in more stable emulsions (Tamnak et al., 2016). The pH had a significant impact on emulsifying properties of the emulsions. For both PPI and G-PPI emulsions, when the pH was close to the isoelectric point (pH 5), the emulsions exhibited poor stability, and phase separation with a transparent serum layer occurred (Figure 3). The G-PPI emulsions showed great emulsion stability in different salt conditions (0.1 – 1 M NaCl), with ES

all above 95%. When the G-PPI protein concentration increased from 1 to 2% in the emulsion, both emulsion capacity and stability were significantly improved. The increased protein concentration promoted oil droplets' surface coverage, enhanced protein adsorption, and effectively inhibited emulsion aggregation (Ladjal Ettoumi, Chibane, & Romero, 2016). The oil/water ratio of emulsion is an important factor that could affect the emulsifying properties. For the modified pea protein G-PPI, when the oil/water ratio was too low or too high, for example, 10/90 or 90/10, the system cannot form a good emulsion, with very low values of emulsion capacity and stability (Figure 3, Table 2). The emulsions with oil/water ratio of 50/50 or 70/30 had the best emulsion stability compared with other ratios, which may be attributed to the larger oil surface area covered by G-PPI protein, increasing droplet size, and apparent viscosity (Table 3).

7.3.4 Apparent viscosity of emulsions

Apparent viscosity of the emulsions containing PPI or G-PPI is shown in Figure 1, and the viscosity values at the shear rate of 100 (1/s) are summarized in Table 3. The emulsions showed shear-thinning behavior between the shear rate of 0.1 -100 s^{-1}, attributed to the breakdown of intermolecular interactions or linkages through droplet particles during the shearing (Zhang et al., 2012). Overall, the G-PPI emulsions exhibited significantly higher viscosity than the PPI emulsions at the same pH, NaCl, or protein concentration (Table 3). This was because the inclusion of guar gum during protein conjugation modification enhanced the formation of new biopolymers through covalent interaction and increased solubility. The smaller particle size (Table 3) and uniform droplet size distribution (Figure 7) of emulsions indicated that the G-PPI had a better ability to facilitate the absorption at the oil/water interface during emulsification (Tamnak et al., 2016). The PPI emulsion showed the highest viscosity at pH 3 (31.09 mPa.s),

while the highest viscosity of G-PPI emulsion was at pH 5 (88.21 mPa.s). This result could confirm that the PPI and G-PPI had the lowest charge at pH 3 and 4.5, respectively (Figure 2), which were attributed to their lower solubility and the formation of aggregates around the isoelectric pH condition.

When the NaCl concentration increased from 0.01 to 1 M, the viscosity of G-PPI emulsion was increased. The emulsion with 0.1 and 1 M NaCl showed significantly higher viscosity at 100 1/s shear rate than the emulsion with 0.01M NaCl. The higher concentration of salt addition had decreased the electrostatic repulsion forces, favored protein aggregation or flocculation through electrostatic and van der Waals attraction. It resulted in higher viscosity with larger droplet size emulsion (Table 3) (Xu, Liu, & Zhang, 2015). When the protein concentration (G-PPI) increased from 1 to 2%, the emulsion viscosity (at 100 1/s shear rate) was increased from 32.89 to 89.04 mPa.s. The pea protein and guar gum conjugation had increased the protein molecular weight and the resistance to flow; thus, the viscosity was significantly increased (Ibanoğlu, 2002). Therefore, a higher concentration of G-PPI in the emulsion increased apparent viscosity. Moreover, the emulsion with an oil/water ratio of 70: 30 showed exceptional higher viscosity (1697.10 mPa.s) than the other emulsions. This was because the expansion of the water-protein matrix caused a large amount of oil to entrapped in the matrix, and the interactions between protein hydrophobic domains and the oil molecules were increased (Zorba, 2006). The higher viscosity of emulsion may limit the motion of droplets and decrease the frequency of collisions among the droplets (Guo & Mu, 2011). These semi solid-like emulsion textures can be potentially further used in the high-internal phase emulsion or other hydrogel applications.

7.3.5 Particle size of emulsions

The average droplet size of the emulsions containing the PPI or G-PPI is summarized in Table 3. Overall, the G-PPI emulsion exhibited a smaller particle size than the PPI emulsions at the same pH, NaCl, or protein concentration. This was because the pea-guar gum conjugate had the amphiphilic structure and provided a bulky steric stabilizing layer around the oil droplet and facilitates its absorption at the oil/water interface, and resulting in smaller droplet size (Pirestani, Nasirpour, Keramat, Desobry, & Jasniewski, 2017; Tamnak et al., 2016). In the meantime, the protein absorption at the oil/water interface is fast enough to efficiently retard the aggregation and coalescence of emulsion. For the emulsion at different pHs, the PPI emulsion showed the largest particle size in pH 5, while the G-PPI emulsion was in pH 3. When the NaCl concentration increased from 0.01M to 1M, the emulsion particle size was increased from 605 to 1084 nm. This was because the protein aggregation or flocculation were formed via van der Waals attraction, which increased the viscosity and droplet size of emulsions (Xu et al., 2015). When the protein (G-PPI) concentration increased from 1 to 2%, the emulsion particle size was decreased from 727 to 588 nm, although there was no significant difference ($p > 0.05$) in the average particle sizes of the emulsions with different protein concentrations. In addition, the emulsion with an oil/water ratio of 70: 30 showed the largest particle size than with other ratios, which was attributed to its higher viscosity and extensive formation of aggregates.

7.3.6 Mayonnaise applications

7.3.6.1 Mayonnaise emulsion capacity and stability

Mayonnaise samples containing different amount of PPI, G-PPI, or egg yolk were prepared, and the emulsion properties were also investigated (Figure 4 and 5). When the protein (PPI or G-PPI) or egg yolk concentration increased, the emulsion capacity and stability increased (Table 4).

Overall, the mayonnaise made by G-PPI exhibited better emulsion capacity and stability than that made from PPI or egg yolk. When the mayonnaise was made from the same concentration of protein or egg yolk (8%), the G-PPI mayonnaise showed much higher stability and viscoelastic properties (Table 4) (Figure 4). Moreover, the egg yolk mayonnaise needs to increase the concentration up to 10%, then could reach similar emulsifying properties compared with G-PPI mayonnaise (8%). The G-PPI protein could be used as an alternative protein in condiments or non-dairy applications to improve the textural and functional properties of products.

7.3.6.2 Viscoelastic properties

The viscoelastic properties (G' and G''), from both frequency and temperature sweeps of the mayonnaise samples, are shown in Figure 6. The elastic modulus (G') and viscous modulus (G'') from frequency sweep at 1 Hz are summarized in Table 4. Obviously, the G-PPI mayonnaise showed significantly higher G' and G'' values at 4, 6, and 8% additions than the other mayonnaises. They also exhibited higher G' than G'', which indicated that the mayonnaise had a solid-like behavior with higher viscosity. However, the PPI and egg yolk mayonnaise showed lower G' and G'' compared with G-PPI, and they revealed higher G'' than G', which exhibited a liquid-like texture. This was attributed to their poorer emulsifying properties.

Because the mayonnaise samples G-PPI 4, 6, and 8%, and egg yolk 10% exhibited solid-like texture, we further investigated their viscoelastic properties with temperature changes. When the temperature increased from 30 to 80 °C, the G' was larger than G'' and was not markedly affected by the temperature, which indicated that the mayonnaise was thermal stable. Two mayonnaise samples with higher protein and egg yolk concentrations showed a peak of 67 and 73 °C for G-PPI 8% and egg yolk 10%, respectively. The protein in the mayonnaise may be further unfolded

and denatured during the heating process, and the molecular were aggregated to form a rigid gel network with a spatial structure, and resulting in an increase of G' and G" (Xiao et al., 2020).

