Enhancement of Pea Protein Solubility and Thermal Stability for Beverage Applications via Endogenous Maillard-Induced Glycation and Chromatography Purification

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Alissa Ann Schneider

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Dedication

I am dedicating this thesis to the many dynamic women in my life—to my grandmas, mom, and dear friends—who have taught me to persist in the face of challenges.

Abstract

Growing demands for non-soy, plant protein sources have guided the rapid expansion of the pea protein ingredient market in recent years. Pea protein has emerged as the most prominent alternative to soy protein, as pea is not a major allergen, non-GM, sustainable, and widely available. Accordingly, a variety of pea protein products are now commercially available, suited for a number of different applications. Pea protein generally exhibits inferior functionality compared to soy protein, however, as a result of its intrinsic protein profile and structure, especially following commercial processing. Namely, pea protein exhibits inferior solubility and thermal stability compared to whey and soy proteins, limiting its application in high-protein, RTD beverages. Enhancement of pea protein solubility under acidic conditions and following thermal treatments is, therefore, of interest.

Controlled, Maillard-induced glycation is a protein modification technique that has the potential to improve pea protein solubility and thermal stability. While glycation of pea protein has been reported, this process has not been developed for an industrial scale, unreacted carbohydrates are rarely removed, and all previous studies have utilized exogenous saccharides (e.g., pectin, gum Arabic, and corn maltodextrin), presenting concerns regarding application in "clean label" products. The starch-rich by-product of pea protein extraction may be further processed to produce an endogenous reducing saccharide, such as maltodextrin, which may react with protein under controlled glycation conditions. Glycation coupled with purification of partially-glycated pea protein has the potential to produce a highly soluble and thermally stable protein ingredient with added-value, having potential for RTD beverage applications.

Therefore, the main objective of this work was to enhance pea protein solubility and thermal stability by producing an endogenously and partially-glycated pea protein (PG-PP) ingredient by completing several goals: (1) develop a method to produce maltodextrin from pea starch with a specific reducing power, (2) initiate and control the early stage of the Maillard reaction to partially-glycate pea protein isolate (PPI) with pea maltodextrin, (3) remove unreacted maltodextrin from the PG-PP via hydrophobic interaction chromatography (HIC) to produce a purified, PG-PP concentrate or isolate,

and (4) characterize the effect of glycation coupled with purification on protein structure and the consequent impact on solubility and thermal stability.

A method to produce maltodextrin from the pea starch-rich by-product obtained during the production of native pea protein isolate (nPPI) was developed by monitoring maltodextrin dextrose equivalent (DE) in response to hydrolysis time, small saccharide removal, and centrifugation to remove large molecular weight residual starch and fiber. The resulting chain-length distribution was evaluated. Maillard-induced glycation was then confirmed and monitored by assessing changes in color, free amino groups, and protein/glycoprotein profiles upon incubation of the produced maltodextrin with nPPI. Next, removal of unreacted maltodextrin by HIC was confirmed by monitoring maltodextrin elution. The purified PG-PP, along with purified PPI controls and reference samples (nPPI and commercial PPI), were then characterized by assessing their composition and protein/glycoprotein profile. Protein thermal denaturation properties, surface properties (surface hydrophobicity and zeta potential), and secondary structures were evaluated, as well. Lastly, protein solubility and thermal stability and protein digestibility were assessed.

The starch-rich by-product was partially hydrolyzed, with hydrolysis conditions optimized to produce maltodextrin with targeted characteristics (DE 15.7, average degree of polymerization 8.3). PPI and maltodextrin were incubated under mild conditions, which initiated and controlled the Maillard reaction to the early stage. PG-PP was formed with minimal browning and protein polymerization, along with moderate free amino group loss. Additionally, PG-PP was purified by removing the majority of unreacted carbohydrates and polymerized proteins via HIC, resulting in a purified water fraction (PW-PG-PP) with a protein content of nearly 60%, reduced surface hydrophilicity, and increased solubility (up to ~91%) and thermal stability at conditions relevant to RTD beverages. Protein digestibility of PW-PG-PP was high and similar to the references. Purification of nPPI control also produced a highly soluble and thermally stable sample with good protein digestibility. HIC removed hydrophobic and polymerized proteins from nPPI, allowing for the fractionation and concentration of hydrophilic proteins in nPPI.

This study proved the concept of “clean-label”, endogenous glycation of pea protein, utilizing endogenously produced maltodextrin and controlled Maillard reaction conditions. Additionally, both endogenous glycation coupled with HIC purification, as well and HIC purification alone, greatly improved the solubility and thermal stability of nPPI under acidic conditions and at a high-protein claim concentration (5% protein), with purified samples having nearly the solubility of whey protein, the gold standard for beverages. These processes also largely maintained the digestibility of PPI. Therefore, glycation and HIC purification created pea protein with potential value for application in high-protein, RTD beverages. Moreover, this work uncovered a PPI fractionation process that has the potential to increase specialty pea protein ingredient value, with the water soluble fraction suitable for beverage applications and hydrophobic fraction suitable for meat analogues. This work also provided foundational information, paving the way for future investigation and process optimization for scaled-up glycation and purification of pea protein.

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Chapter 1: Literature Review

1.1 Introduction

On a global scale, demands for high protein food and beverage products have considerably increased in recent years. Consumers are incorporating more protein into their diets, primarily for the health benefits associated with consuming this essential macronutrient (Formanski, 2020). Food and beverage manufacturers have responded to these demands by accelerating the development of new high protein products in an effort to boost their sales (Kamp, 2020). In developed countries, specifically, the plant-based protein market is rapidly growing in response to changing consumer trends. Plant-based sales are expanding as consumers increasingly identify as vegan, vegetarian, and flexitarian. These populations are largely motivated by the health benefits of plant-based foods, as well as their growing environmental consciousness, animal welfare concerns, and desires to diversify their diets (Formanski, 2020). Accordingly, the value of plant protein ingredients is soaring.

Historically, soy protein has dominated the alternative protein market. However, manufacturers are seeking out new plant protein sources, due to the negative consumer perception of soy as a genetically modified (GM) crop and major allergen in the US. Accordingly, pea protein has emerged as the most prominent alternative to soy protein, as pea is not a major allergen, non-GM, sustainable, and widely available (Brewster, 2020; Formanski, 2020; Tulbek, Lam, Wang, Asavajaru, & Lam, 2017). As the global pea protein ingredient market is projected to reach $555 million in 2028, manufacturers have developed a variety of pea protein ingredients that are suited for a number of different applications (Grand View Research, 2021a). While these ingredients have acceptable nutritional quality, their generally poor functionality in food and beverages limits wide utilization. Poor functionality is attributed to the intrinsic profile and structure of pea protein, especially following commercial processing (Lam, Can Karaca, Tyler, & Nickerson, 2018). Compared to whey and soy proteins, pea protein exhibits inferior solubility and thermal stability, making incorporation of pea protein into high-protein, ready-to-drink (RTD) beverages particularly challenging. Therefore, the functionality of

pea protein must be improved upon to create added-value and successfully expand incorporation of pea protein into various products.

Enhancement of protein functionality can be achieved by altering intrinsic protein characteristics through structural modification. A variety of physical, chemical, and enzymatic processes have been investigated to improve the solubility and thermal stability of pea protein. Of these processes, only enzymatic hydrolysis is commercialized. However, enzymatic hydrolysis is limited by its adverse effect on flavor, as this process results in bitterness and astringency, thus restricting its consumer acceptability (Barac et al., 2012). Therefore, other modification methods that are industrially feasible are needed.

Maillard-induced glycation is an alternative protein modification technique that employs the Maillard reaction, which is a prevalent reaction in many foods that are regularly consumed by humans. This technique involves the controlled formation of stable protein-carbohydrate conjugates in the early stage of the Maillard reaction. Protein glycation is typically achieved by incubating protein with excess reducing carbohydrate under controlled environmental conditions (de Oliveira, Coimbra, de Oliveira, Zuñiga, & Rojas, 2016). The greatest improvement in protein functionality is observed when conjugating a protein with polysaccharides, rather than small saccharides, as the use of polysaccharides limits the reaction rate and thus can be more easily controlled (Zha, Yang, Rao, & Chen, 2020). Recently, pea protein-gum Arabic and pea protein-maltodextrin conjugates have been reported to have improved functionality and flavor (Kutzli, Griener, et al., 2020; Zha, Rao, & Chen, 2021). Accordingly, Maillard-induced glycation has the potential to enhance pea protein functionality and acceptance in food and beverage systems.

Although glycation has been researched for decades with consistent, reported improvements in protein solubility and thermal stability, the current limitations of this technique have prevented its commercialization. Optimization of dry- or wet-heating methods of glycation and protein purification steps are needed to improve the industrial feasibility of this approach. Additionally, poor consumer perception of the exogenous hydrocolloids (e.g., gums, carrageenan, corn dextran, corn maltodextrin) commonly employed in this reaction potentially limit the acceptability of a finished product containing the modified protein ingredient. Thus, efforts to improve the “clean label”

appeal of glycated proteins are needed. Along with protein, pea naturally contains large amounts of starch. This starch component may be converted to an endogenous reducing carbohydrate for which to conjugate with pea protein. Effectively, endogenously produced pea protein- maltodextrin conjugates would mitigate the labeling challenges that conjugates produced with exogenous hydrocolloids potentially face.

Further, purification of a partially-glycated protein, by removal of excess, unreacted carbohydrate is rarely performed. Excess carbohydrate in the modified protein ingredient has the potential to alter the protein functionality, shorten product shelf life, contribute undesirable functionality, and reduce protein purity. Therefore, additional purification of the protein is necessary, to produce a highly functional and valuable protein ingredient. Development of a clean-label process to endogenously glycate pea protein is needed to produce a functionally enhanced pea protein ingredient.

1.2 Hypotheses and objectives

A purified, partially-glycated pea protein (PG-PP) will be produced by initiating Maillard-induced glycation of native pea protein with pea maltodextrin produced from endogenous pea starch. It is hypothesized that controlled, partial hydrolysis of pea starch will produce maltodextrin that can participate in Maillard-induced glycation, when in the presence of pea protein. The controlled and limited application of Maillard-induced glycation will produce PG-PP that will be purified via hydrophobic interaction chromatography to result in a protein concentrate or isolate. Ultimately, it is hypothesized that Maillard-induced glycation of pea protein and subsequent purification of PG-PP will result in enhanced protein solubility and thermal stability. The main objective of this work, therefore, is to enhance pea protein solubility and thermal stability following the partial glycation of pea protein with endogenous maltodextrin produced from pea starch. Specific goals of this work include:

1. Develop a method to produce maltodextrin from pea starch, with a specific reducing power.
2. Initiate and control the early stage of the Maillard reaction to partially-glycate pea proteins with pea maltodextrin.

3. Remove unreacted maltodextrin from the partially-glycated pea protein via hydrophobic interaction chromatography to produce a purified, partially-glycated pea protein concentrate or isolate.
4. Characterize the effect of glycation coupled with purification on protein structure and the consequent impact on protein solubility and thermal stability.

1.3 Significance of plant proteins

1.3.1 Plant protein ingredients demand and market

Demand for protein has rapidly increased in recent years and is projected to continue rising. In 2020, the global protein ingredient market size was estimated at $38.5 billion and is expected to grow at a compound annual growth rate (CAGR) of 10.5% from 2021 to 2028 (Grand View Research, 2021b). This growing market reflects changing consumption patterns. Protein is increasingly a significant part of consumer's diets, as 55% of households in the U.S. say "high protein" is an important attribute that they consider when purchasing food (Nielsen, 2018). With consumers looking to add more protein into their diets, food manufactures have accordingly increased their launch of protein-rich products. According to Mintel Global New Products Database, high/added protein claims on food products have grown 30% from 2015 to 2019 (Kamp, 2020). Additionally, with soaring sales, manufacturers are incentivized to continue filling grocery store shelves with high-protein products. Nielsen (2018) found that products with protein claims have had significantly higher sales than those without a claim. The sales strength of protein products illustrates an opportunity for manufacturers to boost their margins. As demand for protein ingredients continues to increase, manufacturers will likely find investment in the development of functional protein ingredients to be valuable in the coming years.

This insatiable desire for protein is largely driven by the perceived health benefits of consuming protein, regardless of the protein source. The Natural Marketing Institute found that consumers want more protein in their diets to increase their energy, mainly, although managing weight, adding muscle mass, and improving brain health also greatly contribute to this trend (Sloan, 2019). Protein is an essential macronutrient with well-

defined roles in the body. For example, protein consumption is required for essential body functions, as a structural component, and it plays a major role in metabolic processes. This key nutrient is associated with building muscle mass, promoting satiety, aiding in weight management, enhancing glycemic regulation, and improving bone health (Pasiakos, 2015). These health-promoting functions are driving the consumer demand for protein, thus enabling new ingredient and product development.

Although protein sales from both animal and plant sources have experienced growth, consumers are looking for more variety in their protein sources. Especially in developed countries like the U.S., consumers are increasingly adding plant-based protein sources to their diets, while reducing animal protein consumption. This trend is attributed to growing flexitarian, vegetarian, and vegan populations (Ismail, Senaratne-Lenagala, Stube, & Brackenridge, 2020). As a result, plant-based sales are significantly growing. In 2020, grocery sales of plant-based foods grew by 27%, with the retail market for plant-based foods increasing from $5.5 billion in 2019 to $7 billion in 2020 (Cameron & Neill, 2019). A Mintel survey found that consumers are motivated to eat plant-based proteins because they believe it is beneficial to their health, like the taste and variety in their diet, want to lower their environmental impact, and are concerned with animal welfare (Formanski, 2020). However, this movement is primarily influenced by the perceived health benefits of plant-based proteins. Consumers cite that they rely on plant-based protein in order to reduce their intake of undesirable components of animal proteins (e.g., animal fat, cholesterol, hormones, antibiotics, and nitrites) (Formanski, 2020). Further, plant proteins are often consumed as part of a whole food, rather than as purified protein ingredients. Whole food consumption is considered beneficial, as whole sources provide complex carbohydrates, high protein content without high fat content, and bioactive co-nutrients (Ahnen, Jonnalagadda, & Slavin, 2019). Additionally, research suggests that increasing plant protein consumption is associated with improved health, such as reducing the risk of metabolic syndrome, managing diabetes, protecting against some types of cancers, and managing weight (Ahnen et al., 2019). While plant-based proteins have been associated with these health benefits, more research is necessary to understand the impact that plant protein ingredients, rather than whole foods, have on health and how plant protein ingredients compare to animal sources. Aside from positive health

outcomes, plant proteins also serve as acceptable protein alternatives for consumers with food intolerances and allergies to animal proteins, such as eggs and dairy (Ismail et al., 2020).

While the rise of plant-based proteins is largely attributed to consumers' health motivations, another key driver is concern for the environment and sustainability. As consumers, especially younger populations in the US, are increasingly concerned with the impact their diets have on the environment, they are seeking out plant proteins to replace animal proteins (Formanski, 2020). However, it should be noted that environmental consciousness is mainly a concern of consumers in developed countries. These consumers tend to have greater awareness of the impact of food production and consumption on the environment than those in less developed areas (FAO, 2014). Globally, low and middle income countries contribute to increasing protein demands, primarily for animal proteins, due to increasing population sizes and socio-economic changes (e.g., economic development, rising income, and increasing urbanization) (Henchion, Hayes, Mullen, Fenelon, & Tiwari, 2017). While developing countries are increasing their animal protein consumption, developed countries are expanding their diets to include more plant proteins found in legumes, nuts, and other sources to reduce their environmental footprint (Thrane, Paulsen, Orcutt, & Krieger, 2017). Compared to animal proteins, these plant protein sources are produced more efficiently, requiring significantly less water, land, nitrogen, and energy (Nadathur, Wanasundara, & Scanlin, 2017). As consumers are increasingly more concerned about the environment and motivated to reduce their individual impact, plant proteins will continue to gain traction. Soy protein, in particular, is the global leading source of plant protein ingredients.

1.3.2 Soy protein ingredients

Soy-based protein ingredients have long dominated the plant protein market and continue to serve as the most prominent plant protein ingredients, having been extensively researched and utilized for decades. Globally, soy protein is the most common plant protein used in food and beverage formulations, where the global market value for soy protein ingredients is expected to reach $15.3 billion by 2030 (Brewster, 2020; Chauhan & Deshmukh, 2022; Grand View Research, 2021b). The success of these

ingredients can be attributed to the low cost, high availability, high nutritional quality, and relatively good functionality in food and beverage applications.

Soy protein ingredients include soy flour, protein concentrate, isolate, hydrolysate, and texturized protein produced from defatted soy flakes. Defatted soy flakes are milled into flour, which is then used to produce concentrates and isolates. Soy protein concentrate (SPC) is made by removing soluble carbohydrates from the defatted flour to produce a powder with at least 65% protein, on a dry basis (Thrane et al., 2017). Different SPC production methods include the removal of soluble carbohydrates by extraction with acidic water, aqueous alcohol, or protein denaturation with moist heat and water leaching (Riaz, 2011; Thrane et al., 2017).

On the other hand, the production of soy protein isolate (SPI) involves the removal of fiber and soluble carbohydrates to produce an ingredient with >90% protein, on a dry basis (Thrane et al., 2017). Although SPI production methods vary, alkaline extraction and subsequent isoelectric precipitation is the common method. In this method, alkaline water is added to soy flour followed by centrifugation in order to separate the soluble proteins and carbohydrates from insoluble fibers. The soluble carbohydrates are then separated from the proteins by isoelectric precipitation. Proteins precipitate, or separate from the soluble carbohydrates, by decreasing the pH of the extracted solution to approximately 4.2-4.5 and centrifuging. The precipitated protein is then washed and neutralized before spray drying to produce SPI (Thrane et al., 2017).

These protein-rich products may undergo further modification to enhance their nutritional and functional value. Protein hydrolysis is a common protein modification method that is currently used to produce soy protein hydrolysate (SPH) from SPI. Limited enzymatic hydrolysis of soy protein can improve its functionality while preventing excessive bitterness, which is common in more extensive hydrolysis treatments (Akharume, Aluko, & Adedeji, 2021). Further, native soy proteins may be modified to produce textured soy proteins from soy flour, SPC, or SPI via extrusion. This process utilizes high heat, shear, and pressure to physically modify the protein structure (Akharume et al., 2021; Thrane et al., 2017). Texturized soy products can greatly vary in their properties, based on the applied extrusion conditions. However, they are most often used in meat analogs and high-protein snacks and as meat extenders (Riaz, 2011).

The nutritional value and functional properties of soy protein have been major drivers of its commercial success as a food ingredient. Animal proteins, such as dairy and egg proteins, are often considered higher quality protein sources because they contain sufficient amounts of all nine essential amino acids, whereas many plant protein lack these essential amino acids. However, soy protein is comparable to animal-based proteins in nutritional quality. Unlike many other plant proteins, soy protein contains sufficient quantities of all essential amino acids and is highly digestible, with a protein digestibility corrected amino acid score (PDCAAS) ranging from 0.94-1.0 (Thrane et al., 2017). PDCAAS is a measure of protein quality that accounts for the first limiting amino acid and digestibility of a protein, with a maximum value of 1.0. This score is required to calculate the amount of protein (percent daily value) on nutrition labels and make protein claims on food and beverage products (Hughes, Ryan, Mukherjea, & Schasteen, 2011). While antinutritional components (e.g., trypsin inhibitor, lectins and phytates) can interfere with protein digestion, soy protein ingredient processing has been shown to reduce native trypsin inhibitor activity, thus maintaining the protein quality (Deak, Johnson, Lusas, & Rhee, 2008). Likewise, research has found that soy protein quality is mostly preserved after ingredient processing. In fact, soy protein ingredient processing that induces thermal denaturation results in improved digestibility (Deak et al., 2008).

In addition to being a complete, high-quality protein, soy proteins have functional value in food applications. Useful in a variety of foods, soy protein ingredients provide solubility, emulsification, foaming, gelation, water binding, viscosity, and flavor (Thrane et al., 2017). For example, SPC is used in bakery products, soup bases, and plant-based milks, due to the emulsification properties and viscosity provided by the protein and polysaccharide constituents. Further, SPI is suitable in meat and meat analogue applications, such as sausages, because it forms strong gels, binds water, and aids in emulsification (Singh, Kumar, Sabapathy, & Bawa, 2008; Thrane et al., 2017). With a variety of soy-based protein products available, these ingredients are proven to be versatile and functional in many food applications.

While soy proteins are nutritionally and functionally valuable, they are limited by a few factors. Soy primarily faces consumer-based challenges. A major issue with soy protein ingredients is that they contain eight allergenic proteins, with two of these being

the major storage proteins, β-conglycinin and glycinin (Thrane et al., 2017). Soy is one of the "Big 9" food allergens in the US and requires labeling on products, thus limiting its application in foods (US-FDA, 2022). Additionally, over 90% of soybeans produced in the US are GM. While genetic modification has benefited farmers and processors, public concern over GM foods has grown significantly ever since they first hit the market in 1996 (Thrane et al., 2017). In fact, 43% of US consumers avoid GM foods like soy (Formanski, 2019). Consumers also have concerns regarding antinutritional components of soy (e.g., trypsin inhibitor, lectins, phytates, isoflavones) and the use of organic solvents in soy processing (Singh et al., 2008). As a result of these concerns, a significant portion of consumers have been avoiding soy products, with a Mintel survey finding that 18% of US consumers believe that soy foods are unhealthy and 24% of consumers having someone in their household that avoids soy (Formanski, 2019). Poor consumer perception of soy has, however, encouraged research into new plant protein sources, such as pea.

1.3.3 Rising market for pea protein

Recently, pea protein ingredients have displaced soy protein ingredients in a variety of food applications. Pea protein is projected to continue growing in popularity, with a global market valued at $213 million in 2020 and expected to reach $555 million in 2028, growing at a CAGR of 12.7% (Grand View Research, 2021b). Like other plant proteins, this growth can be attributed to consumers choosing plant-based proteins over animal-based proteins, the perceived health benefits of plant proteins, shifting dietary patterns, and rising environmental awareness. Additionally, manufacturers have shifted from soy to pea protein, because it is non-GM and not yet a major allergen, while having sufficient nutritional and functional value (Brewster, 2020; Formanski, 2020). Accordingly, a variety of protein ingredients have been developed, such as flours, concentrates, isolates, hydrolysates, and textured products. With such variety, pea protein has flexibility for use in a wide range of products. Pea protein ingredients are currently found in meat substitutes, bakery products, dietary supplements, and beverages (Grand View Research, 2021b). While pea protein ingredients are increasingly utilized, the inherent characteristics of the protein can present functional challenges in these applications. It is important to further examine the source of these ingredients, from pea

cultivation to protein ingredient processing, to find opportunities to enhance ingredient functionality, thus expanding potential applications.

1.4 Yellow field pea

Peas (*Pisum sativum* L.) are cool-season crops that produce seeds and seed pods that are primarily used for human consumption and to fortify livestock feed (Pavek, 2012). While garden and green peas are harvested before seed maturation for fresh and frozen food markets, field peas are harvested after seed maturation and drying for animal feed and food industry uses (Pavek, 2012; Tulbek et al., 2017). Accordingly, field (dry) peas are marketed as dry grains and are available in whole, split, or ground forms. Several classes of field pea are sold, including yellow and green pea. Pea protein ingredients are most commonly produced from yellow field pea (Lu, He, Zhang, & Bing, 2020; Tulbek et al., 2017).

1.4.1 Field pea cultivation and processing

Field pea is primarily grown in Canada, Europe, Australia, and the US, with Montana and North Dakota being the major US growing sites (Kandel & Endres, 2019). Peas are planted during the winter through early summer and, as a cool-season crop, the seedlings are able to withstand the low temperatures during the winter (Pavek, 2012; Tulbek et al., 2017). However, peas are most productive at 55-64^{o}F and are generally best suited for moderately fertile, well-drained, humus-rich soil with a pH from 5.5 to 7.0 and with 16-39 inches of annual precipitation (Pavek, 2012; Tulbek et al., 2017). Further, dry peas are a sustainable crop that has added-value to farmers, as it can be planted in rotation with small grains and oilseed crops. As a rotational crop, peas break up disease and pest cycles, supply nitrogen, improve soil aggregation, improve soil microbe systems, and help conserve soil water, aside from adding economic diversity (Pavek, 2012). Additionally, as a cover crop, peas are grown to improve and maintain soil productivity. These cover crops are beneficial because they provide added nitrogen in soil, improve the yield of following crops, and reduce erosion, especially during slow growing seasons (Kandel & Endres, 2019; Pavek, 2012).

In addition to providing environmental benefits as a rotational and cover crop, the pea crop contributes to sustainable agriculture by requiring relatively low energy and water inputs. Importantly, pea plants, as legumes, have a symbiotic relationship with *Rhizobium leguminosarum*, a soil bacteria that allows for the plant to fixate atmospheric nitrogen; nitrogen fixation forms ammonia that is necessary for growth (Pavek, 2012; Tulbek et al., 2017). This crop can get up to 90% of its nitrogen needs through the process of nitrogen fixation, thus significantly reducing the need for synthetic nitrogen fertilizers (Kandel & Endres, 2019). Pea produces nearly twice the amount of grain per energy input as spring wheat (Zentner et al., 2004). Numerous studies have demonstrated that pea is an energy efficient crop, as it requires less energy to grow and reduces energy requirements of crops grown in rotation with pea (Tulbek et al., 2017; Zentner et al., 2004). Additionally, peas, like other pulses, require much lower water inputs per gram of protein than other protein-rich foods, like milk, eggs, and chicken. In fact, peas require six times less water per gram of protein than beef (Tulbek et al., 2017). As a high-protein, sustainable crop, field pea production has grown over the past few decades.

Once pea plants have matured and the seeds reach 18-20% moisture, the crop is harvested by combine (Kandel & Endres, 2019). Depending on the moisture content, the whole pea seeds may need further drying, followed by cleaning to remove foreign materials. Pea seeds can be stored at 14% moisture below 60^{o}C, or at 13% moisture when temperature cannot be controlled, to maximize quality and shelf life (Kandel & Endres, 2019; Tulbek et al., 2017). Peas seeds are commonly further processed by dehulling and splitting, before milling to produce pea flour and other products that differ based on composition (Tulbek et al., 2017).

1.4.2 Pea composition

Peas are nutritionally dense, serving as a rich source of protein, starch, dietary fiber, and several vitamins and minerals. The protein concentration of peas ranges from 18% to 30%, averaging around 22%, depending upon the cultivar and environmental conditions (Dahl, Foster, & Tyler, 2012; Lu et al., 2020; Tulbek et al., 2017). Further, peas have 60-65% carbohydrate content, specifically, 37-49% starch, 14-26% dietary fiber, and about 5-9% soluble sugars (Dahl et al., 2012; Tulbek et al., 2017). In contrast

to soy, the fat content of peas is minimal; fat accounts for less than 2% of the dry pea mass (Tulbek et al., 2017). Additionally, vitamin and mineral levels in peas are higher than those in wheat and other cereals. However, antinutrients, such as phytate, can reduce the bioavailability of certain minerals (Dahl et al., 2012; Tulbek et al., 2017). Nonetheless, further processing to produce protein ingredients can degrade antinutrients and alter the vitamin and mineral content (Dahl et al., 2012). With significant amounts of protein, starch, and fiber, manufacturers have found economic value in peas and have developed various pea ingredients and co-products.

1.4.3 Pea-derived ingredients

Peas are an ancient, domesticated crop that has long been consumed in the Middle East and Asia. This crop spread to Europe and eventually to North America, where production has continued to grow over the last few decades. Canada and the United States are now major growers and exporters, exporting an estimated 70% of their peas around the globe (Tulbek et al., 2017; USA Pulses, 2016). Traditional preparations use whole or split forms of peas in soups, salads, snacks, and other prepared dishes (USA Pulses, 2016). With global production of peas continuously growing over the past 30 years, the market for pea-derived products is growing, thus, leading producers to find new applications for peas (Lu et al., 2020). Producers have tapped into the nutritionally and functionally valuable components of pea (i.e., fractionation to produce protein, starch, and fiber-rich ingredients) for use in new, plant-based food products (Boye et al., 2010; Tulbek et al., 2017). Pea ingredient research and development has skyrocketed as a result of increasing demands for plant proteins and the relatively low cost of production (Lu et al., 2020). A variety of pea protein ingredients are now available, including pea flour, concentrate, isolate, hydrolysate, and various by-products.

1.4.3.1 Pea flour and protein concentrate

Following harvesting and initial processing, as discussed in Section 1.4.1, dehulled and split pea seeds are dry milled to produce course or fine flours with approximately 22-28% protein, 40-53% starch, and 6-20% dietary fiber (Tulbek et al., 2017). The milling method and particular parameters may be adjusted to produce a range

of flours with different particle sizes, based on the functional needs of the end-product. Pea flour is frequently utilized in snacks, baked goods, and extruded meat analogue products for protein and fiber enhancement and for its winter-binding, oil-binding, emulsification, gelation, and texturizing properties (Boye, Zare, & Pletch, 2010; Mondor, 2020; Tulbek et al., 2017).

Moreover, pea flour may be further processed to concentrate the proteins using a dry fractionation process, called air classification. Following dry milling, air classification is used to split the finely milled flour into a fine, light (protein) fraction (2-25 µm) and a coarse, heavy (starch) fraction (25-70 µm). This method involves using a spiral air stream to separate the flour based on particle size, bulk density, and powder characteristics (Boye et al., 2010; Mondor, 2020; Pelgrom, Vissers, Boom, & Schutyser, 2013; Tulbek et al., 2017). On a dry basis, the resulting pea protein concentrate (PPC) consists of 48-55% protein and 5-10% starch, while the pea starch concentrate has 10-15% protein and 65-75% starch (Tulbek et al., 2017). Repeated milling and air classification steps can further separate entangled protein and starch granules, improving the yield and purity of the fractions (Boye et al., 2010; Pelgrom et al., 2013). With increased protein content, PPC has water-binding, oil-binding, foaming, and emulsifying properties that are useful in baked goods and meat analogues (Tulbek et al., 2017). While dry fractionation produces valuable PPC and starch ingredients, this technique is currently inadequate for producing protein isolates.

1.4.3.2 Pea protein isolate and hydrolysate

Higher purity pea protein ingredients, such as isolates and hydrolysates, require wet fractionation techniques to remove non-protein components. While labeling guidelines are not formally enforced, pea protein isolates (PPI) are normally at least 80% protein. Alkaline extraction-isoelectric precipitation (AE-IP) and salt extraction are commonly used to produce PPI (Akharume et al., 2021; Mondor, 2020). Briefly, the AE-IP process begins with alkaline extraction, which includes dispersing pea flour in water and adjusting the pH between 7 and 11, where proteins are soluble and starch and insoluble fiber remain insoluble. Following the removal of insoluble materials, the proteins in solution are then precipitated by adjusting the pH of the solution to their

isoelectric point (4-5) to separate the protein from colors, soluble fiber, and other soluble sugars and polysaccharides (Lam et al., 2018; Mondor, 2020; Tulbek et al., 2017). The precipitated proteins are then washed to remove salts, re-dispersed in water, neutralized, and dried (Boye et al., 2010; Lam et al., 2018). On the other hand, salt extraction is used to extract proteins based on the salting-in and salting-out principles. Pea flour is dispersed in a salt solution at low ionic strength, between 0.1 and 1 M, which helps enhance protein solubility, or protein-water interaction (Lam et al., 2018). Insolubles are then removed and the proteins are separated from solubles by salting-out at high ammonium sulfate concentrations, followed by several washes to remove excess salt. Alternatively, solubilized proteins are concentrated using membrane ultrafiltration/diafiltration, followed by drying (Boye et al., 2010; Lam et al., 2018; Mondor, 2020).

Various other protein isolation processes are established, such as micellar precipitation, water extraction, acid extraction, and ultrafiltration. These processes may be used alone, in tandem with, or as modifications to the aforementioned protein extraction methods based on pH and ionic strength (Boye et al., 2010). Salt extraction processes have some advantages over other extraction methods, as extreme pH levels and high temperature are not necessary, reducing the likelihood of protein denaturation and impaired functionality (Lam et al., 2018). Although salt extraction can better preserve native protein structure, this process is typically utilized on a smaller, research scale, while AE-IP processes are more commonplace in the food industry. AE-IP is more feasible on an industrial scale, because salt extraction requires large amounts of salt and water during the extraction and concentrating steps (Lam et al., 2018). With high nutritional value and emulsification and foaming properties, PPI has been added to meat analogues, salad dressings, nutritional supplements for exercise (Lu et al., 2020; Tulbek et al., 2017).

Pea protein hydrolysate (PPH) is a high protein ingredient produced by enzymatically hydrolyzing the proteins in PPI to improve its functionality. Hydrolysates are produced by treating PPI with a protease or a mixture of proteases. Upon enzymatic hydrolysis, peptides and, sometimes, amino acids are produced (Akharume et al., 2021). Due to the broad nature of protease enzymes available and possible reaction conditions, hydrolysates can greatly differ in the physicochemical properties of the released peptides

and consequently in functionality. Further, excessive hydrolysis exposes buried hydrophobic groups, and may result in the production of small, bitter peptides with reduced functionality (Akharume et al., 2021). Therefore, limited enzymatic hydrolysis (degree of hydrolysis <10%) is often targeted to produce an acceptable PPH. Limited enzymatic hydrolysis has been shown to improve pea protein digestibility, as well as solubility, emulsification, and foaming properties (Barać, Pešić, Stanojević, Kostić, & Čabrilo, 2015; Barac et al., 2012; Boye et al., 2010; Lu et al., 2020). Due to the improved functionality and relative ease of manufacture, PPH is now available through a number of ingredient suppliers for use in beverages and other applications (Brewster, 2020).

1.4.3.3 Pea by-products.

With the manufacture of protein ingredients from pea seeds, significant by-product streams are produced. As previously discussed, peas contain significant amounts of starch and fiber, aside from protein. Starch-rich and fiber-rich streams have functional value in food applications, thus have economic value for manufacturers. Starch-rich fractions are produced from the dry- and wet-fractionation processes used to produce PPC and PPI (Tulbek et al., 2017). The coarse, starch-rich fraction produced from air fractionation contains about 65% starch, 8% protein, and 21% fiber (Pelgrom, Boom, & Schutyser, 2015; Ratnayake, Hoover, & Warkentin, 2002). This fraction can be further purified, up to 99% starch, by removing residual protein and fiber with additional washing and filtration steps (Li et al., 2019; Ratnayake et al., 2002; Tulbek et al., 2017). Native pea starch has texturing properties, film forming properties, and better gelling and pasting properties than tapioca and corn starches, making it suitable in coatings, batters, and meat products (Li et al., 2019; Tulbek et al., 2017; Zhou et al., 2019). Further modification of pea starch expands its potential application for use in meat analogs, cosmetics, and packaging materials (Tassoni et al., 2020). As a by-product of protein ingredient production, pea starch is a relatively cheap starch source with added-value for many industrial applications.

Pea fiber is another product of both dry- and wet-fractionation processes. Pea fiber is produced from milled pea hulls or purified from starch-rich fractions (Li et al., 2019; Pelgrom et al., 2015; Tulbek et al., 2017). Product developers can add pea dietary

fiber to foods to increase their nutritional value. Further, fiber can help bind water and provide texture in food systems, like baked goods and extruded snacks (Pelgrom et al., 2015; Tulbek et al., 2017). As pea protein ingredients continue to gain prominence, their processing by-products have increased value, thus, leaving room for the exploration of new applications. Utilization of pea protein ingredients and by-products in new applications requires understanding of their nutritional quality, composition, and functionality.

1.5 Pea protein

1.5.1 Pea protein nutritional quality

The nutritional quality of a protein is affected by its amino acid composition and digestibility, which together are used to determine the PDCAAS value. The PDCAAS of pea protein varies between 0.54-0.89, depending upon the protein source, composition, and processing steps (Ma, Boye, & Hu, 2017; Nosworthy & House, 2017; Rutherfurd, Fanning, Miller, & Moughan, 2014). Regardless of these conditions, pea protein consistently has lower PDCAAS values than soy protein (0.94-1.00) due to differences in amino acid composition and presence of antinutrients. Pea protein has high levels of lysine, but methionine is the limiting amino acid, with sulfur-containing cysteine and essential tryptophan also present in low quantities (Dahl et al., 2012; Ma et al., 2017; Rutherfurd et al., 2014; Tulbek et al., 2017). However, peas can be blended with cereals that are higher in methionine and limiting in lysine; the blending of these two plant protein sources can balance the essential amino acids profile (Nosworthy & House, 2017).

The lower PDCAAS of pea protein is also a result of the presence of antinutrients, such as protease inhibitors, lectins, tannins, and phytic acid (Roy, Boye, & Simpson, 2010; Tulbek et al., 2017). Antinutritional compounds interfere with digestion by binding to proteins and minerals and consequently limiting the accessibility of the digestive enzymes (Tulbek et al., 2017). Depending on the protein source, the protein extraction process may be leveraged to enhance the nutritional quality of protein ingredients. Nosworthy & House (2017) found that PPI had a lower PDCAAS than PPC due to alteration of the amino acid and antinutrient composition during the extraction process

(Ma et al., 2017; Nosworthy & House, 2017). Therefore, protein extraction/concentration procedures may be adjusted to optimize the nutritional quality of the product.

Much of the research investigating the physiological benefits of peas has focused on the consumption of whole peas or pea carbohydrates (e.g., fiber and starch) (Dahl et al., 2012). Consumption of peas, pea flour, and pea dietary fiber may help mediate glycemic response, influence insulin resistance, and enhance cardiovascular and gastrointestinal health (Dahl et al., 2012). More recent studies are specifically evaluating pea protein products, but this research is limited. Two small-scale clinical trials have evaluated the effect of pea protein versus whey protein isolates on several muscle health parameters following strength training (Babault et al., 2015; Banaszek et al., 2019). One study found that pea and whey proteins produced similar outcomes related to body composition, muscle strength, and performance in men and women after consuming these proteins during an 8 week, high-intensity functional training schedule (Banaszek et al., 2019). Likewise, another study evaluated the muscle thickness and strength of younger adult males who consumed 25 g of pea or whey protein twice a day with a resistance training program over 12 weeks (Babault et al., 2015). In this study, both proteins were effective in building muscles with no difference between the whey and pea protein groups. Therefore, pea protein-rich ingredients may have physiological value as alternatives to whey protein. Further research into the long-term health outcomes of pea protein consumption in more diverse groups is needed. While pea protein nutrition and health effects are currently under investigation, manufacturers are increasingly utilizing pea protein ingredients in food products. In order to use these ingredients successfully, the structure of pea proteins and their influence on functional properties must be understood.

1.5.2 Pea protein profile and structure

The functionality of pea protein ingredients depends upon the protein profile and structure. Pea protein is primarily comprised of salt-soluble globulins (55-65%) and water-soluble albumins (18-25%), with minor amounts of prolamins (4-5%) and glutelins (3-4%) (Boye et al., 2010; Gueguen & Barbot, 1988; Lu et al., 2020). The globulin proteins are storage proteins that are subdivided into 11S legumin, 7S vicilin, and 8S

convicilin proteins, accounting for 6-25%, 26-52%, and 4-8% of the total protein, respectively (Lam et al., 2018; Tzitzikas, Vincken, De Groot, Gruppen, & Visser, 2006). Globulins are the proteins of highest interest to the food industry, as they are largely responsible for the functional properties of protein in foods (Tulbek et al., 2017). Whereas, the 2S albumin proteins are metabolic proteins, such as enzymes, inhibitors, and lectins, that have little functional value in foods (Boye et al., 2010; Tzitzikas et al., 2006). Therefore, the structural characteristics of the globulin proteins are of most relevance to the functionality of pea protein and consequently, suitability in various food applications.

Legumin is a hexameric protein (320-400 kDa), comprised of six monomers associated together via noncovalent interactions. Each monomer consists of one acidic (40 kDa) and one basic (20 kDa) subunit linked by a disulfide bond, α–β (Lam et al., 2018; Lu et al., 2020; Tzitzikas et al., 2006). However, there are several different acidic and basic polypeptides that differ in amino acid sequence. Accordingly, the heterogeneous monomers that these polypeptides form are organized into the legumin families *Leg*A, *Leg*J, and *Leg*S. The *Leg*A and *Leg*J families have molecular masses of 60-65 kDa, while *Leg*S has a molecular weight of about 80 kDa (Barac et al., 2010; Tzitzikas et al., 2006). Further, three monomers associate via hydrophobic interactions to form trimers and two trimers assemble via electrostatic interactions and hydrogen bonds to form a hexamer (Tzitzikas et al., 2006). Considering the amino acid composition of legumin, the α-subunit is abundant in glutamic acid and the β-subunit has more alanine, valine, and leucine residues (Lam et al., 2018).

On the other hand, vicilin is a trimer (150-170 kDa), consisting of three monomers (~47-50 kDa) that associate via hydrophobic interactions (Lam et al., 2018; Tzitzikas et al., 2006). Vicilin monomers (α, β, and γ) provide sites for post-translational cleavage, forming various low molecular weight peptides of ~12-36 kDa (Lam et al., 2018; Lu et al., 2020; Tzitzikas et al., 2006). The γ-subunit is also sometimes N-glycosylated near the C terminus (Lam et al., 2018; Tzitzikas et al., 2006). Vicilin subunits lack cysteine residues, therefore they cannot form disulfide bonds. Additionally, vicilin subunits lack methionine and tryptophan, but have high levels of basic (arginine,

lysine) and acidic (aspartic acid, glutamic acid) amino acids (Barac et al., 2010; Lam et al., 2018).