7.3.6.3 Microstructures

The microstructure of emulsions and mayonnaise is shown in Figure 7. The G-PPI emulsion exhibited finer oil droplets with more compact and uniform structures for the emulsion samples, while the PPI emulsion had more polydispersity of oil droplets. This was attributed to the improved emulsifying properties of the G-PPI protein. The protein and guar gum conjugation enhanced the balance of protein hydrophobicity and hydrophilicity and prevented droplets from flocculation or coalescence by increasing steric repulsion (Pirestani et al., 2017). For mayonnaise samples, a higher concentration of G-PPI (6 and 8%) and egg yolk (10%) showed more uniform microstructures. In comparison, the PPI or egg yolk mayonnaise at the lower concentration showed oil droplets with flocculation. This phenomenon was consistent with the result of emulsifying properties and droplet size.

7.4 Conclusions:

Conjugation modification of pea protein with guar gum greatly improved its water and oil holding capacities, solubility, and gelation properties of protein. This modified pea protein (G-PPI) demonstrated excellent emulsifying properties in different emulsion compositions and mayonnaise applications. The emulsions with G-PPI had significantly increased stability, apparent viscosity, and decreased droplet size compared with the PPI emulsions. The pH, NaCl concentration, protein concentration, and oil/water ratio affected the emulsifying properties. The mayonnaise with G-PPI at higher concentrations (6 and 8%) exhibited significantly better emulsification properties and viscoelasticity than that of PPI or egg yolk. This novel and “green” modified pea protein may be used as a healthier emulsifier in different food emulsions. This

study will benefit researchers and food professionals interested in developing and utilizing plant proteins in various food applications.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Figures and Tables

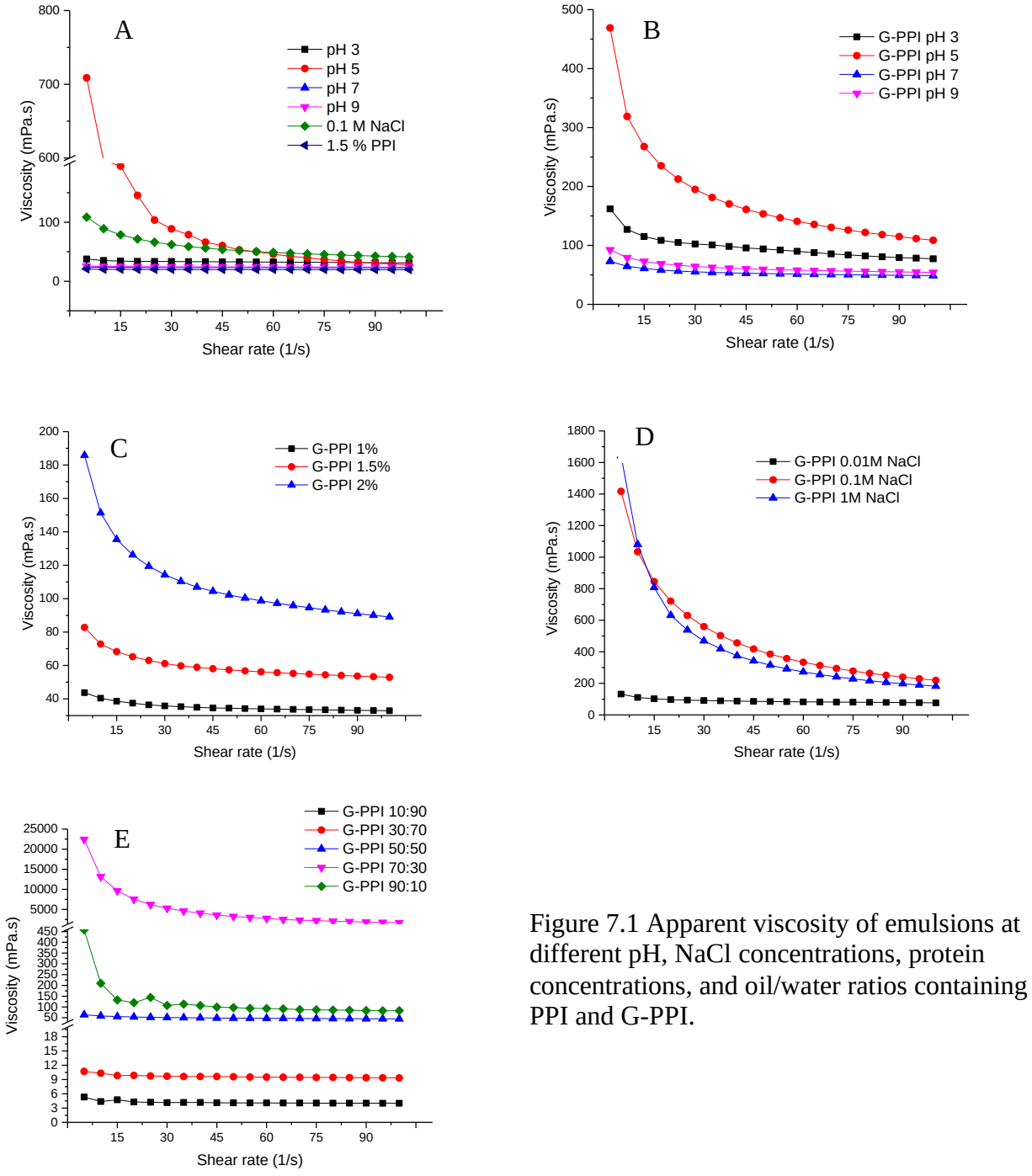


Figure 7.1 Apparent viscosity of emulsions at different pH, NaCl concentrations, protein concentrations, and oil/water ratios containing PPI and G-PPI.

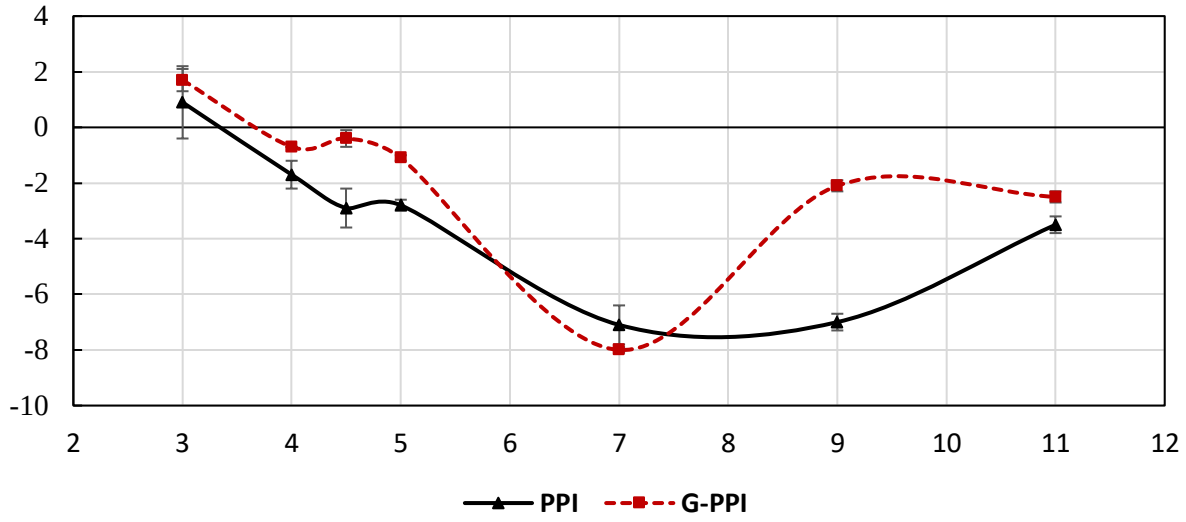


Figure 7.2 Zeta potential of pea protein isolate (PPI) and modified pea protein (G-PPI) at different pH conditions.