Lastly, the convicilin protein is a trimer with a molecular mass of ~210 kDa and a highly charged extended N-terminal (Lu et al., 2020; O'Kane, Happe, Vereijken, Gruppen, & Van Boekel, 2004; Tzitzikas et al., 2006). Convicilin subunits are ~70 kDa each, which form homomeric trimers and, in some cases, heteromeric trimers consisting of vicilin and convicilin subunits (Lam et al., 2018; Tzitzikas et al., 2006). Convicilin subunits are closely homologous with vicilin subunits, leading some researchers to denote them as α-subunits of vicilin (O'Kane et al., 2004). Regardless of this distinction, convicilin is unique in its charged N-terminal extension, and with each monomer having one cysteine residue, thus making it capable of forming disulfide bonds (Lam et al., 2018; Lu et al., 2020).

Given the different composition and structures of each globulin protein, each protein has specific physicochemical properties that dictate their function in foods. For example, legumin has a more rigid/compact structure than vicilin because of its more complex quaternary structure and intermolecular disulfide bridges (Mession, Chihi, Sok, & Saurel, 2015). Therefore, legumin has greater thermal stability and a higher denaturation temperature (75-85^{o}C) than vicilin (65-69^{o}C) (Mession et al., 2015). Due to their more flexible structure, vicilin proteins are more surface active than legumin (Dagorn-Scaviner, Gueguen, & Lefebvre, 1986; Lam et al., 2018). Accordingly, vicilin produces more stable foams and emulsions than legumin (Koyoro & Powers, 1987; Liang & Tang, 2013; Tzitzikas et al., 2006). In addition, the pI of legumin is at a lower pH (4.8) than vicilin (pH 5.5), which impacts their solubility under different pH conditions (Koyoro & Powers, 1987). Convicilin may also be more soluble than the other globulins, due to its highly charged N-terminal extension (Kimura et al., 2008; Lam et al., 2018). However, it is unclear whether legumin has higher surface hydrophobicity than vicilin, due to contradictory findings (Koyoro & Powers, 1987; Lam et al., 2018). With each major globulin protein having distinct physicochemical properties, the particular legumin, vicilin, and convicilin distribution of pea protein impacts its performance in foods. The ratio of vicilin (7S) to legumin (11S) is especially important. The 7S/11S ratio in peas (~1.2-8, vicilin/legumin) is much higher than in soy (~0.47-0.79, β-conglycinin/glycinin),

for example (Tzitzikas et al., 2006). Accordingly, the lower amount of 11S (legumin) proteins substantially and most often negatively influences the protein functionality in food systems. Linking protein structure to functionality is vital in establishing suitable applications for pea protein ingredients.

1.5.3 Pea protein functionality in food applications

The sensory attributes of a food are greatly influenced by the protein functionality in the food system. Protein functionality refers to the physical and chemical properties that influence protein performance in foods, often in terms of solubility, emulsification, foaming, gelling, and water- and oil-holding properties. These measures of performance relate to the interaction of protein molecules with one another and with other food constituents. Functional properties can be broadly classified into three categories: hydration properties, surface properties, and structural properties. These measures of protein functionality are dictated by both intrinsic factors (e.g., amino acid sequence, composition, surface properties, molecular interactions, conformation) and extrinsic factors (e.g., environmental and processing conditions such as pH, temperature, moisture, ionic strength, and pressure) (Damodaran, 2017; Lam et al., 2018). Thus, protein functionality can greatly vary in food applications, depending on the protein structure. Various research groups have investigated the effects of intrinsic and extrinsic factors on protein structure to better understand the impact of structure on functionality, and ultimately identify suitable applications for pea protein ingredients.

1.5.3.1 Hydration properties

Protein hydration properties include those that rely on protein-water interaction, such as solubility, water-binding, and water-holding properties. In food applications, solubility is commonly associated with beverage applications, while water-binding and -holding properties are important in dough and meat products, for example (Akharume et al., 2021; Barać et al., 2015). Hydration properties are notable in foods systems, as they critically influence many other functional properties (e.g., emulsification, foaming, viscosity, and gelation properties, among others). In fact, consumer acceptability of low- and intermediate-moisture food products is highly dependent upon ability of the protein

to interact with water (Damodaran, 2017). Protein-water interaction primarily involves the binding of water molecules to the hydrophilic regions of protein through noncovalent interactions. Charged and polar residues, in particular, contribute most to the binding of water (Damodaran, 2017). However, other intrinsic and extrinsic factors also play a role in the observed solubility, water-binding, and water-holding properties that a protein exhibits.

Protein solubility is highly dependent upon intrinsic surface properties, especially the surface charge and surface hydrophilicity. Charged and hydrophilic residues on the surface cause repulsive electrostatic forces between protein molecules, allowing for protein-water interaction (Barać et al., 2015; Damodaran, 2017; Lam et al., 2018). In addition, the environmental pH, ionic strength, and temperature greatly influence protein solubility (Damodaran, 2017). It is commonly accepted that pea protein has the lowest solubility (<20%) at pH 4-5, regardless of the extraction technique or cultivar (Adebiyi & Aluko, 2011a; Boye et al., 2010; Cui et al., 2020; Lam et al., 2018). At the isoelectric point of pea protein (pH 4-5), proteins have a net zero or low charge load, leading to reduced protein-water interaction and increased protein-protein interactions via hydrophobic bonding (Damodaran, 2017; Lam et al., 2018). However, at pH values below and above the isoelectric point, the protein net charge increases, allowing for increased electrostatic repulsion and protein-water interaction (Damodaran, 2017). PPI is reported to have increasing solubility as the pH level decreases or increases away from the isoelectric point, with the highest solubility at or above pH 7.0, due to an increased net negative charge (Boye et al., 2010; Cui et al., 2020; Gao et al., 2020a; Lam et al., 2018). However, even at pH 7.0, PPI has lower solubility than SPI, due to inherent differences in their protein compositions and surface properties, as PPI has higher surface hydrophobicity and lower surface charge than SPI (Karaca, Low, & Nickerson, 2011).

The effect of pH on protein solubility is particularly important in beverage applications, as high-protein, RTD beverages are commonly formulated under acidic conditions (pH <3.5). Historically, WPI has been considered the gold standard protein for RTD beverage use, as it remains >90% soluble under acidic conditions and following thermal treatment (Boyle, Hansen, Hinnenkamp, & Ismail, 2018; Wang & Ismail, 2012). Such high solubility is attributed to the low surface hydrophobicity of WPI. On the other

hand, plant proteins tend to have comparatively higher surface hydrophobicity, therefore, they exhibit reduced solubility at low pH levels. As acidic conditions are close to its pI, PPI has a high surface hydrophobicity and reduced charge load, thus has relatively low solubility (<25%) near pH 3.0 (Bu, Nayak, Bruggeman, Annor, & Ismail, 2022; Chang, Tu, Ghosh, & Nickerson, 2015; Shand, Ya, Pietrasik, & Wanasundara, 2007). SPI is typically more soluble than PPI under acidic conditions, though, with solubility near pH 3 reported over a wide range of values, ~25-70%, differing based on extraction methodology and solubility testing parameters (e.g., pH and protein concentration) (Barac, Pesic, Stanojevic, Kostic, & Bivolarevic, 2015; Boyle, Hansen, Hinnenkamp, & Ismail, 2018; Chang et al., 2015). Nonetheless, PPI and SPI have higher surface hydrophobicity than WPI, reducing protein solubility and limiting their use in RTD beverage applications, as reduced solubility indicates a lack of beverage stability during storage.

Furthermore, protein solubility is affected by the ionic strength. At specific concentrations, protein solubility may increase, as the salt ions shield electrostatic interactions between protein molecules, enhancing protein-water interactions (Damodaran, 2017; Duong-Ly & Gabelli, 2014). However, at some salt concentrations, salt can reduce protein solubility, as charges on the protein surface are completely shielded by ions, reducing protein-water interactions. Charge shielding reduces the net charge at the protein surface, leading to protein-protein interactions via hydrophobic bonding (Damodaran, 2017; Lam et al., 2018). For example, a 100 mM sodium chloride concentration in water significantly reduced PPI solubility over a range of pH levels and protein concentrations (4% and 8% w/w), while increasing the ionic strength to 200 mM sodium enhanced PPI solubility (Bogahawaththa, Bao Chau, Trivedi, Dissanayake, & Vasiljevic, 2019). Reduced solubility was attributed to enhanced protein-protein interaction by charge shielding, while increased solubility was likely due to the increased solubility of globulin in salt solutions. The particular effect of salt on protein solubility is dependent upon the salt type, salt concentration, and level of protein hydrophobicity (Lam et al., 2018).

Although temperature (up to approximately 50^{o}C) may increase protein solubility, further temperature elevation tends to induce protein denaturation, leading to protein-

protein interaction. In general, thermal treatment results in disruption of protein secondary and tertiary structure, leading to increased intermolecular hydrophobic interaction and disulfide interchange. Increased protein-protein interaction causes protein aggregation and precipitation from solution. Thus, elevated thermal treatment and the resulting denaturation reduces protein solubility (Bogahawaththa et al., 2019; Damodaran, 2017; Lam et al., 2018). Despite this trend, pea protein may form soluble aggregates upon thermal treatment, allowing for high solubility and heat stability under certain conditions (Bogahawaththa et al., 2019). Soluble aggregate formation is highly dependent upon extrinsic factors, such as pH, ionic strength, protein concentration, and thermal treatment (Bogahawaththa et al., 2019; Bu et al., 2022).

Likewise, protein extraction and industrial processing conditions have significant effects on protein solubility, thus overall functionality. Presently, protein ingredient production inherently involves exposure to adverse environments (e.g., pH, heat, solvents, and salts) during the extraction and spray-drying processes that alter intrinsic protein characteristics (Cui et al., 2020). Protein denaturation and aggregation are associated with these processes and are responsible for the relatively low solubility of commercially produced protein isolates (Burger & Zhang, 2019; Hansen, 2020). Further, thermal treatments, such as pasteurization, sterilization, and ultra-high temperature (UHT), are essential for finished product safety and quality, but can reduce protein solubility (Bogahawaththa et al., 2019). Generally, these processing steps are associated with increased surface hydrophobicity and decreased solubility, as proteins unfold and expose the interior hydrophobic moiety at higher temperatures (Lam et al., 2018). In contrast, even with these commercial processing conditions, whey protein isolate (WPI) typically retains high solubility over a wide range of pH values due to its high surface hydrophilicity (Lam, Warkentin, Tyler, & Nickerson, 2017; Wang & Ismail, 2012). Compared to WPI, commercial SPI has lower solubility, while commercial PPI has the lowest solubility, limiting its use in beverage applications (Lam et al., 2017). To enhance the successful incorporation of pea protein into foods and beverages, solubility and thermal stability are key qualities that must be further investigated and improved upon.

Aside from solubility, water-holding is a critical protein hydration function. This property is measured as water-holding capacity (WHC), which refers to the ability of a

protein matrix (e.g., gel or muscle) to entrap and retain water against gravitational forces (Damodaran, 2017; Lam et al., 2018). Like solubility, WHC relies on protein-water interaction by noncovalent interactions, although also involving protein-protein interaction due to the protein matrix. Therefore, intrinsic factors (e.g., amount of hydrophilic residues) and extrinsic factors (e.g., pH, ionic strength, temperature) may similarly impact the WHC (Lam et al., 2018). While WHC refers to water held within a protein matrix, water-binding capacity (WBC) refers to the chemical binding of water to protein, defined as grams of water bound per gram of protein (Damodaran, 2017). In application, WHC impacts the syneresis process (separation of water from a protein matrix) in products like yogurt or meat patties, while WBC impacts other product qualities, such as viscosity, in a variety of products (Zayas, 1997). Although they differ, WHC and WBC are often wrongly used interchangeably in published literature. While there is minimal research and data available regarding the WHC of pea protein, according to the aforementioned definition, several studies have evaluated the WBC, citing results as WHC. Reported WBC values of pea protein are ~2-4 g/g, varying by extraction method and cultivar (Boye et al., 2010; Lam et al., 2018; Stone, Karalash, Tyler, Warkentin, & Nickerson, 2015). Further, commercially produced pea protein is reported to have a much lower WBC (3.1 g/g) than commercial soy protein isolate (12.4 g/g), potentially limiting its use in foods like processed meats (Stone et al., 2015). The hydration properties of pea protein ingredients need improvement in order to increase usage in various foods and beverages.

1.5.3.2 Surface properties

Proteins have surface properties that allow them to stabilize dispersed oil-water and air-water food systems, such as emulsions and foams, respectively. In application, emulsions include oil-in-water (e.g., milk, salad dressings, mayonnaise) and water-in-oil (butter, margarine) systems, while foams consist of air-in-water systems (e.g., ice cream, whipped topping, cake, and meringue). In both emulsions and foams, protein adsorb to the oil-water or air-water interface upon mechanical agitation, aligning hydrophilic/hydrophobic regions accordingly; this alignment minimizes interfacial tension, thus preventing separation of the phases (Damodaran, 2017; Lam et al., 2018).

Highly surface-active proteins are amphiphilic and have molecular flexibility, allowing them to adsorb, migrate, and reorient at the interface, forming a film that stabilizes the interface under stress (Damodaran, 2017; Lam et al., 2018). Molecular flexibility is impacted by intrinsic factors, such as the protein size, charge, and conformation. However, surface activity is most associated with protein conformation. The conformation of a protein relates to the particular flexibility of the polypeptide chain, its response to environmental changes, and the distribution pattern of hydrophilic and hydrophobic groups on the protein surface (Damodaran, 2017; Lam et al., 2018). Thus, emulsification and foaming properties depend upon the protein surface properties, intrinsically, but also in response to extrinsic factors (e.g., pH, ionic strength, and temperature) (Damodaran, 2017).

Emulsification properties of proteins are usually reported by several measures, including the emulsifying activity index (EAI), emulsion stability index (ESI), and emulsion capacity (EC). EAI measures the interfacial area stabilized per unit mass of protein, while ESI measures the resistance of an emulsion to phase separation over time, by evaluating change in emulsion turbidity. EC is a measure of the amount of oil that can be emulsified per unit weight of protein before phase inversion (Damodaran, 2017; Lam et al., 2018; Pearce & Kinsella, 1978). Emulsion formation and stability is highly affected by the pH of the system. At pH levels close to the isoelectric point (pI), emulsions are typically most unstable, as proteins have low solubility, lacking hydration and repulsive electrostatic forces; this promotes oil droplet flocculation, followed by phase separation (Damodaran, 2017). However, emulsification properties increase at pH levels away from the pI, when electrostatic repulsive forces between protein molecules increase (Lam et al., 2018). This pH-dependent trend is consistent with pea protein, as several studies have found emulsifying properties to be poorest near the pI (pH 4-5) (Adebiyi & Aluko, 2011; Barac et al., 2010; Liang & Tang, 2013). Barac et al. (2010) reported reduced EAI of PPI at pH 5.0, with increased activity at pH 3.0 and 8.0, hypothesizing that increased protein-protein interaction near the pI reduced the protein solubility, flexibility, and film formation around oil droplets. Similarly, Adebiyi & Aluko (2011) found increased oil droplet size in PPI emulsions at pH 4.0, compared to small droplet sizes at neutral and alkaline (pH 9.0) conditions. They also found increased emulsion stability at neutral and

alkaline conditions, both measures indicating better emulsification properties at pH levels away from the pI (Adebiyi & Aluko, 2011). Additionally, emulsions are generally most stable at low ionic strengths, as protein electrostatic repulsive forces and solubility are enhanced (Lam et al., 2018). Literature investigating the impact of ionic strength on emulsification properties of pea protein is, however, limited.

The environmental conditions that proteins are exposed to during extraction and further processing (e.g., heat, pH) also impact emulsification properties. It is commonly understood that the partial denaturation of globular proteins can improve their emulsification properties. Following processing, partially unfolded proteins have increased molecular flexibility and surface hydrophobicity, allowing for an enhanced film-forming ability (Damodaran, 2017). The specific effect of extraction method (e.g., ultrafiltration (UF), AE-IP, salt extraction-dialysis (SE), and micellar precipitation (MP)) on emulsification properties is unclear, as numerous studies have found contradictory results; this is likely due to nuances in methodology and starting material (Boye et al., 2010; Lam et al., 2018; Lu et al., 2020; Stone et al., 2015). Boye et al. (2010) found that UF and isoelectric precipitation methods produced pea protein with EAI and ESI values that were not significantly different, while Stone et al. (2015) found slight differences in EC, between PPI produced by AE-IP and by SE. In general, any conditions that lead to the formation of insoluble aggregates greatly reduce emulsification properties (Stone et al., 2015).

Additionally, emulsification properties are directly affected by processing as a result of protein composition. The relative abundance of major, functional globular proteins present in a protein ingredient depend upon the environmental conditions used during processing. As previously discussed, legumin and vicilin are the major protein fractions found in pea protein isolate. The intrinsic properties of these proteins and their relative ratio may impact their film-forming and stabilizing properties (Damodaran, 2017; Lam et al., 2018). A study investigating the effect of partially purified legumin and vicilin isolates on emulsifying properties found that pea legumin had better emulsion forming properties, while vicilin formed more stable emulsions, although these effects are pH-dependent (Liang & Tang, 2013). Barac et al. (2010) also linked increased ESI to a protein isolate with increased legumin content. It was hypothesized that the greater

surface hydrophobicity of legumin led to higher adsorption and stability at the oil-water interface (Barac et al., 2010). Conversely, Lam et al. (2017) found a weak, negative correlation between emulsion stability and legumin/vicilin ratio in PPI produced from several pea cultivars. This correlation was attributed to the smaller size of vicilin and lack of cysteine residues that take part in disulfide interchange, allowing for greater molecular flexibility (Lam et al., 2017; Tzitzikas et al., 2006). The impact of these protein ratios on pea protein emulsification properties needs further investigation. Importantly, though, pea protein reportedly has similar emulsification properties (emulsion stability and EC) to soy protein isolate (Boye et al., 2010; Hansen, 2020; Lam et al., 2018; Stone et al., 2015). Therefore, pea may have potential for food and beverage applications.

Similar to emulsions, the interfacial, surface properties of proteins can help to create and stabilize foams. Protein-stabilized foams have an aqueous continuous phase and a gaseous dispersed phase. Within this system, proteins in the continuous phase adsorb at the air-water interface to reduce surface tension, forming a film that stabilizes the dispersed air bubbles (Damodaran, 2017). Protein foaming properties are usually expressed as foaming capacity (FC) and foam stability (FS), referring to the interfacial area (e.g., volume) that can be created by protein solution and the ability of a protein to stabilize the foam against stresses, respectively (Lam et al., 2018). High FC is typically attributed to high molecular flexibility, as proteins must quickly rearrange and adsorb at the air-water interface to form a foam, while FS is controlled by the strength and flexibility of the film formed at the interface (Lam et al., 2018). Accordingly, high surface hydrophobicity and solubility are considered desirable in forming and stabilizing foams (Barac et al., 2010; Lam et al., 2017). However, the impact of solubility on pea protein foaming properties has been inconsistent, with some studies correlating these properties and others finding no correlation (Adebiyi & Aluko, 2011a; Barac et al., 2010; Cui et al., 2020; Stone et al., 2015). Studies finding no correlation suggest that protein behavior at the air-water interface is most important in foam-forming.

The foamability of pea protein is highly dependent upon various intrinsic and extrinsic factors, which is reflected in the body of research looking into the FC and FS of pea protein. Due to the different methodology used to measure FC and FS, trends are difficult to compare and are often in disagreement. It should be noted that the protein

concentration used in these testing methods often differ and therefore, may significantly alter the protein performance. For example, increasing protein concentration may improve foaming properties due to the increased viscosity of the aqueous phase and enhanced protein-protein interactions required for a stable film (Barac et al., 2010; Damodaran, 2017).

Nevertheless, several studies have found that foamability differs with the particular pea cultivar from which the PPI was produced, likely owing to the differing protein profiles (Barac et al., 2010; Cui et al., 2020; Stone et al., 2015). Although Lam et al. (2017) found no correlation between the vicilin/legumin (7S/11S) ratio and foaming properties, others have shown that higher vicilin concentrations result in improved foaming properties (Dagorn-Scaviner et al., 1986). Vicilin is thought to have better foaming properties than legumin, due to the more flexible structure of vicilin (Lam et al., 2018). Accordingly, as PPI has a higher 7S/11S ratio than SPI, PPI is reported to have similar or better foaming properties (Stone et al., 2015; Tulbek et al., 2017; Tzitzikas et al., 2006). The higher proportion of 7S vicilin in PPI allows for better foam forming properties than SPI, as vicilin quickly moves to the air-water interface to form and stabilize foams (Dagorn-Scaviner et al., 1986). On the other hand, pea protein also tends to have a low FC and FS at pH values near its isoelectric point (pH 4-5), with better values at higher pH levels (Adebiyi & Aluko, 2011a; Barac et al., 2010). The increased charge density of pea protein at higher pH values may enhance protein flexibility and prevent coalescence of air bubbles through increased electrostatic repulsion, while the reduced solubility near its pI likely prevents film formation and/or causes coalescence (Adebiyi & Aluko, 2011; Barac et al., 2010).

Additionally, protein extraction and processing methods can impact foaming properties. Stone et al. (2015) found that lab-produced PPI was affected by extraction method (AE-IP, SE, or MP), although all methods produced PPI with much higher FC and FS values than a commercial PPI, with values closer to those of soy, egg, and whey proteins. The harsh conditions used to produce the commercial PPI and resulting protein aggregation are likely responsible for its reduced foaming properties. Conversely, Boye et al. (2010) found no significant effect of concentration method (isoelectric precipitation vs. ultrafiltration) on the PPI foaming properties tested. Among these studies, there is

generally little consensus on whether pea protein exhibits high FC and FS, especially comparing lab-scale extraction and commercial processing. Consistent testing methodology and further investigation of processing effects are needed to determine whether pea protein foaming properties are suitable for food products.

1.5.3.3 Structural properties

Pea protein gelation and texturization properties rely on protein-protein interactions to form three-dimensional structures that entrap water, fat, and/or other food constituents. These protein structures provide the desirable texture and mouthfeel expected in certain food products like yogurt, tofu, meat, and meat alternatives. Generally, globular proteins undergo denaturation, followed by reformation and realignment via intermolecular interactions to form a protein network (Damodaran, 2017). Extrinsic factors (e.g., heat treatment, pressure, shear, pH, ionic strength, divalent cations, enzymes) can influence protein network formation (Lam et al., 2018; Lu et al., 2020). However, most food gels and texturized proteins are formed by heat treatment.

Heat-induced gelation involves heating of a protein solution above the denaturation temperature to promote the unfolding of native globulins. The unfolded molecules then rearrange and interact via noncovalent interactions and covalent disulfide linkages to form soluble aggregates. These aggregates then associate and form a protein network stabilized by noncovalent interactions and disulfide bridges (Damodaran, 2017; Mession, Sok, Assifaoui, & Saurel, 2013; Vatansever, Tulbek, & Riaz, 2020). While a variety of studies show that pea protein gelation properties are affected by cultivar, extraction method, protein profile, environmental conditions, and heat treatment, pea protein gels are consistently weaker and less elastic than soy protein gels (Lam et al., 2018). This difference is likely due to variation in globulin profile, namely the content of 11S globulin proteins, which contain cysteine residues that are essential in the formation of disulfide bonds. Therefore, the lower percentage of 11S legumin in pea protein hinders its ability to form a gel (Shand et al., 2007; Tzitzikas et al., 2006).

Extrinsic factors can be controlled to generate protein gels with greater strength and elasticity. Various studies have investigated the effect of sodium chloride, pH level, and heating rates on gelation properties, as these factors can be optimized to enhance gel

strength (Mession et al., 2013; Shand et al., 2007; Sun & Arntfield, 2011). For example, increasing temperature and including 1-2% (w/w) sodium chloride can increase gel strength (Shand et al., 2007). Further, protein modification methods, like transglutaminase treatment and cold atmospheric plasma have been used to promote cross-linking and soluble aggregate formation, respectively, improving pea protein gelation properties (Bu et al., 2022; Shand, Ya, Pietrasik, & Wanasundara, 2008).

On the other hand, protein texturization involves the alteration of globulins to form structures that can mimic the texture and mouthfeel of animal protein (Damodaran, 2017). Texturization is primarily achieved using extrusion technology, involving a various moisture, heating, pressure, shear, and cooling conditions (Vatansever et al., 2020). Generally, the extrusion process involves hydration and unfolding of globular proteins, realigning of denatured proteins to form a continuous and viscoelastic mass, and further cross-linking to form protein networks or fibers (Damodaran, 2017; Vatansever et al., 2020). Protein-protein interactions via disulfide linkages and noncovalent interactions (hydrogen bonding and hydrophobic interactions) are the primary drivers of protein fiber formation (Osen, Toelstede, Eisner, & Schweiggert-Weisz, 2015).

Extrusion processes are most often classified as low moisture (<30%) or high moisture (>50%) (Vatansever et al., 2020). Low moisture extrusion produces texturized vegetable protein (TVP) that requires rehydration and has a sponge-like texture. These products are used in high-protein snacks, as meat extenders, and in meat analogues (Osen, Toelstede, Wild, Eisner, & Schweiggert-Weisz, 2014; Vatansever et al., 2020). However, high-moisture extrusion is of most interest, as this processes can produce fibrous, meat-like structures for use as meat analogues. Currently, high-moisture extrusion is used to successfully transform pea protein ingredients into fibrous meat analogues by experimenting with various process variables and/or other additives, such as temperature and the addition of carbohydrates (Chen, Zhang, Zhang, Meng, & Wang, 2021; Osen et al., 2014; Samard & Ryu, 2019). Further refinement and standardization of this processing technique is needed to produce meat analogues with more acceptable texture that closely-resemble whole-muscle meat products. Although extrusion of pea protein has proven successful, pea protein is limited by its other functional properties, as well as its nutritional and sensory qualities.

1.5.4 Limitations of pea protein

As previously discussed, the nutritional quality (PDCAAS) of pea protein lags behind that of soy protein, thus, greater inclusion levels must be used when formulating foods to meet protein claim thresholds. For example, “high” protein claims can only be made for beverages containing ≥4.2% available protein (w/v), based on the 50 g protein daily value recommendations in the US (21 C.F.R. § 101.54, 2022). The relatively lower PDCAAS of pea protein compared to soy protein can be partially attributed to the presence of antinutrients (e.g., protease inhibitors and lectin), which can interfere with digestion, and to the limited content of sulfur containing amino acid, namely methionine (Tulbek et al., 2017). However, protein ingredient processing methods generally reduce the anti-nutritional factor content through thermal treatments and refinement (Joyce Irene Boye & Ma, 2015; Ma et al., 2017).

Importantly, off-flavors and aromas are associated with pea protein, hindering its acceptance in foods and beverages. Volatiles (e.g., alcohols, aldehydes, ketones) that are formed from the oxidation of unsaturated fatty acids by lipoxygenase give pea a characteristic grassy/beany taste, while naturally-occurring saponins in pea are associated with a bitter taste (Gao et al., 2020; Tulbek et al., 2017). On the other hand, soy protein production typically involves defatting, as well as heat processing techniques to inactivate lipoxygenase (Thrane et al., 2017). As pea flour is conventionally not defatted, due to the lower fat content compared to soy, the higher fat content in pea flour compared to defatted soy flour may result in more off-flavor formation (Boye et al., 2010; Maninder, Sandhu, & Singh, 2007). Processing steps (e.g., solvent treatment, thermal treatment, fermentation) can reduce off-flavors, by inactivating lipoxygenase or removing saponins. However, protein functionality is often adversely impacted when these steps are taken, as they can cause protein denaturation and aggregation (Akharume et al., 2021; Lan, Xu, Ohm, Chen, & Rao, 2019). Therefore, other processes have been explored to combat off-flavors. Maillard-induced glycation of PPI with gum Arabic (GA) and solid dispersion-based spray drying of pea protein with a carrier (GA or maltodextrin) have been used to reduce the beany flavors (Lan et al., 2019; Zha, Dong, Rao, & Chen, 2019b; Zha et al., 2020). Researchers using these methods theorize that protein conformational

changes and reduced hydrophobic interactions following these treatments allowed for the release of volatile off-flavors. Further, elevated temperatures used to initiate the Maillard reaction and to spray dry the protein may have "stripped off" volatile off-flavors from the protein and inactivated lipoxygenase (Lan et al., 2019; Zha, Dong, et al., 2019b). In addition to limiting off-flavors, these processes improved protein solubility and emulsification properties. As flavor is the major driver of consumer acceptance, off-flavor reduction is vital, while maintaining or enhancing protein functionality.

Moreover, the poor functionality of pea protein creates formulation challenges, limiting its application in products. Pea protein generally exhibits lower solubility, WHC, surface activity, and gelation properties, when compared to animal and/or soy proteins (Lam et al., 2018). This trend is particularly pronounced in commercially-produced PPI, were harsh extraction and processing conditions denature proteins and cause intense aggregation (Stone et al., 2015). Extreme loss in native structure and subsequent aggregation directly reduces protein solubility, as protein-water interaction is limited. As solubility is a necessary precursor for most other functionalities, it is imperative that pea protein solubility is further investigated and enhanced. Improved solubility may vastly improve the functionality and usability of pea protein ingredients in food and beverage applications, boosting pea protein ingredient value. Specifically, use of pea protein in the growing ready-to-drink RTD beverage sector is of great interest.

1.6 Ready-to-drink (RTD) protein beverages

Given its relatively low solubility and thermal stability, the incorporation of pea protein into high-protein, RTD beverages is especially challenging. In general, the primary indicators of a protein being suitable for RTD beverages include high solubility, good emulsification properties, low dusting potential, and acceptable viscosity. A protein must also be able to withstand the four primary processing steps, including dispersion/hydration, homogenization, thermal processing, and packaging (Paulsen, 2009). Additionally, the beverage must remain stable (i.e., protein remains dispersed and hydrated in solution) over its shelf life. As RTD beverages are typically consumed for their nutritional value, manufacturers aim to formulate products with high amounts of protein. Based on the 50 g protein daily value recommendations in the US, "high" protein

claims can only be made for beverages containing ≥4.2% available protein (w/v) (21 C.F.R. § 101.54, 2022). With such high protein inclusion levels, along with the necessary acidification and thermal processing steps of RTD beverage production, protein-water interactions are limited and protein-protein interactions are enhanced, limiting the product's shelf life (Bogahawaththa et al., 2019; Paulsen, 2009). Therefore, the aforementioned requirements for suitable use in RTD beverages are not currently met by pea protein. The intrinsic characteristics of pea protein, and its response to extrinsic factors (i.e., processing, thermal treatments, environmental conditions), hinder its successful application in RTD beverages and limit the finished product acceptability.

Most foods and beverages are at a neutral (pH 7) or acidic pH (pH <5), although ready-to-drink (RTD) protein beverages are typically acidic (pH <3.5) (Yaozheng Liu, Toro-Gipson, & Drake, 2021; Paulsen, 2009). Acidification of RTD protein beverages is necessary to reduce the severity of the thermal treatments, while maintaining product safety, as heat processing can negatively impact beverage flavor, color, stability, and nutritional value (Paulsen, 2009). As discussed, pea protein has very low solubility in the acidic pH range, near its isoelectric point (pH 4-5). With low solubility under acidic conditions, proteins have a tendency to aggregate and sediment from solution over storage (Lu et al., 2020; Vogelsang-O'Dwyer, Zannini, & Arendt, 2021). A lack of beverage stability, observed through protein sedimentation, is undesirable to consumers both visually and sensorially. Pea protein must have greater solubility under acidic conditions to successfully be incorporated into RTD beverages.

Furthermore, liquid beverages must undergo high-temperature short-time (HTST) pasteurization, ultra-high-temperature (UHT) processing, or retort processing to ensure product safety and stability (Paulsen, 2009). As mentioned, these thermal treatments typically induce protein-protein interactions, leading to insoluble protein aggregate formation and sedimentation from solution (Vogelsang-O'Dwyer et al., 2021). Thus, thermal stability is a crucial factor in creating stable RTD beverages. As it stands, acidification and thermal treatment are major challenges in formulating RTD beverages with pea protein.

To help with stabilizing the protein and limiting precipitation, RTD protein beverages are frequently formulated with hydrocolloids, such as xanthan gum, GA,

carrageenan, and pectin. These hydrocolloids increase the viscosity of the liquid, thus, reduce separation (Lindsay, 2017; Paulsen, 2009). Researchers are also investigating the ability of protein-polysaccharide soluble complexes to improve protein functionality. These soluble biopolymer complexes form when proteins and carbohydrates in are combined in an aqueous medium, at a pH that enhances interaction between positively charged protein patches and negatively charged polysaccharides (Lan, Chen, & Rao, 2018). Pea protein has been complexed with high methoxyl pectin, soybean soluble polysaccharide (extracted from soy pulp), corn fiber gum, carboxymethyl cellulose, and konjac glucomannan, with complexes showing improved solubility under acidic conditions (Lan et al., 2018; Wei et al., 2020; Yin, Zhang, & Yao, 2015). Functionality improvements were attributed to the increase in viscosity, electrostatic repulsion (due to anionic polysaccharides), and steric hindrance among the protein molecules following complexation (Wei et al., 2020). However, further understanding of protein-polysaccharide complexation effect on protein functionality is needed.

Additionally, the use of hydrocolloids, as additives or for forming soluble complexes, has negative consumer perception. Hydrocolloids are not considered “clean label” to many consumers (Asioli et al., 2017). Although this term, “clean label”, is subjective, it may not be economical to formulate products with these hydrocolloids (e.g., gums, carrageenan), as consumers may choose to avoid products containing these ingredients (Asioli et al., 2017; Varela & Fiszman, 2013). Techniques to enhance the functional performance, namely solubility, of pea protein in RTD beverages, while maintaining a consumer-friendly label, therefore, are needed.

1.7 Protein modification to enhance solubility and thermal stability

Enhancement of protein functionality can be achieved by altering intrinsic protein characteristics through structural modification. Changes in protein size, surface charge, hydrophobic/hydrophilic balance, and molecular flexibility can be induced by a variety of methods. Protein modification, broadly, is achieved through physical (e.g., heat, high-pressure, ultrasound, extrusion, cold plasma), chemical (e.g., acylation, esterification, amidation, deamidation, phosphorylation), enzymatic (e.g., hydrolysis and cross-linking), and Maillard-induced glycation techniques (Akharume et al., 2021; Zha, Rao, et al.,

2021). While all of these processes alter the intrinsic characteristics of the protein, specific techniques (or a combination) may be utilized to enhance particular functionalities, given the distinct changes induced.

Considering the functional requirements for proteins in RTD beverages, improvement of pea protein solubility and thermal stability is needed. It is well understood that these functionalities can be enhanced by reducing protein size, increasing surface hydrophilicity, and increasing electrostatic repulsion among protein molecules (Akharume et al., 2021). Several approaches to induce these changes in food proteins have been investigated, including chemical modifications, enzymatic hydrolysis, and Maillard-induced glycation. Chemical methods, such as acylation and phosphorylation, involve a nucleophilic substitution reaction between reactive amino acid side chains (e.g., lysine, serine, threonine) and other molecules of interest (e.g., succinic or acetic anhydride, sodium tripolyphosphate), which alters the net charge of the protein and subsequent noncovalent interactions (Akharume et al., 2021; Damodaran, 2017; Zha, Rao, et al., 2021). Pea protein modified by acylation and phosphorylation had improved solubility (Yanhong Liu et al., 2020; Shen & Li, 2021). For both methods, the increase in protein solubility is attributed to increased protein electronegativity at pH levels above the pI and to the unfolding of the native globular structure (Damodaran, 2017). However, these chemical modification methods have not been commercialized in the US, due to concerns regarding the potential for loss in nutritional value, presence of toxic residues and by-products, negative consumer perception, and regulatory concerns (Akharume et al., 2021; Damodaran, 2017).

Conversely, limited enzymatic hydrolysis of pea protein has been commercialized, producing pea protein hydrolysates with improved functional and nutritional properties (Brewster, 2020). Limited proteolysis can improve solubility, foaming, and emulsification properties for various reason, including reduction in protein size, increased molecular flexibility, and exposure of inner ionizable groups and hydrophobic moieties, ultimately altering the hydrophobic/hydrophilic balance (Zha, Rao, et al., 2021). Although hydrolysis may significantly increase pea protein solubility, especially under acidic conditions, this process may have negative impacts on flavor (Barac et al., 2012; García Arteaga, Apéstegui Guardia, Muranyi, Eisner, & Schweiggert-

Weisz, 2020). High astringency and sourness is associated with high-protein, acidic beverages (Beecher, Drake, Luck, & Foegeding, 2008). A high amount of acid is necessary to lower the beverage pH, as proteins are buffers, resulting in astringency and sourness. The buffering capacity is increased upon protein hydrolysis due to the increase in the available carboxyl and amide groups. Further, bitterness greatly increases with hydrolysis, as this process exposes buried hydrophobic groups and releases small hydrophobic peptides (Akharume et al., 2021; García Arteaga et al., 2020). Additionally, protein hydrolysates may be more likely to react with certain flavor molecules as more internal residues are exposed, as well (Zha, Rao, et al., 2021). The partial impact of hydrolysis on protein-flavor interactions depends upon the changes in protein hydrolysate configuration and surface properties, though. For example, a study found that pea protein hydrolysates had a high binding affinity to octanal and dibutyl disulfide flavors, while reducing 2-octanone and hexyl acetate binding (Wang & Arntfield, 2016). Binding of off-flavors to protein can benefit the protein ingredient sensory properties, however, binding of desirable flavor molecules to protein can reduce the product shelf life (Anantharamkrishnan, Hoye, & Reineccius, 2020). When desirable flavorings in a food product covalently bind to proteins, they can no longer be perceived, therefore, reducing product quality. Increased bitterness and interaction with certain flavor molecules can reduce the overall consumer acceptability. Thus, other modification methods, such as Maillard-induced glycation, must be further explored to produce a pea protein ingredient with improved solubility and limited flavor detriment.

1.8 Maillard-induced Glycation

1.8.1 The Maillard reaction

Maillard-induced glycation involves the formation of stable protein-polysaccharide conjugates that are reported to have improved functionality and flavor (Zha, Gao, Rao, & Chen, 2021). This modification method involves the Maillard reaction, which is a complex set of reactions that are known for the browning and tastes characteristic of toasted bread, roasted vegetables, browned meats, and roasted coffee beans, for example (de Oliveira et al., 2016). This reaction is the namesake of Louis Camille Maillard, who first reported the reaction in 1912. Since then, the reaction has

been further characterized by many, with John Hodge (1953) formally establishing three main reaction stages: initial, intermediate, and advanced stages. The reaction scheme is shown in Figure 1, however, the full reaction mechanism is still under investigation due to its complex nature (Hodge, 1953; Martins, Jongen, & Boekel, 2001).

The initial stage of the Maillard reaction is well characterized, beginning with a condensation reaction between the carbonyl group of a reducing sugar and an available amine group. The ε-amino group of lysine is the most reactive amine site, followed by the α-amino group of terminal amino acids, and, to a lesser extent, groups from histidine, tryptophan, and arginine residues (de Oliveira et al., 2016; Martins et al., 2001; Zha, Rao, et al., 2021). This condensation reaction forms an unstable Schiff base that cyclizes to form N-substituted glycosylamine, which then irreversibly rearranges to form the Amadori compound (also referred to as Amadori rearrangement product (ARP), 1-amino-1-deoxy-2-ketose (Hodge, 1953; Martins et al., 2001; Oliver, Melton, & Stanley, 2006). This stage of the reaction is significant, because the ARP is the first stable product of the reaction and no color changes are observed (de Oliveira et al., 2016). Due the functional benefits associated with this the stage of the reaction, Maillard-induced glycation, as a protein modification method, is ideally limited to early reaction stage.

Compared to the initial stage, the intermediate and advanced stages of the Maillard reaction are much more complex and, consequently, poorly understood. The intermediate stage involves the degradation of the ARP and subsequent reactions. Dehydration and fission of ARP forms colorless reductones and fluorescent compounds; this is followed by Strecker degradation of amino acids and sugar fragments, furfurals, and other products, resulting in brown pigments and off-flavors (de Oliveira et al., 2016; Hodge, 1953; Morales & Boekel, 1999). A number of reactions (e.g., cyclization, dehydration, retroaldolization, rearrangement, isomerization, and condensation) follow, forming advanced glycation end-products (AGEs). The advanced stages of the Maillard reaction are characterized by the formation of brown nitrogenous polymers and co-polymers, called melanoidins, along with nitrogen-free polymers. Generally, the intermediate and advanced stages are associated with off-flavors, browning, and loss in protein nutritional and functional value (Martins et al., 2001; Naik, Wang, & Selomulya, 2021). For these reasons, Maillard-induced glycation must be controlled to the early stage

of the reaction (i.e., protein-polysaccharide conjugation), limiting progression of the intermediate and advanced stages.

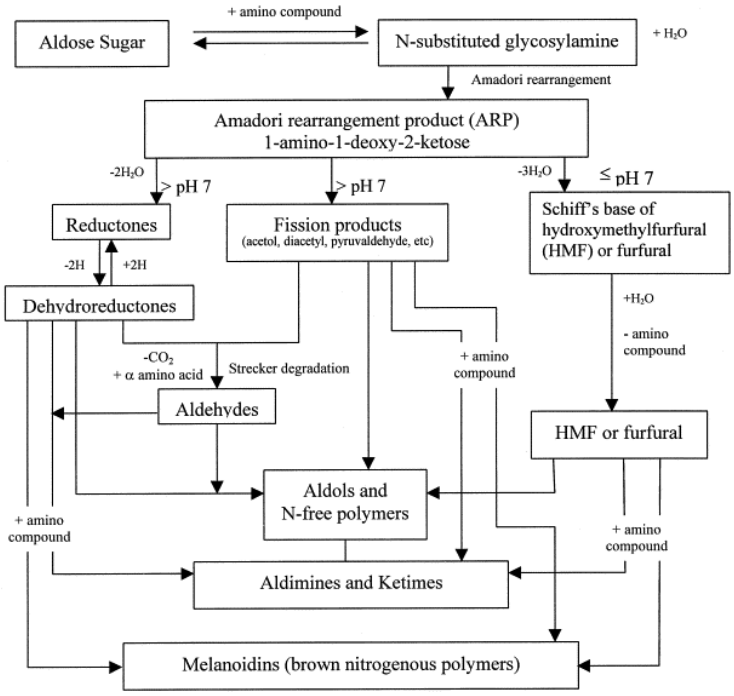

Figure 1. Maillard reaction scheme from Martins et al. (2001), adapted from Hodge (1953).[1]

1.8.2 Controlling the Maillard reaction

Intrinsic and extrinsic reaction conditions may be manipulated to control the Maillard reaction to the early stage, where functional protein-polysaccharide conjugates are formed. The reaction progression depends upon the particular substrates (i.e., protein and aldose/ketose), their ratio, and various extrinsic factors (e.g., reaction pH, temperature, time, water activity); these conditions may be concurrently optimized to produce a glycated protein with the desired properties (de Oliveira et al., 2016).