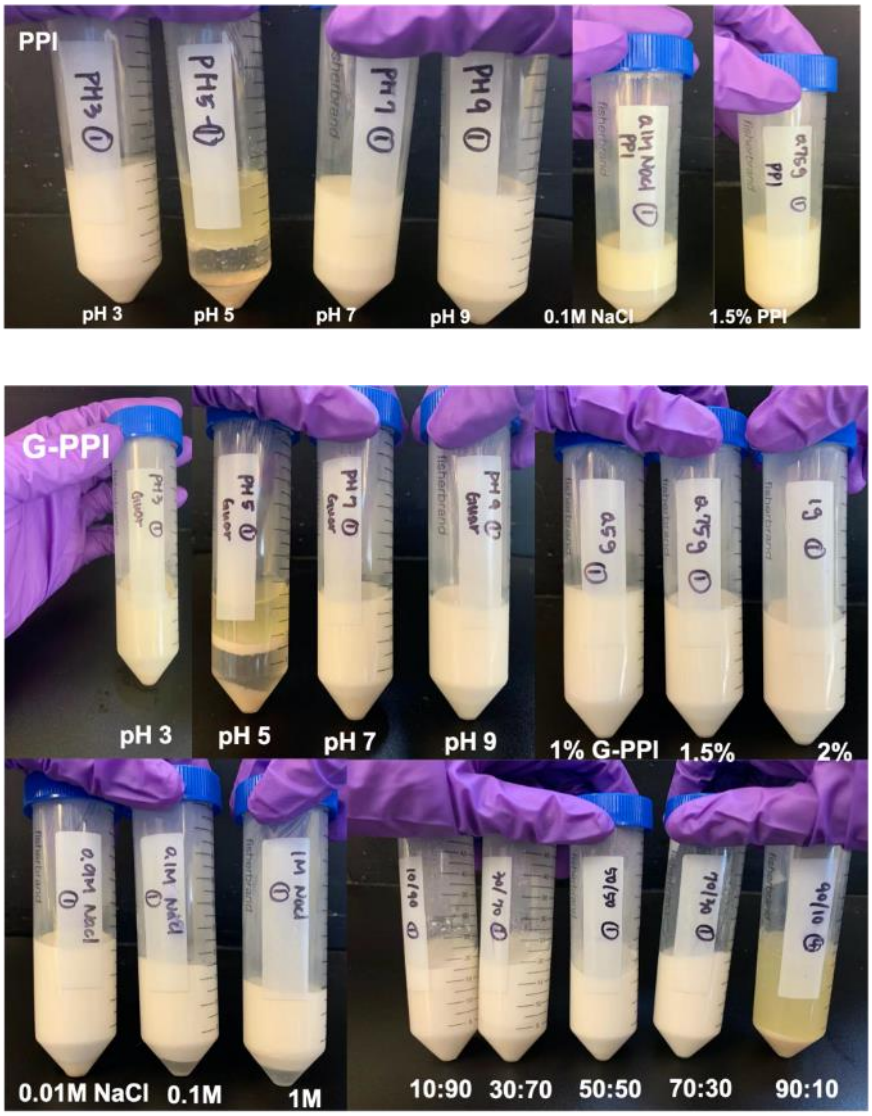


Figure 7.3 Pictures of emulsions after stability tests (i.e., heating and centrifugation treatment) at different pH, NaCl concentrations, protein concentrations, and ratio of oil/water containing PPI and G-PPI.

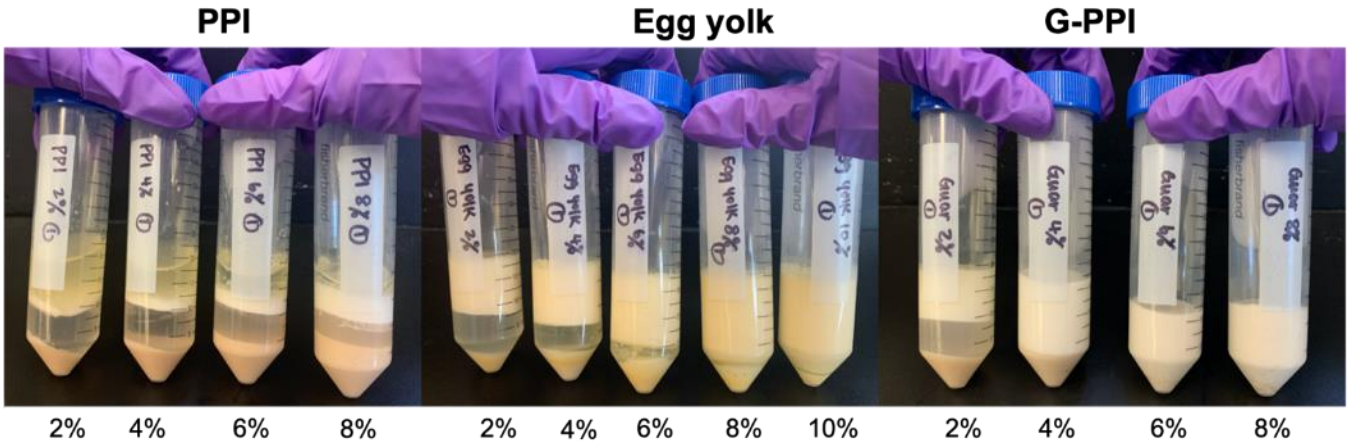


Figure 7.4 Pictures of mayonnaise samples with different protein or egg yolk concentrations after stability tests (i.e., heating and centrifugation treatment).

Figure 7.5 Pictures of mayonnaise samples made from PPI (8%), egg yolk (10%), and G-PPI (8%).