When considering the substrates, the protein source and its structural characteristics influence the reaction progression. The amino acid composition and protein conformation (i.e., denaturation state and rigidity/flexibility) impact the

[1] Reprinted from Trends in Food Science & Technology, Volume 11, Sara I.F.S. Martins, Wim M.F. Jongen, and Martinus A.J.S. van Boekel, A review of Maillard reaction in food and implications to kinetic modeling, p. 364-373, Copyright (2001), with permission from Elsevier.

availability of reactive amines to participate in the reaction (de Oliveira et al., 2016; Zha, Gao, et al., 2021). The reaction rate also depends on the nature of the reducing saccharide (also referred to as reducing sugar or carbohydrate), with aldoses, ketoses, disaccharides, oligosaccharides, and polysaccharides each having distinct impacts. The saccharide type, size, and structure dictate its reactivity with anime groups (Zha, Gao, et al., 2021). Larger polysaccharides, such as maltodextrin and dextran (a large polysaccharide produced by *Leuconostoc mesenteroides*), have a lower reducing power and react much more slowly than smaller sugars (e.g., mono- and disaccharides). A study evaluating the conjugation of pea protein and different saccharides (glucose; lactose; maltodextrin of 5, 10, and 18 DE) by the Maillard reaction found that ARP formation decreased with increasing saccharide size and decreasing DE (reactive carbonyl groups) (Zha et al., 2020). This difference in reactivity is attributed to the greater steric hindrance of large polysaccharide chains and the reduced amount of reactive carbonyl groups (de Oliveira et al., 2016; Jiménez-Castaño, Villamiel, & López-Fandiño, 2007; Laroque et al., 2008). Lastly, the relative abundance of reactive amine groups and carbonyl groups impacts the reaction rate. The combination of protein and saccharide substrates is usually reported as a mass or molar ratio, and increasing the relative amount of saccharide is associated with increased reaction rate (Martinez-Alvarenga et al., 2014). Thus, the ratio of carbonyl groups to available amine groups may be optimized in combination with other reaction parameters, to efficiently produce glycoproteins.

Control of the Maillard reaction is also achieved by manipulating external reaction parameters. The pH level is an important factor, as the protonation stage of the reactive groups is dictated by pH, with the carbonyl group being reactive when protonated and the amine group reactive when unprotonated (Martins et al., 2001). With this in mind, the rate of the Maillard reaction generally increases with increasing pH, favored at more alkaline pH levels (pH <10) (Huber & BeMiller, 2017; Martins & Van Boekel, 2005). Additionally, temperature plays a critical role in the reaction progression, as the reaction rate increases with increasing the temperature (de Oliveira et al., 2016; Martins et al., 2001). High temperatures increase the reactivity of carbonyl and amino groups, due to the reaction kinetics, and result in unfolding of the protein structure exposing more reactive amine groups (Martinez-Alvarenga et al., 2014). However,

temperature is usually held below the denaturation temperature of the native protein to limit loss in protein structure and potential unwanted protein polymerization. A balance between reduced time and elevated temperature of incubation is usually found around 40-60^{o}C, with 60^{o}C being the most common reaction temperature (Zhang et al., 2019). Lastly, water activity (a_W) also impacts the propagation of the Maillard reaction, with the reaction occurring between 0.3 to 0.8 a_W, and the rate of the reaction increasing with a_w (de Oliveira et al., 2016; Martinez-Alvarenga et al., 2014). Reduced reaction rates are observed at a_W below ~0.3 and above ~0.8, which is related to limited diffusion between substrates and inhibition of the condensation reaction, respectively (Martinez-Alvarenga et al., 2014). Regulation of reaction pH, temperature, a_W, and time, therefore, is necessary to control the rate and extent of the Maillard reaction for the purpose of producing glycated proteins while limiting the formation of AGEs.

1.8.3 Methods of Maillard-induced glycation

As control of the Maillard reaction to the early stage is needed to form glycated proteins, there are two common methods of preparation: wet-heating and dry-heating. Wet-heating is characterized by the mixing of protein and carbohydrate in an aqueous environment, usually a buffer, and heating to a certain temperature to initiate the Maillard reaction. The ratio of protein and carbohydrate, solids content, pH level, temperature, and time parameters can be controlled using this technique (de Oliveira et al., 2016; Zhang et al., 2019). Benefits of wet-heating include a short reaction time and a well-dispersed system of reactants. However, water can inhibit the formation of glycated protein, as the first stage of the Maillard reaction is a condensation reaction. Excess water shifts the reaction equilibrium, preventing progression of the reaction to the Amadori product and subsequent later stages. Additionally, uncontrolled, elevated temperatures risk denaturation and polymerization of proteins (de Oliveira et al., 2016; Zha, Gao, et al., 2021; Zhang et al., 2019).

On the other hand, dry-heating is typically carried out by mixing protein and carbohydrate in a buffer at a particular pH, followed by lyophilization and incubation of the dried protein-carbohydrate mix under controlled relative humidity (RH) and temperature conditions. The protein-carbohydrate ratio, buffer, pH, and incubation

parameters (temperature, RH, time) are controllable during dry-heating (Zha, Gao, et al., 2021; Zhang et al., 2019). Compared to wet-heating, this method benefits from better stability and control of the progression of the Maillard reaction, although longer reaction times are associated with dry-heating, especially with globular/rigid proteins (de Oliveira et al., 2016; Zha, Gao, et al., 2021). In addition, non-thermal techniques to produce these conjugates are more recently emerging, involving pulsed electric fields, ultrasound, extrusion, high pressure, and electrospinning (Zha, Gao, et al., 2021). These and other novel techniques may be used alone or in tandem with wet- or dry-heating methods to produce glycated protein. As it stands, though, wet- and dry-heating techniques are the most prevalent methods of inducing the Maillard reaction to modify protein structure and enhance protein functionality.

1.8.4 Modification of protein structure

Protein-carbohydrate conjugates are formed following the initiation of the Maillard reaction, but degraded in subsequent stages of the Maillard reaction, as discussed. Therefore, it is important to limit the reaction to its early stage, where the glycated protein has new structural properties that translate to enhanced functionality. The glycated protein, simply put, is a protein molecule with carbohydrate(s) covalently attached, mostly on lysine residues. Generally, one or two carbohydrates can bind to proteins in their more structured, native form, while several can bind to a denatured protein, as more amine groups are exposed and available to react when denatured (de Oliveira et al., 2016). Accordingly, protein structure and functional behavior depends upon the number of carbohydrates attached to a protein (Zha, Gao, et al., 2021). Carbohydrate size also impacts glycated protein structure and function, as larger polysaccharides tend to impart more steric hindrance than mono- and disaccharides, increasing solubility (Zha et al., 2020). Glycated protein structure and functionality is also affected by its denaturation state, as elevated temperatures and glycation may induce partial unfolding of the protein (Wang & Ismail, 2012). The effects of unfolding on protein structure highly depend on the extent of denaturation and protein source (i.e., intrinsic properties). Overall, the conformational changes induced in glycated proteins

likely contribute to the changes in functionality, thus, it is important to link protein structure to functionality.

Generally, Maillard-induced glycation decreases the surface hydrophobicity of proteins, by the covalent attachment of hydrophilic carbohydrates. The hydrophilic carbohydrates block hydrophobic regions on the surface of the protein and increase the capacity of the molecule to interact with water via non-covalent interactions (Kutzli, Beljo, Gibis, Baier, & Weiss, 2020; Wang & Ismail, 2012). The addition of carbohydrates also increases steric and electrostatic repulsion among protein molecules, enhancing solvation (de Oliveira et al., 2016; Naik et al., 2021). The induced steric hindrance helps limit protein-protein interactions, while the hydrophilic nature facilitates protein-water interaction.

Likewise, the protein surface charge (ζ-potential) is affected by glycation, but also is dependent upon pH level and ionic strength. Free amine groups are blocked upon glycation, reducing the positive charge below the pI of the protein or increasing the negative surface charge above the pI (Kutzli, Beljo, et al., 2020; Zha, Dong, Rao, & Chen, 2019a). Additionally, certain linked carbohydrates lend a greater magnitude of charge, as anionic polysaccharides (e.g., GA) have deprotonated carboxyl groups over a wide pH range (Pirestani, Nasirpour, Keramat, & Desobry, 2017; Zha, Dong, et al., 2019a). Anionic polysaccharides can contribute to a greater surface charge, thus enhancing solubility over a wide range of pH values (Zha, Dong, et al., 2019a). In addition, researchers have found that the isoelectric point (pI) shifts to a more acidic pH upon glycation, due to the blockage of free amino groups, which are normally protonated at pH levels below their pK_a (Kutzli, Beljo, et al., 2020; Martinez-Alvarenga et al., 2014; Wang & Ismail, 2012). The shift in pI may allow for glycated proteins to have greater solubility near their native state pI and over a wider pH range.

Furthermore, protein secondary structures shift following glycation, with β-sheet structures increasing and α-helix, β-turns, and/or random coils decreasing, according to studies of glycated whey and canola proteins (Pirestani et al., 2017; Wang, He, Labuza, & Ismail, 2013). Pirestani et al. (2017) theorized that glycation may reduce intramolecular hydrogen bonding, increasing intermolecular interactions; thus, explaining the increase in β-sheet formation. Enhanced thermal stability of glycated proteins may be

associated with the increased β-sheet content, as β-sheets are more thermally stable than other secondary structure configurations (Damodaran, 2017; Wang & Ismail, 2012). Similarly, the denaturation temperature of proteins may increase following glycation, as found with glycated whey and canola proteins (Pirestani, Nasirpour, Keramat, Desobry, & Jasniewski, 2018; Wang & Ismail, 2012). An increased denaturation temperature indicates that the glycated protein is more thermally stable. The various changes in protein structure following glycation can enhance its functionality through enhanced protein-water interaction and enhanced thermal stability.

1.8.5 Effects of glycation on protein solubility and thermal stability

Modification of the protein via Maillard-induced glycation is associated with improvements in its functionality, primarily solubility and thermal stability. Historically, glycation has primarily been applied to animal-based proteins (e.g., whey, casein, and egg white proteins), but with the increased interest in plant-based proteins, glycation of plant proteins (e.g., soy, canola, wheat, rice, peanut, and oat proteins) has been further investigated over the past decade (de Oliveira et al., 2016; Naik et al., 2021). And more recently, glycation of pea protein has been investigated by a few research groups, with promising results.

The glycation of pea protein (PPI, PPC, and PPH) with GA has been investigated, utilizing 1:4 mass ratios (w/w) of protein ingredient to GA at pH 7 and incubating under dry-heating conditions (79% RH; 60 ^{o}C; for 0, 1, 3, and 5 days) (Zha, Dong, et al., 2019b, 2019a; Zha, Yang, Rao, & Chen, 2019). The solubility of PPI-GA conjugate was higher than that of the PPI control (~7% solubility), with the greatest solubility observed for conjugates produced after 24 hours of incubation (~16% solubility) (Zha, Dong, et al., 2019a). A much higher solubility was observed with PPC-GA conjugates, having the greatest solubility after 3 days of incubation (~41% solubility) (Zha, Dong, et al., 2019b). Similarly, increased solubility was observed following PPH-GA glycation, with 1 day of incubation contributing to increased PPH solubility from ~19% to ~26% (Zha, Yang, et al., 2019). These improvements in solubility were attributed to the enhanced steric hindrance of conjugates, improving protein-water interaction and limiting protein-protein interaction. However, these studies evaluated the solubility of 0.1% (w/v) solutions of the

unpurified samples (containing ~80% GA) in 10 mM phosphate buffer (pH 7.0). In application, much greater protein concentrations are used to make protein claims on food and beverage products, which are often at acidic conditions, rather than neutral. Thus, the solubility of glycated pea protein needs investigation at protein concentrations and conditions (e.g., pH, thermal treatments) relevant to food and beverage applications.

In another study, the structural and functional effects of grafting various saccharides (glucose; lactose; and maltodextrin of 5, 10, and 18 DE) to PPI (Zha et al., 2020) under wet-heating conditions were evaluated. A mass ratio of 5:1 PPI to saccharide was prepared in carbonate buffer solution (pH 10, 1:2 w/v) and heated at 80 ^{o}C for 12 or 24 hours. All saccharides improved the solubility of PPI, but larger saccharides improved solubility the most, with PPI-maltodextrin (DE 18) having the greatest solubility (~38%) at pH 7. This increase in protein-water interaction was associated with increased surface hydrophilicity and steric hindrance, along with partial denaturation (induced by alkaline conditions) helping to "loosen" the rigid native protein structure (Zha et al., 2020).

Similar results were found when PPI-maltodextrin electrospun fibers were dry-heated (75% RH; 65 and 70 ^{o}C; for 12 and 24 hours), with solubility increasing following glycation, measured at 0.25% (w/w) solids concentrations (Kutzli, Griener, et al., 2020). However, this study also demonstrated increased solubility of glycoproteins over pH 2-7. These results are significant, as solubility was enhanced near the native PPI pI (pH 4-5) (Kutzli, Griener, et al., 2020). Maillard-induced glycation, therefore, has the potential to increase solubility over a wide range of pH levels. Further, Tamnak et al. (2016) evaluated PPI-pectin conjugates produced under dry-heating conditions (79% RH; 60^{o}C; for 0, 6, 7, and 48 hours) in 1:1, 2:1, 3:1, and 1:2 w/w ratios of pectin to PPI. Solubility of 1% (w/w) solids solutions at their natural pH was determined. Glycation of PPI with pectin maintained or reduced solubility, compared to mixed PPI and pectin prior to incubation (Tamnak, Mirhosseini, Tan, Ghazali, & Muhammad, 2016). This reduction was attributed to protein denaturation and polymerization and high molecular weight of PPI-pectin conjugates. Further investigation of the impact of Maillard-induced glycation on PPI solubility is needed, and, as mentioned, future studies would benefit from evaluating solubility of proteins at concentrations relevant to food and beverage applications.

While a number of studies have evaluated pea glycoprotein solubility, none have investigated the effect of thermal treatments on solubility. Literature is currently limited, in regard to the thermal stability of glycated pea protein. However, as discussed in earlier, it is possible that glycation could improve pea protein thermal stability. Previous studies demonstrated an increase in thermally stable β-sheet structures and denaturation temperature following glycation of whey and canola proteins (Pirestani et al., 2017, 2018; Wang et al., 2013; Wang & Ismail, 2012). Further investigation into the effect of glycation on pea protein thermal stability is needed, as enhanced thermal stability has the potential to improve the prospects of pea protein use in beverage applications. Nevertheless, while the potential benefits of Maillard-induced glycation are significant, its current limitations have prevented further scale-up efforts and commercialization.

1.8.6 Limitations

Discussion of the limitations of Maillard-induced glycation is warranted, as this modification technique has been researched for nearly three decades without known commercialization. In part, this echoes major concerns regarding the control of the Maillard reaction. As discussed, controlling the reaction extent is of high importance, as the intermediate and advanced stages of the Maillard reaction result in browning, off-flavor production, polymerization, and loss in protein nutritional and functional value (Naik et al., 2021). From a quality perspective, reaction intermediates formed in the intermediate and advanced stages (e.g., Strecker aldehydes, bitter flavors, burnt flavors) are undesirable in certain applications, like high protein beverages (Lund & Ray, 2017). Similarly, the production of melanoidins in the advanced stage of the reaction leads to undesirable sensory properties in some applications and shelf life reduction. Protein polymerization in the advanced stage also impairs ingredient functionality, limiting the potential utilization by food and beverage manufacturers (Lund & Ray, 2017). To maintain protein ingredient quality, control of the Maillard reaction is, thus, necessary. Additionally, commercialization of this modification technique has not occurred due to the considerable method development needed to scale-up production. Primarily existing as a research-based technique, the Maillard-induced glycation method has not been well-standardized. Further, as a necessary step to increase protein ingredient purity and value,

removal of unreacted carbohydrates has not been extensively researched. Scaled-up trials and cost analyses of Maillard-induced glycation must be completed before commercialization is possible.

Further, the value of a protein ingredient is largely tied to its nutritional value. It is in the interest of manufacturers to ensure that Maillard-induced glycation does not significantly reduce the nutritional value of protein ingredients. However, research on the impact of controlled, Maillard-induced glycation on protein quality is somewhat limited and unclear. Regardless, it is well-established that lysine residues are the primary site of conjugation (de Oliveira et al., 2016). As an essential amino acid, lysine loss can significantly affect the nutritional value of glycated protein by reducing the PDCAAS if lysine become the limiting amino acid following glycation (Nooshkam, Varidi, & Verma, 2020; Tuohy et al., 2006). Additionally, bulky polysaccharides linked to protein and protein cross-linking (during the advanced stage of Maillard reaction) may reduce protein digestibility, due to limited enzyme access. The effect of glycation on protein digestibility is, however, contested. Wang & Ismail (2012) found whey protein isolate-dextran to have ~9% greater *in vitro* digestibility than that of native whey protein isolate. This was attributed to partial unfolding of the protein upon Maillard-induced glycation, which may increase digestive enzyme accessibility (Wang & Ismail, 2012). Others, however, have found rapeseed protein isolate-dextran conjugates have reduced digestibility (Qu et al., 2018). Differences in the digestibility of glycated proteins are likely caused by differences in the extent of glycation (i.e., number of saccharides attached to the protein molecule), the size of the linked saccharides, and changes in protein conformation (Wang & Ismail, 2012). Additionally, the impact of lysine loss on protein quality may depend upon the protein source. For example, lysine is the reported limiting amino acid for rice, wheat, and oat proteins, while sulfur-containing methionine and cysteine are limiting for pea protein (Rutherfurd et al., 2014). Protein sources with higher lysine content, such as pea protein, have the potential to mitigate loss in quality due to lysine blockage.

Furthermore, research on the impacts of the Maillard reaction on human health is ongoing, although, compounds formed in the later stages of the Maillard have been linked to negative health effects. While AGEs formed *in vivo* have been linked to inflammation and various chronic diseases (e.g., diabetes, chronic heart failure,

atherosclerosis, renal failure, and Alzheimer's disease), the impacts of dietary AGEs on health are controversial and often conflated with those produced *in vivo* (Bastos & Gugliucci, 2015; Lund & Ray, 2017). Dietary AGE formation is dependent upon the protein source, although pyrraline and N^{ε}-carboxymethyl-lysine (CML) are commonly studied (Aljahdali & Carbonero, 2019). Studies have suggested that dietary AGEs play a role in causing chronic inflammatory diseases, though a vast amount of studies continue to investigate uptake patterns and mechanisms of dietary AGEs (Bastos & Gugliucci, 2015; Lund & Ray, 2017; Uribarri et al., 2005). Interestingly, reaction intermediates and AGEs (e.g., reductones and melanoidins) also have reported antioxidant activity, which suggests positive health outcomes (Lund & Ray, 2017; Naik et al., 2021). Further clarity on the impact that dietary AGEs and other Maillard reaction intermediates have on health is needed. Importantly, though, Maillard-induced glycation is ideally limited to the formation of protein-carbohydrate conjugates; AGEs should not be observed when the reaction is well-controlled to the early stage.

As discussed, control and limitation of the Maillard reaction is highly dependent upon the primary substrates: protein and carbohydrate. While diverse carbohydrate types have been employed in this reaction, all previous studies have utilized exogenous carbohydrates; that is, carbohydrates derived from sources independent of the protein source are used in the conjugation reaction (e.g., pea protein-GA, pea protein-pectin, and whey protein-dextran conjugates). The exogenous carbohydrates employed in this reaction are commonly hydrocolloids. As consumer perception of hydrocolloids is typically poor, usage of exogenous carbohydrates presents possible acceptability challenges when the modified protein is incorporated into foods (Section 1.6) (Asioli et al., 2017). Efforts to improve the "clean label" appeal of glycated proteins are, therefore, needed. Fortunately, pea contains significant amounts of carbohydrates (60-65% of pea flour), which is primarily pea starch (37-49% of pea flour) (Dahl et al., 2012; Tulbek et al., 2017). The starch component of pea may be further processed to produce carbohydrates with reactive carbonyl groups, such as maltodextrin. Thus, pea protein has the potential to be glycated with an endogenous carbohydrate source, producing pea protein-pea maltodextrin conjugates that have "clean label" appeal. To further explore

this concept of endogenous glycation, additional examination of pea starch and maltodextrin is needed.

1.9 Pea Starch

1.9.1 Pea starch structure and composition

Pea starch granule morphology, molecular structure, and composition has been heavily reviewed (Hoover, Hughes, Chung, & Liu, 2010; Ratnayake et al., 2002; Ren, Yuan, Chigwedere, & Ai, 2021; N. Singh, 2021). Briefly, smooth pea starch granules are primarily oval shaped, however, round, elliptical, and irregularly shaped granules have also been observed (Ratnayake et al., 2002; Wang, Yu, & Yu, 2008). The granule surface is typically smooth, but dry fractionation processes may incur minimal, physical damage to the surface (Hoover et al., 2010; Pelgrom et al., 2013).

The two primary components of starch granules are amylose and amylopectin. Amylose mainly consists of α-(1→4) linked glucose units, with slight branching via α-(1→6) linkages, while amylopectin consists of chains of linear α-(1→4) linked glucose units connected by α-(1→6) linkages. Amylopectin chains are larger and more heavily branched than amylose chains (Hoover et al., 2010; Ren et al., 2021). These components associate in a way that give starch granules a semi-crystalline structure. The branched amylopectin chains form double helices, which associate to form clusters; these clusters pack together to form crystalline lamella. This structure consists of alternating crystalline and amorphous regions, with the amorphous region mainly being amylopectin branch points (Hoover et al., 2010; Ren et al., 2021). Amylose is primarily found in the amorphous region, which is mainly in the center of pea starch granules, while the crystalline amylopectin is mainly located in the outer granule region (Hoover et al., 2010; Li et al., 2019; Wang et al., 2008). Pea starch typically has a higher amylose content (~38-41%) than other common starches, like maize and tapioca (~2-30% amylose) (Li et al., 2019; Ren et al., 2021). This difference in amylose composition, along with differences in morphology and structure, contribute to the unique functionality and resistance to hydrolysis of pea starch, compared to other common starches.

1.9.2 Pea starch functionality

Starch functionality primarily relates to the behavior of starch granules in water and the effect that heating and cooling processes have on its behavior. Importantly, gelatinization refers to the order-to-disorder phase transition that starch undergoes when heated in excess water. This transition involves the diffusion of water into the granule, which causes swelling and loss in crystalline order. Upon gelatinization, double helices uncoil and dissociate, and amylose leaches from the granule (Hoover et al., 2010; Ren et al., 2021). Using differential scanning calorimetry (DSC), several studies have found that the gelatinization temperature of pea starch is lower than those of maize and tapioca starches. For example, the gelatinization onset temperature (T_o) of pea starch is ~58-62 ^oC, peak temperature (T_p) is ~63-72 ^oC, and conclusion temperature (T_c) is ~71-81 ^oC (Chung, Liu, Hoover, Warkentin, & Vandenberg, 2008; Li et al., 2019). The gelatinization temperature relates to the thermal stability of the crystalline structures, thus, the differences between pea starch and other sources is attributed to the different organization of their crystalline structures (polymorphs) in the granules (Li et al., 2019). However, after gelatinization and upon cooling, amylose and amylopectin chains re-associate to form a more ordered structure; this is called retrogradation (Ren et al., 2021). Pea starch exhibits a higher degree of retrogradation than maize and tapioca starches, which is likely due to the higher amylose content of pea starch and differences in distribution of the amylopectin branch-chain-lengths among the different sources (Li et al., 2019; Ren et al., 2021).

Moreover, granular swelling and pasting properties are related to gelatinization and retrogradation properties. When starch is heated in an aqueous environment, starch granules swell, causing amylose to leach out of the granule. Generally, pea starch has restricted granular swelling and minimal leaching (until reaching ~60 ^oC), due to the high amylose content and strong interactions among starch chains (Hoover et al., 2010; Ren et al., 2021). When continuing to apply heat and shear, starch granules continue to swell and leach amylose and small amylopectin molecules. A viscous paste forms, containing hydrated amylose and/or amylopectin, along with granule ghosts and fragments (Hoover et al., 2010; Ren et al., 2021). This physical process is referred to as “starch pasting” and is usually evaluated based on rheological measurements (e.g., pasting curves using a

Rapid Visco Analyzer (RVA) instrument). Compared to common maize and tapioca starches, pea starch tends to have lower peak and breakdown viscosities, but higher final and setback viscosities; this is attributed to their higher amylose content, which restricts granule swelling upon heating and promotes retrogradation (Chung et al., 2008; Li et al., 2019; Ren et al., 2021). Pasting properties may also relate to amylose chain orientation, starch chain length, and the strength of starch chain interactions within the granule (Hoover et al., 2010). It is clear that the molecular structure and composition of starch dictates its functional properties. However, the functionality of pea starch should be further investigated in food systems, as pea starch has industrial-value as a significant by-product of pea protein production (see Section 1.4.3.3). Hydrolysis is a common modification method to add value to starch products.

1.9.3 Starch hydrolysis

In North America, increasing production of pea protein has motivated manufacturers to further process and modify the pea starch by-product in an effort to develop functional ingredients with added-value. Starch hydrolysis is a common industrial practice, used to make various products, such as maltodextrins and syrups. Hydrolysis is achieved by treating starch with acid and/or enzymes; these agents hydrolyze the glycosidic linkages that hold starch chains and granules together (Ratnayake et al., 2002).

Acidic hydrolysis is a traditional starch modification method, dating back to the 19th century. This process involves treatment of granules with dilute acid solutions (hydrochloric or sulfuric acid) for various time periods, either following gelatinization or below the gelatinization temperature (Wang & Copeland, 2015; Zhou et al., 2019). Generally, it is thought that amorphous regions of the granule are hydrolyzed quickly, as they are more accessible, while crystalline regions are more slowly attacked by acids, due to the high degree of packing (Wang & Copeland, 2015). A few studies investigating the acid-catalyzed hydrolysis of pea starch have reported that acid treatment results in the generation of shorter chains, a decrease in crystalline structure, and an erosion or complete destruction of starch granules (Wang, Blazek, Gilbert, & Copeland, 2012; Wang & Copeland, 2015; Zhou et al., 2019). Regardless, it is known that acid hydrolysis

randomly cleaves the starch molecule, while enzymatic hydrolysis acts on specific starch chain sites (Zhou et al., 2019). Some products, like maltodextrin (Section 1.9.4), are defined by their size/reducing power, therefore, may benefit from a more controlled, enzymatic hydrolysis method.

Enzyme-catalyzed hydrolysis relies on many reaction parameters that can be controlled by manufacturers. The reaction progression relies on numerous factors, including the enzyme (e.g., source, type, and enzyme to substrate ratio), starch characteristics (e.g., starch source, granule morphology, molecular associations, composition, and crystallinity), and extrinsic conditions (e.g., cofactor concentration, time, temperature, and pH) (Ratnayake et al., 2002). Industrially, these factors are manipulated to design processes that generate specific products of interest.

In general, various enzymes catalyze the breakdown of starch (e.g., amylases, isoamylases, glucanosyltransferases, and debranching enzymes), but amylases are most commonly used in the food industry (Hoover & Zhou, 2003; Robyt, 2009). While there are many types of amylases, microbial α-amylases are commonly used to produce starch hydrolysates, as microbial enzymes can be highly purified and produced on a large scale (Robyt, 2009). Enzymes are carefully chosen, as different sources produce different hydrolysates. For example, controlled hydrolysis using α-amylase from *Bacillus amyloliquefaciens* primarily produces maltotriose, maltohexose, and maltoheptose; these are considered maltodextrin products (Hoover & Zhou, 2003; Robyt, 2009). Thus, the use of different enzymes requires research and optimization.

Considering the action of enzymes on starch, α-amylase breaks down both amorphous and crystalline regions of the granule (Zhou, Hoover, & Liu, 2004). However, the action of the enzyme depends upon starch source and structure. Different types of granules, like small smooth pea starch granules, are more resistant to hydrolysis by α-amylase than larger granules, due to the high amylose content and physical state of the granule (Bertoft, Manelius, & Qin, 1993). Further, a study comparing the digestibility of several native starches found that pulses have less rapidly and slowly digestible starch and more resistant starch than normal and waxy maize starches (Li et al., 2019). This observation was attributed to the pulse starches having higher amounts of amylose, less porous granular structures, and larger proportions of longer amylopectin branch chains

(Bertoft et al., 1993; Li et al., 2019). Generally, the native structure and composition of pulse starches make them less susceptible to enzymatic hydrolysis than common maize starches. Starch granules are typically gelatinized prior to enzymatic hydrolysis, to enhance enzyme access to the internal starch chains.

In general, enzymatic hydrolysis first involves gelatinization, in order to disrupt the granule and increase the substrate accessibility to the enzyme; this increases the reaction rate (Baks, Bruins, Matser, Janssen, & Boom, 2008; Uthumporn, Zaidul, & Karim, 2010). However, in a study that evaluated the hydrolysis of a few native starches (corn, cassava, mung bean, sago) at sub-gelatinization temperatures (35 ^{o}C) using α-amylase and glucoamylase, sugars from the amorphous region of the granules were produced over the course of 24 hours (Uthumporn et al., 2010). After 12 hours of hydrolysis mung bean starch had a DE of ~12, and ~36 after 24 hours. Small, pinhole structures on the surface of the native mung bean starch granules likely allowed the enzymes to enter the granules (Uthumporn et al., 2010). These results show promise for pea starch hydrolysis at sub-gelatinization temperatures, for increased energy efficiency of the saccharification process. Further exploration of this hydrolysis technique is needed, as manufacturers could use this process to more efficiently produce starch hydrolysates.

1.9.4 Maltodextrin production

Maltodextrin is a commercial starch hydrolysate that consists of linear and branched carbohydrates, and is distinguished by having a dextrose equivalent (DE) between 3 and 20. DE refers to the reducing power of a carbohydrate, as a percentage of the reducing power of pure dextrose (i.e., dextrose has a DE of 100) (Huber & BeMiller, 2017; Kennedy, Knill, & Taylor, 2012). As a product of partial starch hydrolysis, maltodextrin is composed of maltooligosaccharides of varying chain length, or degree of polymerization (DP) 2-20; maltodextrins are also classified by having an average DP >5. Larger maltodextrins have lower DE values, as DE is inversely related to average molecular weight (Huber & BeMiller, 2017). In practice, ingredient manufacturers produce maltodextrins of varying DE and carbohydrate profiles, suited for diverse applications.

On an industrial scale, maltodextrin is produced by controlled starch hydrolysis, which is accomplished via enzymatic and/or acidic treatments. In practice, controlled enzymatic hydrolysis using α-amylase is the most common method, as acid hydrolysis, alone, produces too much glucose and haziness in solution (Kennedy et al., 2012). The enzymatic process begins by preparing an aqueous starch slurry (up to 40% w/w) and heating it to ~70-90 ^{o}C, near neutral pH, followed by incubation with a bacterial α-amylase until the desired DE is reached (Kennedy et al., 2012; Marchal, Beeftink, & Tramper, 1999). This process simultaneously gelatinizes and liquefies the starch. The liquefied product is then autoclaved at 110-115 ^{o}C to completely gelatinize the starch, and it is possibly further hydrolyzed to reach the desired DE, upon cooling (Kennedy et al., 2012). In some circumstances, however, acidic and enzymatic hydrolysis are used in combination. A combined process begins with the acidic hydrolysis (using hydrochloric acid) of a starch slurry, to DE 5-15, followed by neutralization and a second round of hydrolysis with α-amylase. Following the hydrolysis step (enzyme or acid-enzyme), the product is clarified to remove residual fiber, protein, and lipids by acidification (pH ~4.5) and filtration. Further refinement of the product may be completed using processes such as carbon refining. The refined solution is then concentrated with a vacuum evaporator for liquid maltodextrin products, or spray dried for powder maltodextrin products (Kennedy et al., 2012).

Powder and liquid forms of maltodextrin are available to use in foods and beverages as stabilizers, bulking agents, fat or sugar replacers, texture/mouthfeel enhancers, and/or carriers for granulated or encapsulated products (Ren et al., 2021). In addition to providing a multitude of functionalities, maltodextrin has a bland taste and contributes very little or no sweetness to products (Kennedy et al., 2012). These characteristics make maltodextrin a valuable food ingredient for manufacturers. As discussed in Sections 1.8 and 1.9, maltodextrin also functions as a reducing carbohydrate. Thus, maltodextrin can be used to modify proteins, via Maillard-induced glycation. Past studies have found that protein-maltodextrin conjugates have enhanced solubility, however, the conjugates are normally evaluated in an excess of maltodextrin (Kutzli, Griener, et al., 2020; Martinez-Alvarenga et al., 2014; Zha et al., 2020). Purification of the modified protein, or removal of unreacted carbohydrate, is needed.

1.10 Purification of partially-glycated (PG) protein

Removal of unreacted carbohydrate from the glycated and non-glycated (partially-glycated) proteins is necessary for several reasons. First, removal is needed to accurately evaluate protein functionality. When in the same system, carbohydrates have the potential to impact protein behavior by competing with protein for water interactions, potentially limiting protein solvation in beverage applications. Additionally, excess carbohydrate may impart its unique functionality, like adding viscosity and body, which may be undesirable in certain applications. Second, excess carbohydrate would allow for the Maillard reaction to continue and progress to the intermediate and advanced stages upon storage of the modified protein ingredient and the product. As discussed, it is imperative that the Maillard reaction is controlled to the early stage, to maximize protein functionality and quality. Lastly, excess carbohydrate in the protein ingredient would undermine its value as a "healthy" ingredient. Manufacturers typically add protein ingredients to products for both their functional and nutritional qualities; excess saccharide would "dilute" the protein content and add undesired, additional calories. Therefore, methods of removing unreacted carbohydrates need exploration.

While the overwhelming majority of Maillard-induced glycation studies do not purify proteins following glycation, a few have used filtration and chromatographic techniques for purification purposes. A number of studies have used membrane filtration, size exclusion chromatography, ion-exchange chromatography, and/or affinity chromatography to further purify glycated proteins (Allelein, Arunkumar, & Etzel, 2012; Bund, Allelein, Arunkumar, Lucey, & Etzel, 2012; Gu et al., 2009; Jiménez-Castaño et al., 2007; Qi, Yang, & Liao, 2009; Zhu, Damodaran, & Lucey, 2010). However, these studies focused on purification of glycated proteins from non-glycated proteins, not necessarily to remove unreacted saccharides; in fact, saccharide composition is rarely reported.

In one study, whey protein isolate (WPI) and dextran were mixed in a mass ratio of 1:3 (w/w) and subjected to wet-heating to produce WPI-dextran conjugates (Bund et al., 2012). Unreacted (whey) proteins were removed by isoelectric precipitation and the soluble whey protein-dextran and unreacted dextran components were partially separated

via cation exchange. The purified whey protein-dextran conjugate was reported to be about 50% protein (2x concentrated from initial mixture) and the remainder being dextran, although it is not clear whether the dextran fraction is fully linked to protein or partially free (Bund et al., 2012). This study also calculated only about 18% protein yield from their process. So, while these methods can help purify the protein-saccharide conjugate for further characterization, the protein yield can be quite low. Combining both glycated proteins and non-glycated proteins to produce a partially-glycated (PG) protein product, is of interest, as to reduce protein waste.

A PG-protein (WPI-dextran) with improved solubility was produced by Wang & Ismail (2012). In this study, WPI and dextran were initially mixed in a 1:4 mass ratio, subjected to Maillard-induced glycation, and the PG-protein was purified via hydrophobic interaction chromatography (HIC). The PG-protein had ~60% protein and ~30% carbohydrate, meaning the initial mix (1:4 mass ratio, 20% protein) was concentrated by a factor of three upon unreacted dextran removal (Wang & Ismail, 2012). This method of protein purification has promise, provided HIC fractionation produces high protein yields. However, the additional cost and time of this process must be considered. On a commercial scale, implementation of this process would likely reflect in an increased product cost. Although, the functionally-enhanced protein ingredient would, presumptively, have increased value in application. The increased functional value of a purified, PG-protein ingredient has the potential to offset additional purification processing costs.

1.11 Conclusions

As consumer demand for non-soy, plant protein is growing, the pea protein ingredient market has rapidly expanded in recent years. While the nutritional value of pea protein appears acceptable, the native protein structure and composition lend to functional challenges in application. These functional challenges are largely attributed to the poor solubility of pea protein, particularly following essential industrial processing steps involving thermal treatments. Poor functionality limits the application of pea protein in many products, but is markedly pronounced in RTD, high protein beverages due to the acidic environment and thermal processes used to manufacture these products.

However, the native pea protein structure can be modified to enhance its solubility and thermal stability. Controlled, Maillard-induced glycation is a protein modification technique that has the potential to improve pea protein solubility and thermal stability. Specifically, conjugation of protein with polysaccharides, rather than small saccharides, incurs the greatest solubility enhancement.

While pea protein has been glycated with several polysaccharides, the process has not been developed nor optimized for an industrial scale. Additionally, all previous studies have utilized exogenous saccharides (e.g., pectin, GA, and corn maltodextrin), which present concerns regarding application in "clean label" products. However, pea naturally contains significant amounts of starch that may be further processed to produce an endogenous reducing saccharide. Partial hydrolysis of pea starch is likely to produce polysaccharides, such as maltodextrins, which may react with pea protein under controlled glycation conditions. Thus, a pea protein-pea maltodextrin conjugate with enhanced solubility may be produced. Additional purification of these conjugates and non-glycated proteins has the potential to produce a highly soluble and valuable protein ingredient that is suitable for diverse applications. Therefore, development of a process to endogenously glycate pea protein and produce a purified ingredient is needed. Further structural and functional characterization of the purified, PG-pea protein ingredient is necessary to prove this concept has value in producing a protein ingredient with enhanced solubility and thermal stability for beverage applications.

Chapter 2: Materials and Methods

2.1 Materials

Yellow pea flour was provided by AGT Foods (Regina, SK, Canada) and commercial pea protein isolate (cPPI, ProFam® Pea 580, 79.5% protein) was provided by Archer Daniels Midland (ADM) (Decatur, IL, USA). These samples were stored at -20°C when not in use. Bacterial α-amylase (BAN® 480 LS, 528 KNU-B/g activity) was kindly provided by Novozymes North America, Inc. (Franklinton, NC, USA) and stored at 4°C. SnakeSkin™ dialysis tubing (3.5 kDa molecular weight cut-off (MWCO)), Sartorius Vivaflow® 200 Crossflow Cassettes (3 kDa MWCO), Corning™ transparent UV 96-well plates, silicone microplate mats, CarboPac™ PA100 ion-exchange column (4 x 250 mm) and accompanying guard column (4 x 50 mm), Imperial™ Protein Stain, a Pierce™ BCA assay kit, and a Pierce™ glycoprotein staining kit were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Criterion™ TGX™ 4-20% precast gels, Laemmli sample buffer, 10X Tris/Glycine/sodium dodecyl sulfate running buffer, and Precision Plus Protein™ molecular weight (MW) marker were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Octyl Sepharose™ 4 Fast Flow hydrophobic interaction chromatography (HIC) resin was purchased from GE Healthcare Bio-Sciences (Uppsala, Sweden). Aluminum crucibles (40 µL, with pin) for DSC were purchased from Mettler-Toledo (Columbus, OH, USA). Folded capillary cells for zeta potential were purchased from Malvern (Malvern, UK). Costar® opaque black 96-well plates, UV 96-well plates, polypropylene 96-well plates, o-phthaldialdehyde, and 8-anilino-1-naphthalenesulfonic acid (ANS) were purchased from MilliporeSigma (St. Louis, MO, USA). A Protein Digestibility Assay Kit (K-PDCAAS) was purchased from Megazyme International Co. (Bray, Ireland). All other chemicals were reagent grade or higher and were purchased from Thermo Fisher Scientific or MilliporeSigma.

2.2 Preliminary method development

Initially, the scope of this work involved *in situ*, Maillard-induced glycation of the proteins in pea flour. It was hypothesized that the starch granules in pea flour could be subjected to *in situ* enzymatic hydrolysis to produce a treated pea flour (TPF) containing maltodextrins, while largely maintaining the native structure of proteins within the flour.

Further, it was hypothesized that the TPF, containing both protein and maltodextrin (among other pea flour components), could be incubated under controlled external conditions to induce Maillard glycation. To test these hypotheses, preliminary testing was completed to produce endogenous maltodextrin, *in situ*. Ultimately, it was deemed that the conditions needed to hydrolyze native starch granules would inevitably lead to extensive denaturation and polymerization of the native legumin proteins, thereby likely limiting protein functionality (**Figure 5**, **Appendix A**). Additionally, the presence of high molecular weight (HMW) starch and fiber in the flour caused challenges with later protein purification following hydrophobic interaction chromatography (HIC). Accordingly, the method was adjusted to isolate protein from the other pea flour components (primarily starch and insoluble fiber, referred to as the starch-rich fraction) through a common, pH-based extraction method (Section 2.3) and separately hydrolyze endogenous starch granules to produce maltodextrin (Section 2.4).