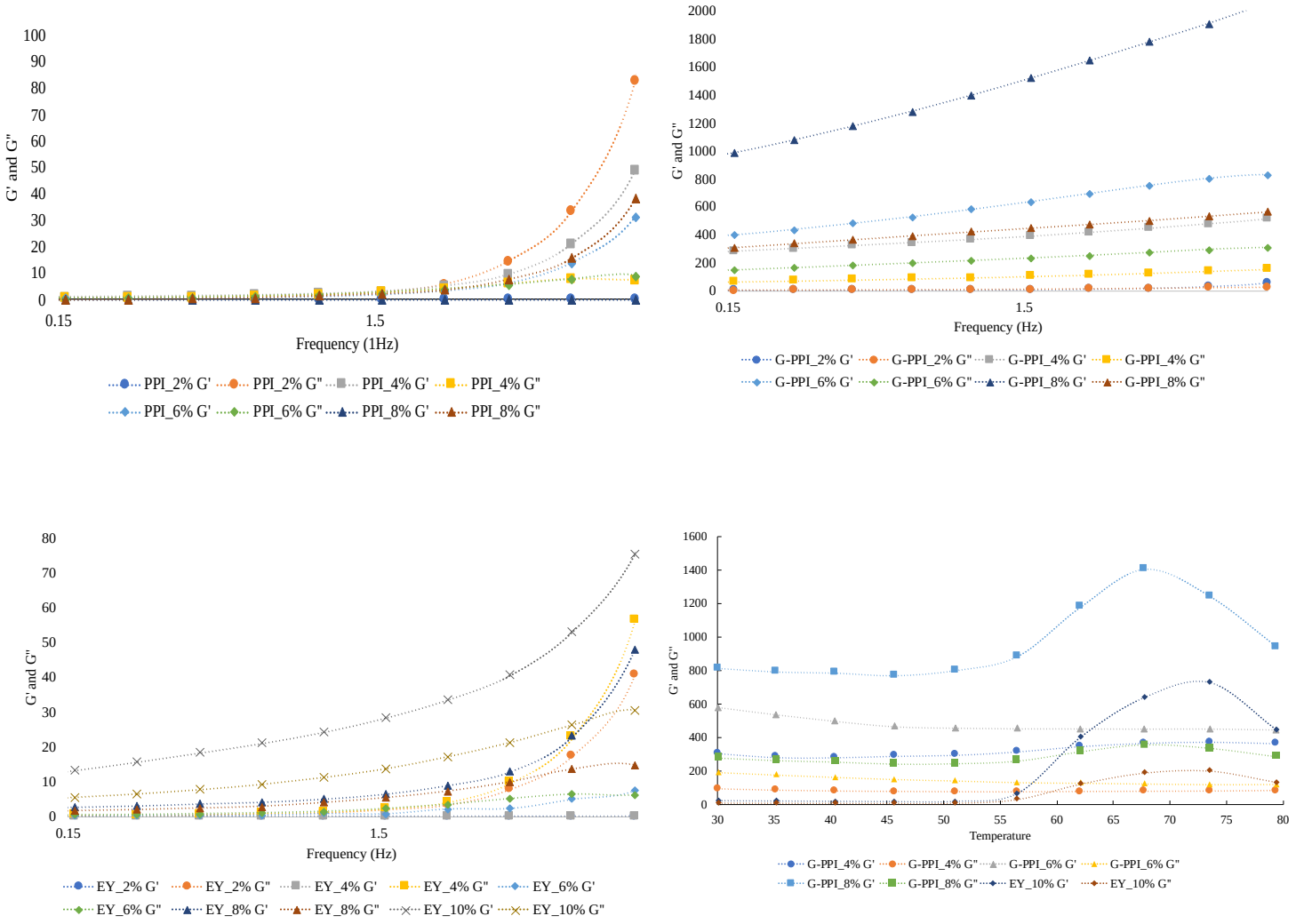


Figure 7.6 Viscoelastic properties (G' and G"), including frequency and temperature sweeps of mayonnaise samples.

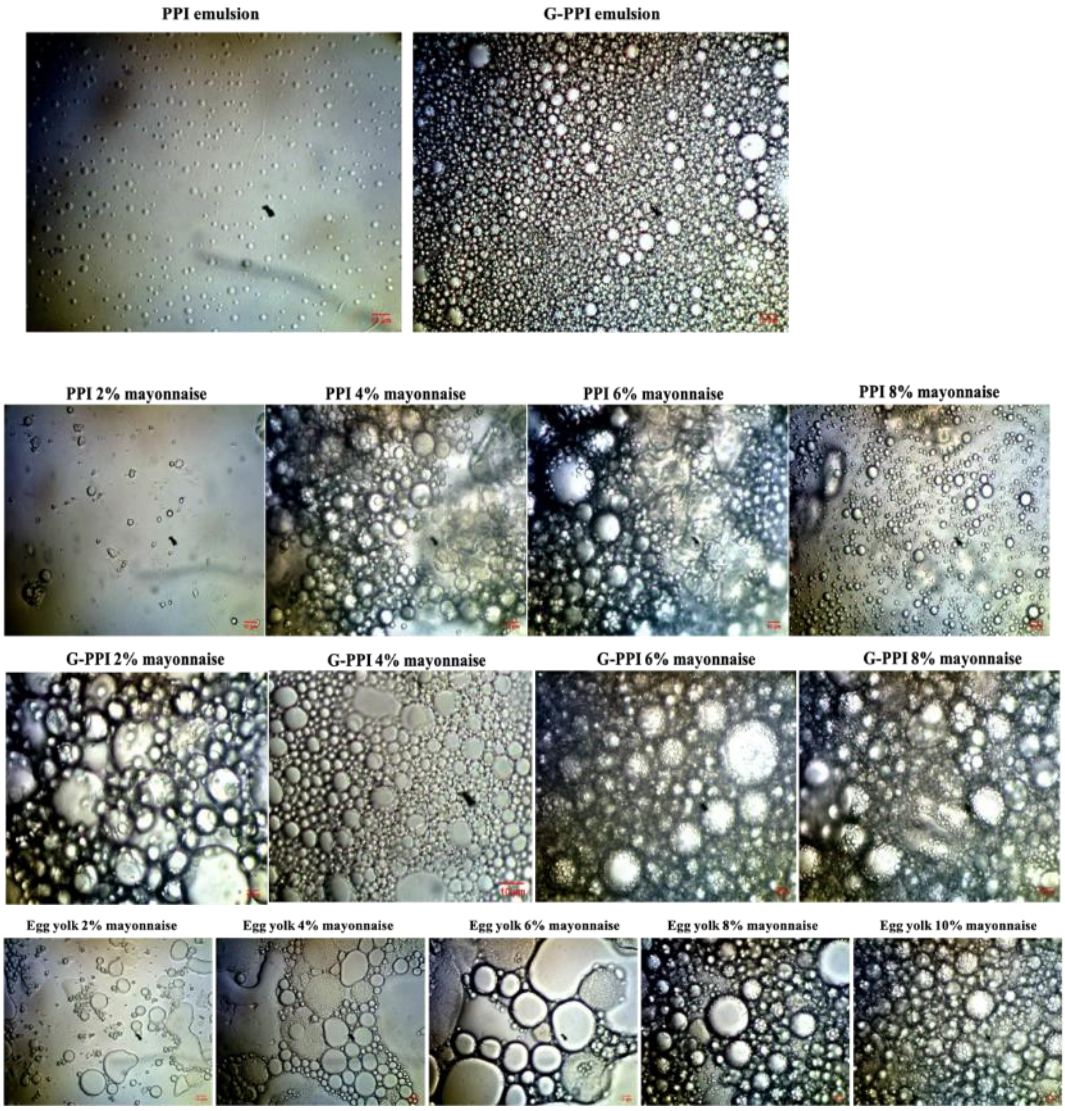


Figure 7.7 Microstructures of oil-in-water emulsions with PPI (1.5%) and G-PPI (1.5%), and mayonnaises made from PPI, egg yolk, and G-PPI at different concentration.

Table 7-1 Functional properties of pea protein isolate (PPI) and modified pea protein (G-PPI).

Samples	WHC(g/g)	OHC(g/g)	Solubility(%)	LGC(%)
PPI	4.1 ± 0.0^{b}	1.4 ± 0.0^{b}	22.9 ± 0.5^{b}	17^{a}
G-PPI	5.0 ± 0.0^{a}	2.5 ± 0.0^{a}	64.7 ± 2.0^{a}	12^{b}

*Water and oil holding capacities (WHC, OHC), solubility (pH 7), and least gelation capacity (LGC).

**Means with different letters in each column indicate significant differences ($p< 0.05$).

Table 7-2 Emulsifying properties including emulsion capacity (EC) and stability (ES) of emulsions at different pH, NaCl concentrations, protein concentrations, and oil/water ratios.

PPI						
pH	pH 3	pH 5	pH 7	pH 9	0.1M NaCl	1.5% PPI
EC (%)	89.7±5.1^{b}	44.7±3.7^{d}	83.6±1.6^{b}	97.6±0.6^{a}	64.8±3.2^{c}	84.9±0.2^{b}
ES (%)	67.2±1.7^{bc}	0	62.7±1.1^{bc}	69.8±2.1^{a}	60.6±4.7^{d}	65.2±1.5^{bcd}
G-PPI						
pH	pH 3	pH 5	pH 7	pH 9		
EC (%)	98.9±0.2^{a}	73.6±1.0^{c}	97.1±0.3^{b}	98.0±0.5^{ab}		
ES (%)	88.9±2.1^{b}	0	89.4±0.7^{b}	93.7±0.9^{a}		
NaCl Conc.	0.01M	0.1M	1M			
EC (%)	99.0±0.2^{b}	99.5±0.1^{a}	99.2±0.3^{ab}			
ES (%)	96.6±1.2^{ab}	97.6±0.4^{a}	95.7±0.4^{b}			
Protein Conc.	1%	1.5%	2%			
EC (%)	90.0±1.3^{c}	97.7±0.5^{b}	99.5±0.0^{a}			
ES (%)	73.3±1.6^{c}	92.7±0.7^{b}	98.2±0.3^{a}			
Oil/water ratio	10/90	30/70	50/50	70/30	90/10	
EC (%)	13.3±1.6^{b}	99.1±0.0^{a}	98.8±0.3^{a}	100±0^{a}	0	
ES (%)	21.7±3.7^{c}	58.9±1.1^{b}	98.0±0.4^{a}	100±0^{a}	0	

*PPI: pea protein isolate; G-PPI: modified pea protein; *Means with different letters in each row indicate significant differences ($p< 0.05$).

Note: For PPI emulsions, pH 3-pH 7 emulsion samples: 1.5% protein concentration, 0 M NaCl, 50/50 O/W; 0.1 M NaCl emulsion sample: 1.5% protein, and 50/50 O/W at original PPI pH (~pH 7.8); 1.5% emulsion sample: 0 M NaCl, 50/50 O/W at original PPI pH.

For G-PPI emulsions, pH variation samples: 0 M NaCl, 1.5% protein, 50/50 O/W; NaCl concentration variation samples: 1.5% protein, 50/50 O/W at G-PPI original pH; Protein concentration variation samples: 0 M NaCl, 50/50 O/W at G-PPI original pH; Oil/water ratio variation samples: 1.5% protein, 0 M NaCl at G-PPI original pH.

Table 7-3 Apparent viscosity (shear rate, 100 s^{-1}) and average particle size of emulsions at different pH, NaCl concentrations, protein concentrations, and oil/ water ratios containing pea protein isolate (PPI) and modified pea protein (G-PPI).

PPI						
pH	pH 3	pH 5	pH 7	pH 9	0.1M NaCl	1.5% PPI
Viscosity (mPa.s)	31.09±0.42^{b}	27.68±1.42^{c}	22.80±0.68^{d}	24.00±0.93^{d}	41.34±0.23^{a}	18.93±0.55^{e}
Diameter (nm)	2231.95±392.94^{bc}	4904.15±753.71^{a}	1664.80±71.42^{cd}	1309.65±9.55^{cd}	3380.65±184.06^{b}	1493.15±80.12^{d}
G-PPI						
pH	pH 3	pH 5	pH 7	pH 9		
Viscosity (mPa.s)	60.77±4.15^{b}	88.21±6.40^{a}	48.62±1.32^{b}	54.16±1.58^{b}		
Diameter (nm)	1767.10±308.30^{a}	888.15±41.51^{b}	934.40±157.12^{b}	872.95±33.30^{b}		
NaCl Conc.	0.01M	0.1M	1M			
Viscosity (mPa.s)	76.60±1.34^{b}	174.48±7.06^{a}	149.17±9.16^{a}			
Diameter (nm)	605.00±16.83^{b}	885.35±87.89^{a}	1084.05±73.47^{a}			
Protein Conc.	1%	1.5%	2%			
Viscosity (mPa.s)	32.89±0.47^{c}	52.90±2.11^{b}	89.04±1.05^{a}			
Diameter (nm)	727.30±93.06^{a}	803.30±67.88^{a}	588.95±55.79^{a}			
Oil/water ratio	10/90	30/70	50/50	70/30	90/10	
Viscosity (mPa.s)	4.01±0.07^{b}	9.34±0.04^{b}	44.85±1.34^{b}	1697.10±117.95^{a}	82.76±5.97^{b}	
Diameter (nm)	377.25±43.20^{c}	969.50±6.08^{ab}	567.55±39.39^{bc}	1494.00±277.33^{a}	668.70±131.66^{bc}	

*Means with different letters in each row indicate significant differences (p< 0.05).

Note: For PPI emulsions, pH 3-pH 7 emulsion samples: 1.5% protein concentration, 0 M NaCl, 50/50 O/W; 0.1 M NaCl emulsion sample: 1.5% protein, and 50/50 O/W at original PPI pH (~pH 7.8); 1.5% emulsion sample: 0 M NaCl, 50/50 O/W at original PPI pH.

For G-PPI emulsions, pH variation samples: 0 M NaCl, 1.5% protein, 50/50 O/W; NaCl concentration variation samples: 1.5% protein, 50/50 O/W at G-PPI original pH; Protein concentration variation samples: 0 M NaCl, 50/50 O/W at G-PPI original pH; Oil/water ratio variation samples: 1.5% protein, 0 M NaCl at G-PPI original pH.

Table 7-4 The emulsifying properties, including emulsion capacity (EC) and stability (ES) of mayonnaise at different protein or egg yolk concentrations, and their viscoelastic properties (G' and G") at 1 Hz.

	PPI 2%	PPI 4%	PPI 6%	PPI 8%	
EC	11.9±2.1^{b}	50.3±0.6^{a}	50.9±1.2^{a}	50.9±1.2^{a}	
ES	6.6±0.9^{d}	10.5±1.1^{c}	13.0±0.4^{b}	25.8±1.2^{a}	
G'	0.39±0.02^{b}	2.41±0.37^{a}	0.88±0.36^{b}	0.00±0.00^{b}	
G"	1.35±0.06^{a}	1.99±0.31^{a}	1.80±0.03^{a}	1.49±0.28^{a}	
	Egg yolk 2%	Egg yolk 4%	Egg yolk 6%	Egg yolk 8%	Egg yolk 10%
EC	63.3±0.9^{d}	69.8±2.1^{c}	84.2±3.7^{b}	93.8±0.7^{a}	97.9±0.3^{a}
ES	59.0±4.3^{d}	62.9±0.8^{d}	72.2±1.0^{c}	88.3±0.5^{b}	95.7±0.7^{a}
G'	0.00±0.00^{c}	0.00±0.00^{c}	0.77±0.00^{c}	4.95±0.22^{b}	22.79±2.13^{a}
G"	1.22±0.13^{c}	1.33±0.02^{c}	1.48±0.15^{c}	4.09±0.02^{b}	11.04±0.18^{a}
	G-PPI 2%	G-PPI 4%	G-PPI 6%	G-PPI 8%	
EC	71.8±4.3^{c}	87.6±1.3^{b}	94.5±2.0^{a}	98.3±0.2^{a}	
ES	54.8±5.0^{c}	80.8±3.4^{b}	91.6±5.2^{a}	97.5±0.7^{a}	
G'	7.74±0.34^{b}	334.89±46.45^{b}	485.46±137.16^{ab}	1161.57±335.92^{a}	
G"	7.12±0.18^{b}	89.60±7.32^{b}	176.14±55.30^{ab}	361.51±87.25^{a}	

*Means with different letters in each row indicate significant differences ($p< 0.05$).