2.3 Preparation of pea protein isolate and pea starch pellet

Native pea protein isolate (nPPI) and a starch-rich fraction were produced from yellow pea flour, following a pH-based extraction method (alkaline solubilization with isoelectric precipitation), as outlined by Bu et al. (2022). Pea flour was dispersed in a tenfold volume of double distilled water (DDW) and adjusted to pH 7.5 with 2 M NaOH. The dispersion was stirred for one hour at room temperature, then centrifuged at 5000 x g for 30 minutes to precipitate insoluble starch and fiber. The pellet was re-suspended in a tenfold volume of DDW and adjusted to pH 7.5 for another one hour solubilization, followed by centrifuging at 5000 x g for 30 minutes. The remaining pellet (starch-rich fraction, or pea starch pellet (PSP)) was collected and lyophilized. The supernatants from both solubilizations were combined and adjusted to the average pea protein isoelectric point (pH 4.5), followed by centrifuging at 5000 x g for 10 minutes to precipitate the protein. The protein pellet was re-suspended in DDW (1:4 w/v), neutralized, dialyzed against DDW with 3.5 kDa MWCO tubing, and lyophilized. The protein content of nPPI (86.0%) and PSP (2.8%) was determined by the Dumas method (AOAC 990.03), using a Leco® FP828 nitrogen analyzer (LECO, St. Joseph, MI, USA), with a conversion factor of 6.25.

2.4 Production of pea maltodextrin

Partial enzymatic hydrolysis of the lyophilized PSP was optimized to produce pea maltodextrin, with the goal of conjugating the maltodextrin with nPPI via Maillard-induced glycation and purifying the modified protein via HIC. Preliminary research found success with a commercial corn maltodextrin of ~10 dextrose equivalent (DE). Thus, hydrolysis conditions were optimized to produce a maltodextrin product from PSP with sufficient reducing power (approximately 10-20 DE) that was suitable for protein glycation.

The maltodextrin production procedure was optimized by evaluating the impact of several parameters (i.e., enzyme incubation time, methods of removing small saccharides, and the addition of a centrifugation step to remove large molecules) on maltodextrin DE. All trials began with preparing a PSP suspension by mixing 8.6 g PSP dry basis (d.b.) with 100 mL of 2 mM $CaCl_2$ DDW. The suspension was heated in a Brabender® Micro Visco-Amylo-Graph (MVAG) (C.W. Brabender® Instruments, Inc., Hackensack, NJ, USA) to gelatinize the starch granules and form a slurry by increasing the sample temperature to 95°C at a rate of 10°C/min, and holding at 95°C for 5 minutes. The slurry was transferred to a preheated 250 mL jacketed beaker and stirred on a magnetic stir plate until it was cooled to 75°C. The slurry was held at 75°C and continued stirring while 80 µL α-amylase (1.1% enzyme dose (g enzyme/g PSP d.b.)) was added with continued stirring. After the slurry was incubated with the enzyme for various times (5, 10, 20, 30, 40, 50, or 60 minutes), the enzyme was inactivated by adjusting the pH to 3.0 with 2 M HCl and holding for 5 minutes at 75°C. The sample was then cooled to room temperature on ice and adjusted to pH 7. The neutralized sample was either left as is or centrifuged at 5000 x g for 10 minutes to remove HMW PSP components. Additionally, samples were then dialyzed (3.5 kDa MWCO tubing) or ultrafiltered (3 kDa MWCO membranes) against DDW to remove small saccharides in some trials. Samples were lyophilized and stored at -20°C prior to analysis. From these initial trials, the final (optimized) maltodextrin production procedure was determined and repeated ~20 times to bulk the final maltodextrin product for glycation.

2.4.1 Maltodextrin Characterization

2.4.1.1 Dextrose equivalent by micro-Somogyi-Nelson

The dextrose equivalent (DE) of the produced maltodextrin was determined by the micro-Somogyi-Nelson assay using a dextrose standard curve (0.1-0.6 mM) (**Figure 6**, **Appendix B**) and as outlined by Shao & Lin (2018) with no modification. Reagents were prepared according to Somogyi and Nelson, with no modifications (Nelson, 1944; Somogyi, 1952). Solution I was prepared by dissolving sodium potassium tartrate tetrahydrate (7.5 g), sodium carbonate (15 g), sodium bicarbonate (10 g), and sodium sulfate (90 g) in DDW and diluting to 500 mL with DDW, while Solution II was prepared by dissolving copper sulfate pentahydrate (4 g) and sodium sulfate (36 g) in DDW and diluting to 200 mL with DDW. The arsenomolybdate color reagent was prepared by dissolving ammonium molybdate (25 g) in ~450 mL DDW and adding 21 mL concentrated sulfuric acid; this was followed by dissolving sodium arsenate dibasic pentahydrate (3 g) in 25 mL DDW and mixing with the ammonium molybadate solution. The color reagent was brought to a final volume of 500 mL with DDW and incubated at 37^{o}C for 36 hours, before storing in a brown bottle away from light. In triplicate, samples were prepared by weighing 10 mg maltodextrin in microcentrifuge tubes and dissolving in 1 mL DDW, followed by vortexing, stirring for 1 hour, and diluting 40 fold with DDW. Diluted samples and dextrose standards (0.1-0.6 mM) (45 µL) were loaded into polypropylene 96-well plates, along with DDW blank wells. The working reagent was freshly prepared by mixing four parts of Solution I and one part Solution II, then adding an aliquot (45 µL) to each well. The prepared plate was covered with a silicone microplate mat and taped with a layer of aluminum foil. The plate was shaken for 10 seconds to mix reagents and then heated in a boiling water bath for 20 minutes, according to the method developed by Shao & Lin (2018). After heating, the plate was cooled under running cold water for 5 minutes and arsenomolybdate color reagent (45 µL) was added to each well. The color was allowed to develop for 15 minutes. Prior to reading the absorbance, bubbles were removed by pipetting the solution in each well up and down several times. The absorbance of the plate was measured at 600 nm in a microplate reader (Biotek, Winooski, VT, USA) and the DE was calculated using **Equation 1**. A sample calculation is found in **Appendix B**.

Equation 1. Dextrose equivalent.

$$Dextrose\ equivalent = \frac{dextrose_{sample}\ (g)}{sample\ (g)} * 100$$

Where:

"$dextrose_{sample}$ (g)" = the mass of dextrose in the sample, determined from dextrose standard curve

"sample (g)" = the mass of the sample

2.4.1.2 Chain-length distribution by High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD)

The chain-length distribution of maltodextrin produced, produced following the optimized procedure, was completed based on the method of Okyere, Boakye, Bertoft, & Annor (2022) with some adjustments in sample preparation. Maltodextrin (2.0 mg) was dissolved in 90% DMSO (100 µL), in septuplicate, and stirred overnight at room temperature. The solution volume was adjusted by adding 90% DMSO to obtain a final concentration of 2 mg maltodextrin/mL and subsequently filtered through a 0.45 µm nylon filter. A filtered aliquot (25 µL) was analyzed by high-performance anion-exchange chromatography (HPAEC) using a Dionex™ ICS-5000+ HPAEC system (Dionex Corporation, Sunnyvale, CA, USA) coupled with a pulsed amperometric detector (PAD), CarboPac™ PA100 ion-exchange column (4 x 250 mm) and accompanying guard column (4 x 50 mm). The samples were eluted at a flow rate of 1 mL/min and following a gradient elution using 150 mM sodium hydroxide (eluent A) and 150 mM sodium hydroxide containing 500 mM sodium acetate (eluent B) as follows: 0-9 min, 15-36% B; 9-18 min, 35-45% B; 18-110 min, 45-100% B; 100-112 min, 100-15% B; and 112-130 min, 15% B (Annor, Marcone, Bertoft, & Seetharaman, 2014; Okyere et al., 2022). Areas under peaks were manually integrated and corrected to carbohydrate concentration according to Koch, Andersson, & Åman (1998) and the average degree of polymerization was estimated according to Bertoft, Piyachomkwan, Chatakanonda, & Sriroth (2008). Ultrapure (18.2 MΩ-cm) water was used for preparation of all eluents and samples (Barnstead™ Smart2Pure™ water purification system, Thermo Fisher Scientific

Inc., Waltham, MA, USA). A sample chromatogram (**Figure 7**) and calculations are shown in **Appendix C**.

2.5 Preparation of partially-glycated pea protein isolate (PG-PP)

Native pea protein isolate (nPPI) was mixed with maltodextrin in a 1:4 ratio (w/w), dissolved in potassium phosphate buffer (0.01 M, pH 7) in a 1:4 ratio (w/v) of powder (g) to buffer (mL). The buffer solution was adjusted to pH 7.0 and lyophilized. The lyophilized powder (nPPI and maltodextrin, or nPPI+MD) was transferred to petri dishes and evenly spread in an approximately 0.0415 g/cm^2 thick layer, in at least triplicate. The samples in petri dishes were incubated in a constant climate chamber (HPP260, Memmert®, Büchenbach, Germany) set at 49.0% relative humidity (RH) and 60^{o}C for 24 hours to initiate and limit the Maillard reaction (Walter, Greenberg, Sriramarao, & Ismail, 2016; Wang & Ismail, 2012). Glycation pre-trials were run for 24 and 48 hours, and the 24-hour incubation time was chosen to initiate the Maillard reaction, while limiting the loss of free amino groups. Following incubation, the protein content of partially-glycated pea protein (PG-PP) samples (17.8%) was determined by the Dumas method and the samples were stored at -20^{o}C when not in use.

2.5.1 Assessment of glycation extent

2.5.1.1 Color analysis

The color of all protein samples (nPPI, nPPI+MD, PG-PP) was measured in at least triplicate, using a Chroma Meter CR-221 (Minolta Camera Co., Osaka, Japan), as outlined by Bu et al. (2022). The colorimeter was calibrated with a white CR-221 calibration plate (Minolta) prior to sample analysis and measurements were recorded using the CIE (International Commission on Illumination) 1976 L* a* b* color system, where L* indicates lightness on a scale from 0 (black) to 100 (white); positive a* values represent redness; negative a* values represent greenness; positive b* values represent yellowness; while negative b* values represent blueness. Equal amounts of sample were weighed in petri dishes to obtain similar sample thickness for all readings. Sample photos were taken for reference (**Figure 8**, **Appendix D**).

2.5.1.2 Percent free amino groups

Percent of free amino groups and percent loss in free amino groups after incubation was determined for all protein samples (nPPI, nPPI+MD, PG-PP) based on the o-phthaldialdehyde (OPA) method outlined by Goodno, Swaisgood, & Catignani (1981), with a few modifications in sample and reagent preparation. For each sample, 1% (w/v) solutions were prepared in 3% sodium dodecyl sulfate (SDS) (w/v), in at least triplicate. The samples were shaken for 1 hour and then centrifuged at 15,682 x g for 10 minutes. The supernatants of nPPI samples were diluted 10-fold and those of nPPI+MD and PG-PP samples were diluted 2.5-fold with 3% SDS. The protein content of the diluted supernatant was determined using the Pierce™ BCA assay kit following the manufacturer's instructions, using a bovine serum albumin (BSA) standard curve (**Figure 9**, **Appendix E**). L-lysine standard solutions (5-125 µg/mL) were prepared in 3% SDS. The OPA reagent was prepared by dissolving sodium tetraborate (2.012 g) and SDS (0.5 g) in DDW, followed by adding OPA (0.080 g) dissolved in ethanol (4 mL), adding βME (200 µL) and bringing to 100 mL with DDW. Diluted samples and lysine standards (50 µL) were loaded onto a UV 96-well microplate, along with 3% SDS blank wells. The OPA reagent (200 µL) was added to each well and the absorbance was read at 340 nm on a microplate reader (Biotek, Winooski, VT, USA). The free amino group concentration (µg/mL) of each sample was based on the L-lysine standard curve (**Figure 10**, **Appendix E**) and adjusted for sample protein content (µg/mL) to calculate the percent (%) free amino groups, as shown in **Equation 2**. Percent (%) loss in free amino groups of PG-PP was determined by **Equation 3**. A sample calculation is shown in **Appendix E**.

Equation 2. Percent (%) free amino groups.

$$\%\ Free\ amino\ groups = \frac{Free\ amine\ content\ (\frac{ug}{mL})}{Protein\ content\ (\frac{ug}{mL})} * 100$$

Where:

Free amine content (µg/mL) = calculated from L-lysine standard curve

Protein content (µg/mL) = calculated from BSA standard curve of the BCA Assay

Equation 3. Percent (%) loss in free amino groups.

$$\%\ Loss\ in\ free\ amino\ groups = \frac{\%\ free\ amino\ groups_{nPPI} - \%free\ amino\ groups_{PG-PP}}{\%\ free\ amino\ groups_{nPPI}} * 100$$

2.5.1.3 Protein and glycoprotein profiling by gel electrophoresis

The protein and glycoprotein profiling of all protein samples (nPPI, nPPI+MD, PG-PP) was performed using sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) as outlined by Boyle, Hansen, Hinnenkamp, & Ismail (2018), with modification in sample preparation. Samples were dispersed in DDW (0.4% protein w/v) for 1 hour. Laemmli buffer was added to a sample aliquot in a 1:1 ratio to analyze the sample under non-reducing conditions. To evaluate the sample under reducing conditions, a ratio of 1:0.95:0.05 of sample to Laemmli buffer to β-mercaptoethanol (βME) was used. Both samples, under non-reducing and reducing conditions, were heated in a boiling water bath for five minutes and cooled to room temperature. Precision Plus Protein™ MW marker (9 µL) and samples (5 µL, ~10 µg protein) were loaded onto a Criterion™ TGX 4-0% precast Tris-HCl gradient gel and electrophoresed at 200V. Two gels were run at the same time. One gel was stained for protein with Imperial™ Protein Stain (Coomassie brilliant blue R-250) and destained with DDW. The other gel was stained for glycoprotein with the Pierce™ glycoprotein staining kit (periodic acid-Schiff method) and destained with 3% acetic acid and DDW. Both gels were imaged using the Molecular Imager Gel XR system (Bio-Rad Laboratories).

2.6 Removal of unreacted maltodextrin and protein purification by hydrophobic interaction chromatography (HIC)

Removal of unreacted maltodextrin from the protein (PG-PP) was completed using HIC. A Shimadzu Scientific Instruments high-performance liquid chromatography (HPLC) system, equipped with a LC-6AD pump system, SPD-20AV UV/Vis detector, and CMB-20A communication module (Shimadzu Corp., Kyoto, Japan), was used, along with a GE HiScale™ 50/20 column (GE Healthcare Bio-Sciences, Uppsala, Sweden). The column was packed with Octyl Sepharose™ 4 Fast Flow HIC resin up to an approximately 7.5 cm bed height (~150 mL total column volume (CV)) and equilibrated

with 2 M ammonium sulfate solution (pH
7.0).

To complete initial, small-scale production, the column was fitted with 20 µm net rings and the PG-PP sample was prepared for injection by dispersing PG-PP (0.5 g) in 10 mL of 1 M ammonium sulfate. The resulting ~1% protein (w/v) dispersion was adjusted to pH 8 to ensure solubilization and stirred at a medium speed for 1 hour. Following washing of the column with 3 CV DDW and equilibration with 2 CV 2 M ammonium sulfate, an aliquot (8 mL) of the dispersed sample was injected onto the column and run at a flow rate of 15 mL/min with UV detection set at 280 nm. The sample was first eluted with a 2.5 CV 2 M ammonium sulfate wash to remove unreacted carbohydrates, followed by a 2.5 CV DDW wash, and a 3 CV 0.1 M NaOH wash/cleaning. This procedure was repeated 30 times to gather a sufficient amount of protein for preliminary compositional characterization. The DDW fraction was collected to capture water-soluble proteins (glycated and non-glycated), adjusted to pH 7.0, and transferred to dialysis tubing (3.5 kDa MWCO tubing). The sample was dialyzed against DDW at 4^{o}C, changing the dialysate every 2-4 hours over the span of ~2 days and using a ratio of ~350 mL dialysate:1 mL sample. Lastly, the dialyzed DDW fraction was lyophilized and the resulting powder was stored at -20^{o}C. This product is referred to as the small-scale purified, water-fraction of PG-PP (PW-PG-PP).

Efforts to increase PW-PG-PP output involved scaling-up the removal of unreacted maltodextrin by fitting the column with 80 µm net rings and increasing the amount of protein injected onto the column. The PG-PP sample was prepared at approximately 3% protein (w/v) by dispersing PG-PP (1.5g) in 10 mL 1 M ammonium sulfate, adjusting to pH 8, and stirring for 1 hour. The column was washed and equilibrated as previously detailed, and an aliquot (8 mL) of the 3% protein sample was injected at a flow rate of 15 mL/min with UV detection set at 280 nm. The sample was first eluted with a 3 CV 2 M ammonium sulfate wash to remove unreacted carbohydrates, followed by a 3 CV DDW wash, and a 3 CV 0.1 M NaOH wash/cleaning. The volume of ammonium sulfate and DDW washes was increased to account for the increased amount of PG-PP injected. This procedure was repeated until enough protein was collected for all structural and functional testing (approximately 30 times). As with small-scale

production, the DDW fraction (PW-PG-PP) was collected, adjusted to pH 7.0, dialyzed, lyophilized, and stored at -20^{o}C. Additionally, the 0.1 M NaOH fractions from 6 runs were collected and treated as PW-PG-PP fractions were, to evaluate differences in the protein structure between the two fractions. The lyophilized powder is referred to as the purified, NaOH fraction of PG-PP (PN-PG-PP). Removal of unreacted carbohydrates from PG-PP was monitored by collecting 10-minute interval fractions of eluent and measuring the total carbohydrate content (glucose equivalent). The total carbohydrate content in each fraction was determined according to the phenol-sulfuric acid method outlined by Nielsen (2017), diluting the fraction aliquots in DDW as necessary and using a glucose standard curve (10-50 µg/mL) (**Figure 11**, **Appendix F**). A sample calculation of total carbohydrate content is shown in **Appendix F**.

Furthermore, nPPI was purified according to the scale-up HIC method detailed above, to serve as a purified protein control to the partially-glycated sample. The DDW fractions of approximately 30 nPPI injections (8 mL of 3% protein) were collected and treated as detailed above. The resulting powder is referred to as the purified, DDW-fraction of nPPI (PW-PPI). The 0.1 M NaOH fractions from 6 of these runs were collected and treated as the DDW fractions were, to evaluate differences in protein structure. This product is referred to as the purified, NaOH fraction of nPPI (PN-PPI). Sample chromatograms of PG-PP and nPPI runs are available in **Appendix G** (**Figures 12** and **13**).

2.7 Characterization of purified protein

2.7.1 Protein, total carbohydrate, and ash content

The protein contents of reference samples (cPPI, nPPI) and purified samples (PW-PPI, PN-PPI, PW-PG-PP, PN-PG-PP) were determined following the Dumas method. The total carbohydrate content, expressed as percentage total carbohydrates (glucose equivalent), was determined according to the phenol-sulfuric acid method outlined by Nielsen (2017), diluting the samples in DDW as necessary and using a glucose standard curve (10-50 µg/mL). Ash content was determined using the AOAC dry ashing method (AOAC 942.05). All analyses were run in duplicate.

2.7.2 Protein and glycoprotein profiling by gel electrophoresis

The protein and glycoprotein profiling of all samples (cPPI, nPPI, PW-PPI, PN-PPI, PG-PP, PW-PG-PP, PN-PG-PP) was performed using SDS-PAGE, as described in Section 2.5.1.2.

2.7.3 Thermal denaturation by differential scanning calorimetry (DSC)

The protein denaturation temperature and enthalpy of samples (cPPI, nPPI, PW-PPI, PW-PG-PP) were determined using a DSC instrument (DSC 1 STARe System, Mettler Toledo, Columbus, OH, USA), according to the method outlined by Bu et al. (2022) without modification. Samples, in triplicate, were dispersed in DDW (20% protein, w/v) and stirred overnight at room temperature. An aliquot of each sample (20 µL, delivering approximately 4 µg protein) was transferred to an aluminum pan, sealed, and analyzed alongside an empty, sealed reference pan. The pans were held at 25^{o}C for 5 minutes, then heated at a rate of 5^{o}C/min to 110^{o}C. Thermograms were recorded and peaks were manually integrated to obtain the denaturation temperature (peak) and enthalpy of denaturation for each sample using Mettler Toledo's STARe Software version 11.00. Sample thermograms (**Figure 14**) are shown in **Appendix H**.

2.7.4 Protein surface properties

The surface hydrophobicity of all samples was determined using a spectrofluorometric method that utilizes an 8-anilino-1-naphthalenesulfonic acid (ANS) probe, as outlined by Bu et al. (2022), with no modification. In triplicate, 0.05% protein (w/v) samples (20 mL) were prepared, solubilized in 0.017 M:0.165 M citric acid/sodium phosphate buffer (pH 7), and stirred for two hours. Protein solutions were diluted with the citric acid/sodium phosphate buffer in concentrations ranging from 0.005-0.050% protein (w/v). Sample (200 µL) were loaded into a 96-well black opaque well plate, along with buffer blank wells. The relative fluorescence index (RFI) was measured at excitation and emission wavelengths of 400/30 (wavelength/bandwidth) and 460/40 nM, respectively, and 40 gain using a microplate reader (Biotek, Winooski, VT, USA). The ANS probe solution (20 µL, 12.6 µg/mL) was added to all wells, followed by covering the plate with aluminum foil and allowing the plate to sit for 15 minutes before measuring RFI again.