Table 7-5 Average particle sizes of pea protein isolate (PPI) and modified pea protein (G-PPI) at different pH conditions.

	D10 (nm)	D50 (nm)	D90 (nm)
		PPI	
pH 3	625.39±55.69[a]	888.13±20.33[a]	1333.78±92.43[a]
pH 4	130.25±0.85[b]	153.96±6.32[d]	182.64±13.54[b]
pH 4.5	758.92±116.15[a]	1102.67±123.14[a]	1679.75±93.97[a]
pH 5	808.13±21.24[a]	1122.58±79.65[a]	1628.46±213.67[a]
pH 7	262.92±24.23[b]	550.77±23.99[b]	1154.95±223.82[a]
pH 9	190.41±107.32[b]	400.31±44.80[bc]	973.42±348.66[ab]
pH 11	31.04±2.71[b]	244.89±10.39[cd]	975.14±368.91[ab]
		G-PPI	
pH 3	133.77±19.53[b]	184.10±19.93[ab]	276.84±0.73[a]
pH 4	33.08±0.85[c]	197.31±9.02[ab]	309.63±37.58[a]
pH 4.5	338.27±126.31[a]	383.46±143.99[a]	1253.27±991.61[a]
pH 5	47.54±1.97[c]	130.38±9.48[b]	240.29±17.09[a]
pH 7	18.73±0.08[c]	74.81±0.57[b]	283.92±30.40[a]
pH 9	29.65±3.52[c]	183.45±10.58[ab]	823.99±22.25[a]
pH 11	33.71±1.01[c]	174.10±16.76[ab]	751.89±257.83[a]

*Means with different letters in each row indicate significant differences ($p< 0.05$).

Chapter 8 - Conclusions and future perspectives

8.1 Conclusions

The demand for proteins continues to increase due to their nutritional benefits, the growing world population, and rising protein deficiency. Plant-based proteins represent a sustainable source to supplement costly animal proteins. This study aimed to investigate the effect of different drying methods on the functional and physicochemical properties of quinoa protein isolate; improve the functional properties of pea protein isolate through different modification approaches, and evaluate the modified pea protein used in meat patty and mayonnaise applications.

Previous studies related to the pea protein composition, functionality, modification, and food applications were extensively reviewed in Chapter 1. In chapter 2, the protein from freeze drying method had the highest emulsification capacity and stability as well as oil absorption capacity due to its higher surface hydrophobicity. Gels prepared from the freeze-dried protein had higher elastic and viscous modulus than that from spray- and vacuum-dried proteins. Conclusions from functional properties were well supported by protein structural features from SDS-PAGE, sulfhydryl and disulfide analysis, secondary structure, surface hydrophobicity, and thermal characterization. In chapter 3, we investigated the effect of acylation or/and conjugation on pea protein functionalities, and we found that the sequential acylation and conjugation modifications had exceptional synergistic effects on protein emulsification, oil holding capacity, and gelation properties. Because of the concerns of using chemicals such as acetic anhydride or succinic anhydride during acylation modification; therefore, in chapter 4, we developed clean-label approaches for protein functional enhancement. Combining enzymatic and conjugation

modifications have delivered some synergistic effects and produce more functional protein ingredients. In chapter 5, we successfully manipulated pea protein inter- and intra-molecular interactions (e.g., disulfide bond, hydrogen bonding, hydrophobic interaction, peptide bond) with specific denaturation agents and achieved enhanced functional properties. Pea protein denaturation with sodium sulfite, urea, SDS, or trypsin all greatly improved protein solubility and oil holding capacity compared with the original protein. Trypsin hydrolyzed protein possessed excellent foam capacity and greater gelation property. The proteins unfolded with urea or SDS showed improved emulsification properties. Urea or SDS denatured proteins exhibited better water holding capacity and gelation property. In chapter 6, extending beef patties with PPI or PGG reduced cooking loss, and thus increasing cooking yield, but also led to decreased juiciness and beef flavor scores and increased off-flavor score. The beef patties containing PGG also showed much softer and tender texture compared with the control patty, which would be advantageous features for some elders with such sensory preference. In chapter 7, the modified pea protein (G-PPI) was successfully used as a natural emulsifier in the oil-in-water emulsion and mayonnaise applications. The emulsion with G-PPI significantly increased stability, apparent viscosity, and decreased droplet size compared with the PPI emulsions. The pH, NaCl concentration, protein concentration, and oil/water ratio significantly affected the emulsifying properties. The mayonnaise with G-PPI at higher concentrations (6 and 8%) exhibited significantly higher emulsifying properties and viscoelasticity than that of PPI or egg yolk mayonnaise. In summary, the newly developed pea proteins through green modifications may expand their uses in various food applications and better meet the increasing demand for more functional plant proteins.

8.2 Future perspectives

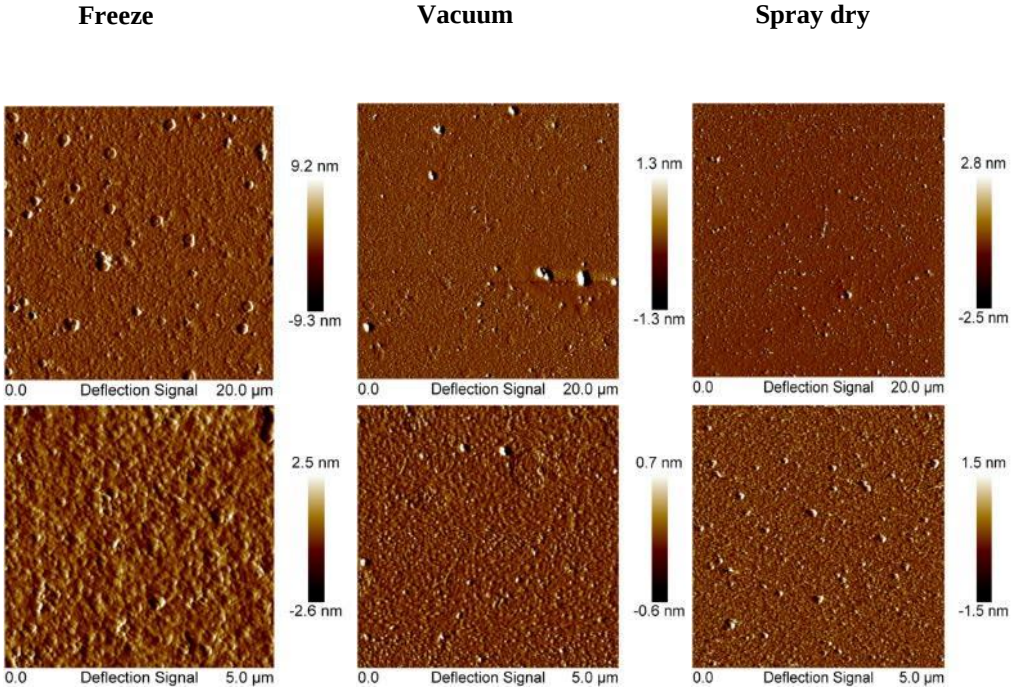
Besides the studies that have been discussed in this dissertation, other research fields could be considered and worth to be further investigated: 1) optimize the modification methods, for example: use few modifiers, or less time and electricity during the processing, to produce similar functionally enhanced pea protein. 2) develop functionally enhanced pea protein and other plant proteins through physical modification with advantages in clean-label, mild reaction, safety, and efficiency aspects. 3) Continue to deeply investigate and characterize the solid-like high-internal phase emulsions, and understand the mechanism behind that. 4) develop modification method to improve pea protein foaming capacity and stability, which could be utilized in the bakery applications. 5) Further research is needed to eliminate or reduce the off flavor of pea proteins or food products containing the proteins.

Appendix A -

Chapter 2-Table S1: Amino acid composition of quinoa proteins from different drying methods.

Amino acid	**Freeze dry (mg/g)**	**Spray dry (mg/g)**	**Vacuum dry (mg/g)**
Alanine	31.73±0.27[a]	20.40±0.17[b]	17.77±1.02[c]
Glycine	44.12±1.58[a]	30.22±0.50[b]	33.30±1.59[b]
Valine	47.14±8.16[a]	45.50±1.26[a]	47.03±0.33[a]
Leucine	68.23±7.78[a]	67.12±0.35[a]	69.58±2.57[a]
Isoleucine	48.01±8.16[a]	47.74±0.53[a]	47.92±0.88[a]
Threonine	28.86±5.15[a]	26.29±0.14[a]	29.66±2.54[a]
Serine	28.21±4.25[a]	28.32±1.89[a]	28.55±1.53[a]
Proline	28.45±1.38[a]	27.89±0.08[a]	28.54±0.97[a]
Methionine	22.66±4.41[a]	21.35±1.03[a]	23.24±3.37[a]
Phenylalanine	38.58±1.56[a]	36.48±1.84[a]	37.46±0.99[a]
Lysine	38.25±6.83[a]	36.24±1.17[a]	37.59±1.94[a]
Histidine	37.96±4.87[a]	35.69±0.14[a]	38.65±1.35[a]
Tyrosine	34.49±5.06[a]	32.38±0.08[a]	36.19±0.67[a]
Cysteine	2.62±0.12[b]	3.05±0.10[a]	2.42±0.01[b]
Tryptophan	4.18±0.78[a]	4.04±0.94[a]	3.62±0.12[a]
Cystine	22.24±1.87[a]	18.61±1.42[a]	21.12±1.84[a]
Aspartic acid	76.88±2.54[a]	70.49±2.23[a]	80.43±3.03[a]
Glutamic acid	58.01±2.84[a]	55.86±2.30[a]	58.13±0.34[a]

*Means with different letters within each amino acid denote significant differences ($p < 0.05$).

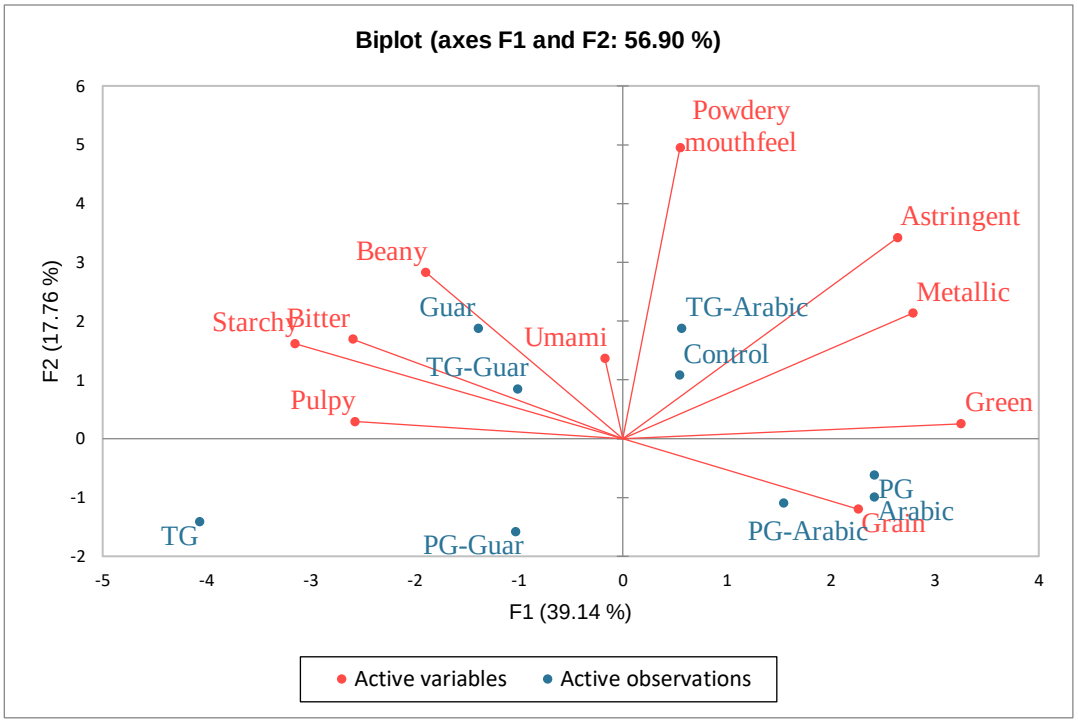


Chapter 2-Figure S1: AFM images of quinoa proteins from different drying methods.

Chapter 2-Figure S2: Quinoa protein powders from different drying methods.

Chapter 4-Table S1: Sensory descriptive analysis score of pea and the modified pea proteins.

Sample	Beany	Starchy	Grain	Green	Pulpy	Powdery mouthfeel	Umami	Astringent	Bitter	Metallic
Control	6	6	5	3	0	5.5	2	2.5	2.5	1.5
PG	5.5	5	6	3	0	5.5	0	2.5	2	1.5
TG	6	6.5	5	2.5	5	5	2	2	3	0
Guar	6.5	7	4.5	2.5	0	5.5	2	2.5	2.5	1.5
Arabic	6	4	6	3	0	5	2	2.5	2	1.5
PG-Guar	6	6	4.5	2.5	0	5	0	2	2	1.5
PG-Arabic	5	5	5	3	0	5	2	2.5	2.5	1.5
TG-Guar	6	6	5.5	2.5	3	5.5	0	2.5	3	1.5
TG-Arabic	6	5	5	3	3	6	2	2.5	2.5	1.5



Chapter 4-Figure S1: Principal component analysis (PCA) biplot shows the relationship between modified pea proteins and sensory attributes.

Cleanouts: Mozzarella cheese, unsalted crackers.

FLAVOR:

Beany: A slightly brown, musty, slightly nutty and starchy flavor associated with cooked beans.

Reference: Bush's Best Pinto Beans= 7.5 (f)

Preparation: Drain beans and rinse with de-ionized water. Serve in 3.25 oz cups.

Grain: A general term used to describe the aromatic which includes musty, dusty, slightly brown, slightly sweet and is associated with harvested grains and dry grain stems.

Reference: Cereal Mixture (dry) = 8.0

Preparation: Mix 1 cup of each General Mills Rice Chex, General Mills Wheaties and Quaker Quick Oats. Put in a blender and "Pulse" blend into small particles. Serve 1 tsp in 1 1 oz cup.

Green (grass)**:** A green aromatics associated with newly cut-grass and leafy plants; characterized by sweet and pungent character.

Reference: Fresh parsley water = 7.0 (f)

Preparation: Fresh parsley water: 50 g chopped fresh curly parley soaked in 600 mL room temperature de-ionized water for 15 minutes, filtered. Serve in 1 oz cups.

Green (Pea Pod): A green aromatic associated with fresh green peapods. May include beany, increased pungent, musty/earthy, bitter and astringent.

Reference: Kroger Frozen Lima Beans = 9.0 (f)

Preparation: Serve about 10 beans (thawed) in 3.25 oz cups.

Mouthfeel, Powdery: The feeling of undissolved starch from vegetable product such as potatoes and beans, left in the mouth after swallowing

Reference: Bush's Best Pinto Beans = 7.0

Preparation: Drain beans rinse with de-ionized water. Serve in 3.25 oz cups.

Pulpy: The quantity or amount of perceivable pulp. Evaluated by manipulating the sample with the tongue 3-5 times in the mouth.

Reference: Tropicana Grovestand Orange Juice (carton) = 5.0

Preparation: Serve in 3.25 oz cups.

Starchy: The dry aromatics associated with starch and starch based grain products such as wheat, rice, oats and other grains.

Reference: Argo Corn Starch in Water = 3.0 (f)

Preparation: Mix 2 g corn starch in 200 mL water. Serve in 1 oz cups.

Umami: A general term for aromatics associated with juices from cooked seafood, meat and/ or vegetables.

Reference: Botton Mushroom Broth = 2.0 (f)

Preparation: Add 2 cups water and 2 medium-size button mushrooms into a small sauce pan, bring to a boil and then boil for 5 minutes. Strain through a coffee filter and serve the liquid in 1 oz cups.

Astringent: The dry, puckering mouth feel associated with an alum solution in the mouth.

Reference: 0.05 % Alum Solution = 2.5

Reference: 0.07 % Alum Solution = 3.5

Bitter: The fundamental taste factor associated with a caffeine solution.

Reference: 0.02 % Caffeine Solution = 3.5

Reference: 0.035 % Caffeine Solution = 5.0

Metallic: The flavor aromatics described as flat associated with iron, copper, and silver spoons.

Reference: 0.10 % Potassium Chloride Solution = 1.5

Sweet: A fundamental taste sensation of which sucrose is typical.

Reference: 2 % Sucrose Solution = 2.0

Reference: 3 % Sucrose Solution = 3.0

Chapter 6-Table S1: Color of protein powders (PPI and PGG).

	L*	a*	b*
PPI	81.71±0.02[a]	2.57±0.02[a]	17.78±0.01[a]
PGG	76.78±0.02[b]	2.32±0.02[b]	15.90±0.01[b]

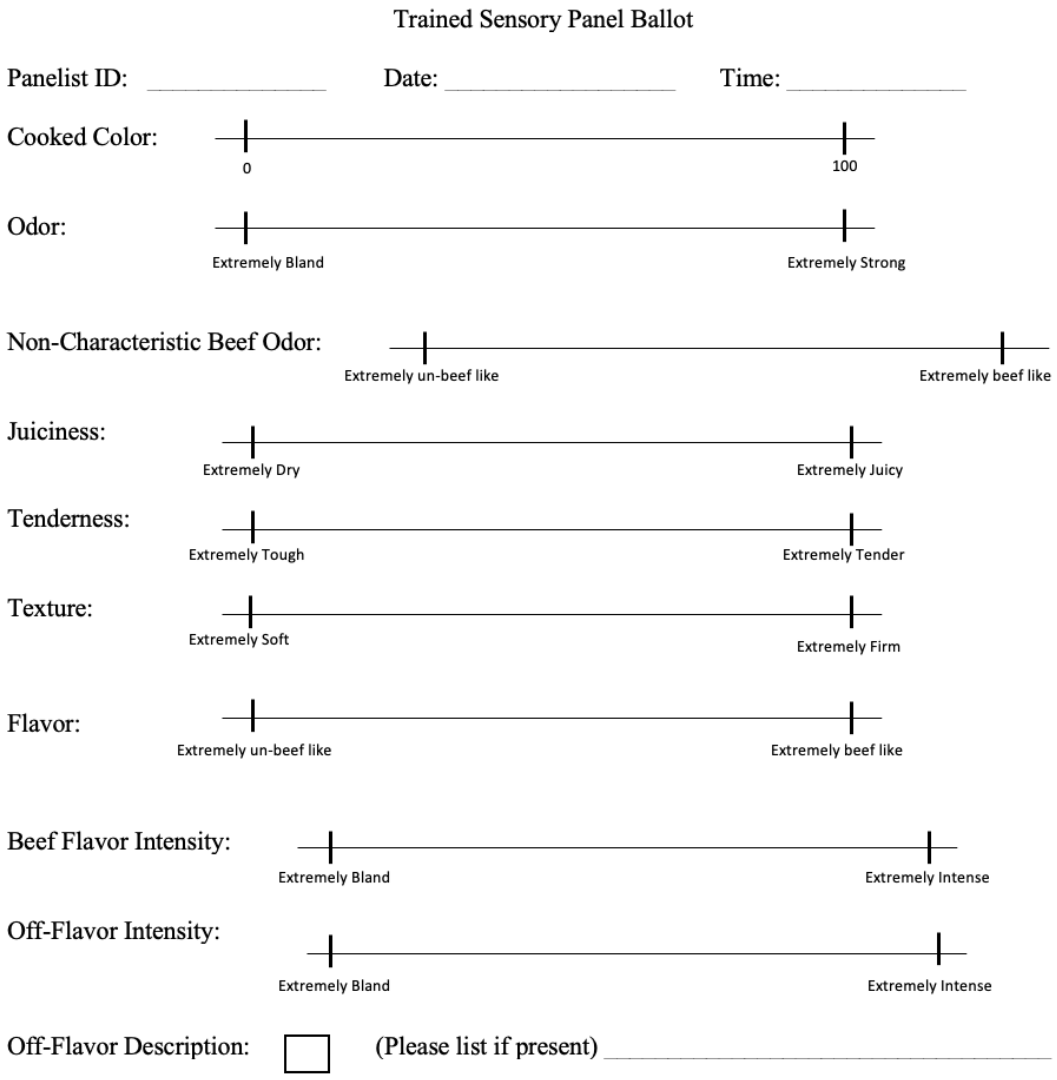
*Means with different letters in each column indicate significant differences ($p< 0.05$).

Chapter 6-Table S2: Descriptive sensory scores of beef patties with PPI or PGG.

	Juiciness	**Tenderness**	**Texture**	**Flavor**	**Beef flavor intensity**	**Off flavor**
Control	55.9[a]	65.7[a]	68.2[a]	86.1[a]	48.1[a]	1.3[a]
2.5% PPI	42.0[b]	62.1[ab]	67.9[a]	29.2[b]	15.8[b]	31.1[b]
5% PPI	35.3[b]	60.0[b]	68.5[a]	12.3[cd]	8.4[c]	47.2[c]
2.5% PGG	49.3[a]	71.2[c]	51.5[b]	17.6[bc]	9.5[bc]	44.0[bc]
5% PGG	44.2[b]	72.9[c]	43.9[b]	7.3[d]	4.2[d]	57.8[d]

*Means with different letters in each column indicate significant differences ($p < 0.05$).

Chapter 6-Figure S1: Photos of protein powders (PPI and PGG).



Chapter 6-Figure S2: Trained sensory panel ballot for beef patty evaluation.