Net RFI was plotted against percent protein concentration and the slope was used as an index of protein surface hydrophobicity. A sample calculation and plot (**Figure 18**) is shown in **Appendix I**.

Zeta potential was measured using a dynamic light scattering instrument (Malvern Nano Z-S Zetasizer), following the method outline by Bu et al. (2022), at both 3.4 and 7.0 pH levels. Protein solutions (0.1% protein, w/v) were prepared in DDW, in triplicate, and stirred for two hours. The pH of each sample was adjusted to pH 3.4 or 7.0 prior to analysis. An aliquot (1 mL) of each solution was dispensed into a folded capillary cell and inserted into the Zetasizer. After equilibrating for 30 seconds, the electrophoretic mobility was measured by three sub-rep readings taken every 10 seconds for each replicate. Zeta potential was determined using Malvern's Zetasizer software (version 7.13) following the Smoluchowski model.

2.7.5 Protein secondary structures by attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR)

ATR-FTIR spectra of samples (cPPI, nPPI, PW-PPI, PW-PG-PP) were recorded using a Fourier transform infrared spectrometer (Thermo Scientific™ Nicolet™ iS50 FTIR) following the method outlined by Bu et al. (2022), with no modification. Aliquots (~400 mg) of the powder samples were placed in a desiccator for no less than 36 hours to dehydrate the sample and minimize moisture interference with the instrument measurements. The samples were placed on a diamond ATR and scanned from 400-4000 cm^{-1} by DLaTGS detector. The resulting ATR spectra were converted to transmission spectra using OMNIC® software and the second derivative of the Amide I band (1600 cm^{-1} to 1700 cm^{-1}) was obtained by PeakFit v.412 software to identify secondary structures and determine the distribution of α-helix, β-sheet, β-turn, and random coil structures. A sample original spectrum (**Figure 19**) and a sample second derivative of Amide I band (1600 cm^{-1} – 1700 cm^{-1}) (**Figure 20**) are shown in **Appendix J**.

2.7.6 Protein solubility and thermal stability

The solubility of samples (cPPI, nPPI, PW-PPI, PW-PG-PP) was determined using the method described by Bu et al. (2022), with modification in the pH value. In

order to assess the suitability for acidic, high-protein beverages, the samples were evaluated at pH 3.4, rather than pH 7.0. Protein solutions (5% protein in DDW, w/v) were prepared, in triplicate, and stirred for one hour at room temperature. The pH was adjusted to 3.4 using 2 M HCl and allowed to stir for another hour at room temperature. The samples were adjusted to pH 3.4, as necessary, before placing aliquots (1 mL) into microcentrifuge tubes. Aliquots were assessed at room temperature and after thermal treatment (80ºC for 30 minutes). Both heated and non-heated samples were centrifuged at 15,682 x g for 10 minutes. The protein content of the supernatants and initial protein solutions were determined following the Dumas method. Protein solubility was expressed as the percentage of soluble protein (present in the supernatant) compared to the total protein (present in the initial sample), as shown in **Equation 4**. A sample calculation is shown in **Appendix K**.

Equation 4. Percent (%) solubility.

$$\%\ solubility = \frac{\%\ protein_{supernatant}}{\%\ protein_{initial}} * \ 100$$

2.7.7 Protein digestibility

The *in vitro* protein digestibility of samples (cPPI, nPPI, PW-PPI, PW-PG-PP) was determined using a commercial Protein Digestibility Assay Kit (K-PDCAAS) from Megazyme International Co., Bray, Ireland) and partial analysis of amino acid composition (**Table 6**, **Appendix L**). Partial amino acid (all amino acids besides cysteine, methionine, and tryptophan) analysis was performed by the Agricultural Utilization Research Institute (AURI®, Marshall, MN, USA) using a modified, performic acid oxidation with acid hydrolysis-sodium metabisulfite method (AOAC 996.12).

2.8 Statistical analysis

Analysis of variance (ANOVA) and t-tests were performed using IBM SPSS Statistics software version 27.0 for Windows (SPSS Inc., Chicago, IL, USA). Tukey-Kramer Honest Significant Difference (HSD) multiple means comparison test was used to determine significant differences ($P \leq 0.05$) among means (**Tables 8**-**28**, **Appendix**

M). Two-sample, unpaired t-tests were used to determine significant differences ($P \leq 0.05$) between the means of two different samples.

Chapter 3: Results and Discussion

3.1 Targeted production of maltodextrin

With all other experimental conditions kept constant, hydrolysis time, use of ultrafiltration or dialysis (small saccharide removal), and centrifugation (to remove large molecular weight starch and fiber) were each evaluated by measuring their effect on the resulting maltodextrin DE. At first (trial 1), only the effect of starch hydrolysis time (10-60 minutes in 10-minute increments) was evaluated without small saccharide removal or centrifugation. As expected, the maltodextrin DE increased with increasing reaction time (**Table 1**). Increasing the reaction time allowed for the α-amylase to further hydrolyze α-(1→4) glycosidic linkages of starch chains, thereby creating more chains with reducing ends and increasing DE over time (Yusraini, Hariyadi, & Kusnandar, 2013). The 10-minute reaction time was chosen for the next trial, as a DE of 10-20 was the target, and further removal of small saccharides was likely to reduce the DE. This DE range was targeted to slow the Maillard reaction rate, while limiting incubation time. Zha et al. (2020) found that glycation of PPI was more controlled with maltodextrins than with small saccharides (glucose and lactose), as small saccharides have greater reducing power than maltodextrin. Maltodextrins of DE 10 and 18 also induced greater improvement in protein solubility and thermal stability than small saccharides.

Secondly (trial 2), the effect of dialysis and ultrafiltration (UF) on maltodextrin DE was evaluated, in an effort to reduce the small saccharide (i.e., mono- and disaccharides) content and the resulting DE. Small saccharides increase the rate of the Maillard reaction and its progression to the undesirable, advanced stages (Zha et al., 2020). Small saccharides are more reactive due to their size and increased relative abundance of reactive carbonyl groups, compared to larger oligo- and polysaccharides (Zha, Gao, et al., 2021). Therefore, removal of small saccharides was necessary. Both dialysis and UF significantly reduced the initial DE, with no significant differences between the two (**Table 1**). In both of these processes, smaller sugars passed through semipermeable membranes (3.5 kDa or 3 kDa size pores), effectively reducing the small saccharide content and the DE. Rather than UF, dialysis was chosen to add to the maltodextrin production procedure simply due to the relative ease of the dialysis process.

Table 1. Dextrose equivalent of maltodextrin production as affected by starch hydrolysis time (trial 1), ultrafiltration and dialysis (trial 2), and centrifugation (trial 3).

Starch Hydrolysis[1]			**Removal of Small Carbohydrates**		**Reducing power**
Trial	Time	Centrifugation	Ultrafiltration	Dialysis	DE[2]
1	10	No	No	No	24.1^{a3D4}
	20	No	No	No	28.7^{b}
	30	No	No	No	33.0^{c}
	40	No	No	No	40.5^{d}
	50	No	No	No	49.8^{e}
	60	No	No	No	64.0^{f}
2	10	No	No	No	21.0^{bC}
	10	No	Yes	No	13.5^{aA}
	10	No	No	Yes	14.6^{aAB}
3	5	Yes	No	Yes	14.6*
	10	Yes	No	Yes	16.2^{B}

[1] Hydrolysis completed on gelatinized pea starch paste at 75°C with 1.1% enzyme (g enzyme/g starch-rich by-product (dry basis)) in all trials; [2] Dextrose equivalent; [3] Lowercase letters indicate significant differences among means ($n \geq 3$) within each of Trial 1 and 2 according to the Tukey-Kramer multiple means comparison test ($P < 0.05$); [4] Uppercase letters indicate significant differences among means ($n \geq 3$) within 10-minute hydrolysis times according to the Tukey-Kramer multiple means comparison test ($P < 0.05$); * Designates a significant difference between means in Trial 3 as tested by a two-sample unpaired t-test ($P < 0.05$).

Lastly (trial 3), an adjustment to the hydrolysis reaction time, in conjunction with the addition of a centrifugation step, was evaluated. Centrifugation was investigated as a method to remove high molecular weight (HMW) components (namely starch and fiber) present in the PSP that originated from pea flour. Preliminary trials revealed challenges with sample injection through the HIC system, likely due to the presence of insoluble, HMW PSP components (e.g., large starch molecules and insoluble fiber) (data not shown). With centrifugation and removal of these HWM components, the maltodextrin DE was expected to increase as DE was determined on a mass basis, i.e., the relative amount of reducing saccharides in a given mass would increase. Thus, a 5-minute hydrolysis time was investigated to produce maltodextrin with a lower DE, alongside the 10-minute time previously tested. The 5-minute hydrolysis time with centrifugation produced maltodextrin with a significantly lower DE than the 10-minute hydrolysis with centrifugation, similar to the trend observed in trial 1 (**Table 1**). As expected, the addition

of the centrifugation step significantly increased the maltodextrin DE, as HMW compounds were sedimented and removed from the soluble maltodextrin chains (**Table 1**). The 5-minute hydrolysis time, therefore, was chosen as the final hydrolysis time, as the maltodextrin product had a DE in the mid-range of the targeted values (10-20 DE).

A bulk maltodextrin sample was then produced and characterized by determining its DE and chain-length distribution. Although the repeated process had a slightly higher DE (15.7) than the sample produced during the initial trial (**Table 1**), the values were close and within the targeted range, and the difference was only ~5%, which is within expected experimental variation. Further, the maltodextrin chain-length distribution (**Figure 2**) revealed that nearly 75% of the chains fell between 2-20 DP, with an average of approximately 8.3 (~1.3-1.5 kDa). The term "maltodextrin" refers to a starch hydrolysis product composed of maltooligosaccharides of primarily 2-20 DP, with an average DP >5, and between 3-20 DE (Huber & BeMiller, 2017; Kennedy et al., 2012). Therefore, the developed process was successful in producing a maltodextrin product with the desired characteristics.

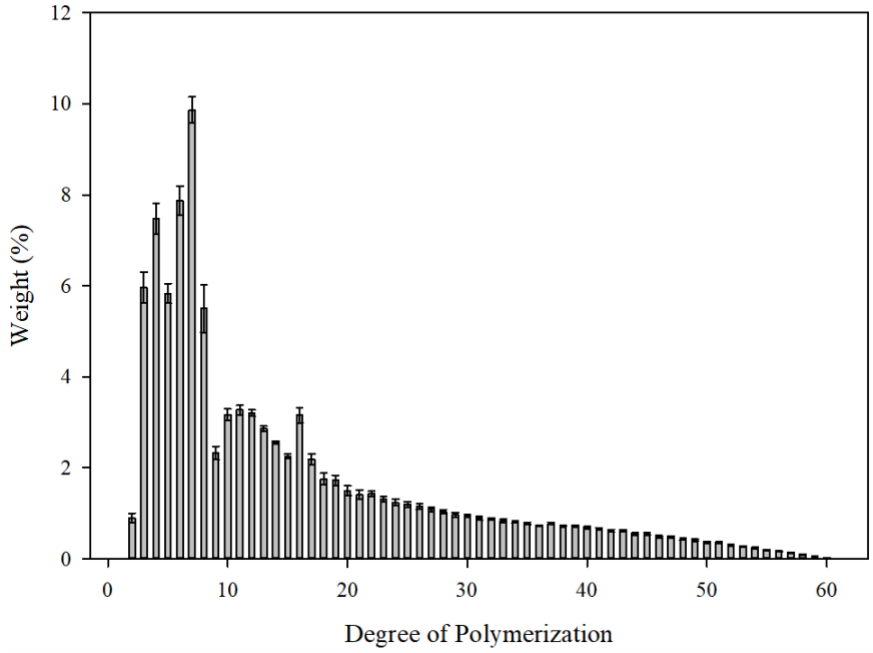

Figure 2. Chain-length distribution of maltodextrin as determined by HPAEC-PAD. Error bars represent standard error (n = 7).

3.2 Impact of Maillard-induced glycation on key characteristics

3.2.1 Effect of Maillard-induced glycation on color

Mixing of nPPI with maltodextrin resulted in a product (nPPI+MD) with a slightly lower lightness (L*), along with slightly less green (a*) and yellow (b*) color when compared to nPPI (**Table 2**). While nPPI was analyzed as a reference, nPPI+MD prior to incubation was analyzed as a control to monitor the extent of the Maillard reaction. A significant yet modest decrease in lightness (L*) was observed following incubation, with no significant differences in a* or b* values (**Table 2**). Although the lightness of PG-PP was significantly lower than that of nPPI+MD, the difference is numerically very small and visually indiscernible (**Figures 8a-d**, **Appendix D**). This slight decrease could be attributed to minor physicochemical changes during incubation (Cruz-Tirado, Martins, Olmos, Condotta, & Kurozawa, 2021). Larger decreases in lightness (e.g., increased browning) are typically observed when protein-carbohydrate mixtures are incubated for longer times under more severe environmental conditions (e.g., 79% relative humidity and 80^{o}C) that favor higher Maillard reaction rates (Martinez-Alvarenga et al., 2014; Zha, Dong, et al., 2019b; Zha et al., 2020). Browning of various glycated pea protein products has been observed in previous studies utilizing dry-heating conditions (79% RH, 60^{o}C, ≤5 days; 75% RH, 70^{o}C, ≤24 hours) or wet-heating conditions (80^{o}C, ≤24 hours), especially upon increased incubation time (Kutzli, Griener, et al., 2020; Zha, Dong, et al., 2019b; Zha et al., 2020). However, the mild dry-heating conditions used in this experiment (49% RH, 60^{o}C, 24 hours) appeared to have limited the progression of the Maillard reaction to advanced stages. While all previous studies of pea protein glycation have either lacked color data or observed browning, this study found limited change in color, both visually and by L*a*b* measurement. In the present study, limited and partial glycation of pea protein with endogenous pea maltodextrin was achieved following a controlled approach that mitigated undesirable reaction products, potentially enhancing the resulting protein quality and functionality.

3.2.2 Effect of Maillard-induced glycation on percent free amino groups

Free amino groups content was monitored before and after incubation to assess the extent of Maillard glycation. The was no statistical difference in free amino content of

nPPI and nPPI+MD, as expected (**Table 2**). Upon incubation for 24 hours, the free amino groups content significantly decreased, equating to a 29.6% loss in free amino groups in PG-PP compared to nPPI (**Table 2**). This loss was attributed to maltodextrin chains covalently linking to available ε-amino groups of lysine residues in the early stage of the Maillard reaction (Zha, Gao, et al., 2021). Comparable results were reported by Zha, Dong, et al. (2019b) when conjugating pea protein concentrate with gum Arabic, which resulted in approximately 20% loss in free amino groups after 24 hours of incubation at 79% RH and 60°C, and 25.9% after 72 hours. Further, the extent of free amino group loss in the present study is relatively moderate, as other studies have found much greater levels of blockage (up to ~60%) when employing more intense Maillard reaction conditions (e.g., 65-80°C, 70-79% RH) and more reactive carbohydrates (e.g., mono- and disaccharides or higher DE maltodextrin) (Kutzli, Griener, et al., 2020; Zha, Dong, et al., 2019b; Zha et al., 2020). Moreover, loss of free amino groups sometimes could occur because of protein polymerization upon incubation at high temperatures.

Table 2. Color (L* a* b*) and free amino groups (%) of native pea protein isolate (nPPI), combined nPPI and maltodextrin before incubation (nPPI+MD), and partially-glycated pea protein (PG-PP).

		Color			**Free Amino Groups**
Samples		L*	a*	b*	Free Amino Groups (%)
	nPPI	86.75^{c1}	-0.36^{b}	+20.00^{b}	6.88^{b}
Before Incubation	nPPI+MD	84.62^{b}	-1.91^{a}	+15.14^{a}	6.77^{b}
After Incubation	PG-PP	82.69^{a}	-1.84^{a}	+15.78^{a}	4.84^{a}

[1] Lowercase letters indicate significant differences among means ($n \geq 3$) in each column according to the Tukey-Kramer multiple means comparison test ($P < 0.05$).

High loss in free amino groups is undesirable, as large polysaccharides and protein cross-linking can limit the access of digestive enzymes to the protein, thereby reducing protein digestibility and nutritional value (Nooshkam et al., 2020; Tuohy et al., 2006). Additionally, lysine is an essential amino acid and the primary site of glycation on proteins, contributing to a concern for proteins that have lysine as the limiting amino acid (e.g., oat, rice, wheat) (de Oliveira et al., 2016; Rutherfurd et al., 2014). Lysine, however, is not the limiting amino acid in pea protein, so glycated pea protein with moderate levels

of lysine loss (approximately <50%) may mitigate loss in protein quality. In this study, a balance between a sufficient level of glycation and moderate loss in free amino groups was met.

3.2.3 Effect of Maillard-induced glycation on protein and glycoprotein profiles

SDS-PAGE was performed to confirm glycation and to monitor protein profile changes upon mixing nPPI with maltodextrin and upon incubation. The protein profiles of nPPI and nPPI+MD were similar under both non-reducing and reducing conditions (**Figures 3a** and **3b**; Lanes 2-3 and 5-6). All major protein subunits (convicilin, legumin (acidic and basic), and vicilin subunits) were present in both samples with similar respective band intensity and proportion (Barac et al., 2010; Lam et al., 2018). However, glycoprotein staining (non-reducing conditions) revealed the presence of HMW glycoproteins (>250 kDa) in both nPPI+MD and PG-PP samples (**Figures 3c**; Lanes 11-12). Since no major differences in nPPI and nPPI+MD protein profiles were observed, these HMW glycoproteins in both nPPI+MD and PG-PP could be attributed to residual proteins in the maltodextrin produced from the PSP, that could have formed conjugates during the production of maltodextrin. The thermal treatments (i.e., gelatinization, hydrolysis, enzyme inactivation) involved in the production of maltodextrin may have induced the Maillard reaction, resulting in the glycation of residual PSP proteins. The glycated PSP proteins potentially remained soluble and in the maltodextrin supernatant following centrifugation, thus were present in both nPPI+MD and PG-PP.

Furthermore, SDS-PAGE visualization confirmed that glycation occurred upon the incubation of nPPI+MD. All major protein subunits (convicilin, legumin (acidic and basic), and vicilin subunits) shifted to a higher molecular weight distribution upon glycation, visible under both non-reducing (**Figure 3a**; Lane 4) and reducing conditions (**Figure 3b**; Lane 8), and confirmed by glycoprotein staining (**Figure 3c**; Lane 12). Higher molecular weight bands confirm glycation, as conjugation of native protein subunits with maltodextrin chains would result in glycoprotein molecules with greater molecular weight. Moreover, glycation was also confirmed by the presence of longitudinal smearing within PG-PP lanes, as the molecular weights of glycated subunits would have been heterogeneously distributed. The broadness of glycoprotein molecular

weight banding depends upon the size and number of chains linked to each protein subunit, as maltodextrin is composed of maltooligosaccharides with varying chain lengths. Previous studies noted similar increases in molecular weight and heterogeneous distribution of glycated proteins (Kutzli, Griener, et al., 2020; Walter et al., 2016; Wang & Ismail, 2012). Additionally, the glycated convicilin, vicilin, and legumin acidic subunit bands had greater intensity and more elevated molecular weight than the glycated legumin basic subunit band (**Figures 3a** and **3b**; Lanes 4 and 8). Similarly, Walter et al. (2016) found minimal change in the banding of the 11S (glycinin) basic subunit in partially-glycated soy protein, compared to the 7S and 11S acidic subunits. The particular amino acid composition and protein conformation impact the availability of reactive amine groups that can participate in the Maillard reaction. The higher lysine content of 7S vicilin and 8S convicilin subunits may allow for these subunits to more readily form conjugates with maltodextrin chains than the 11S legumin subunits (Lam et al., 2018; Zha, Gao, et al., 2021).

Additionally, no heavy banding was observed at the top of the PG-PP lane under non-reducing conditions (**Figure 3a**; Lane 4), contrary to previous observations where pea protein polymerization and/or the linkage of large polysaccharides (i.e., gum Arabic) have occurred under more severe glycation conditions (e.g., 65-80^{o}C, 70-79% RH) (Kutzli, Griener, et al., 2020; Zha, Dong, et al., 2019b; Zha et al., 2020). In this study, the absence of heavy banding suggested that the mild conditions used to initiate the Maillard reaction resulted in limited propagation to advanced stages, thus, prevented the formation of HMW protein polymers that may have low solubility. Given the minimal change in color, moderate loss in free amino groups, and lack of protein polymerization in PG-PP, it is concluded that the Maillard reaction was successfully controlled to the early stage. The targeted Amadori products were formed, as pea proteins were glycated with endogenous pea maltodextrin.

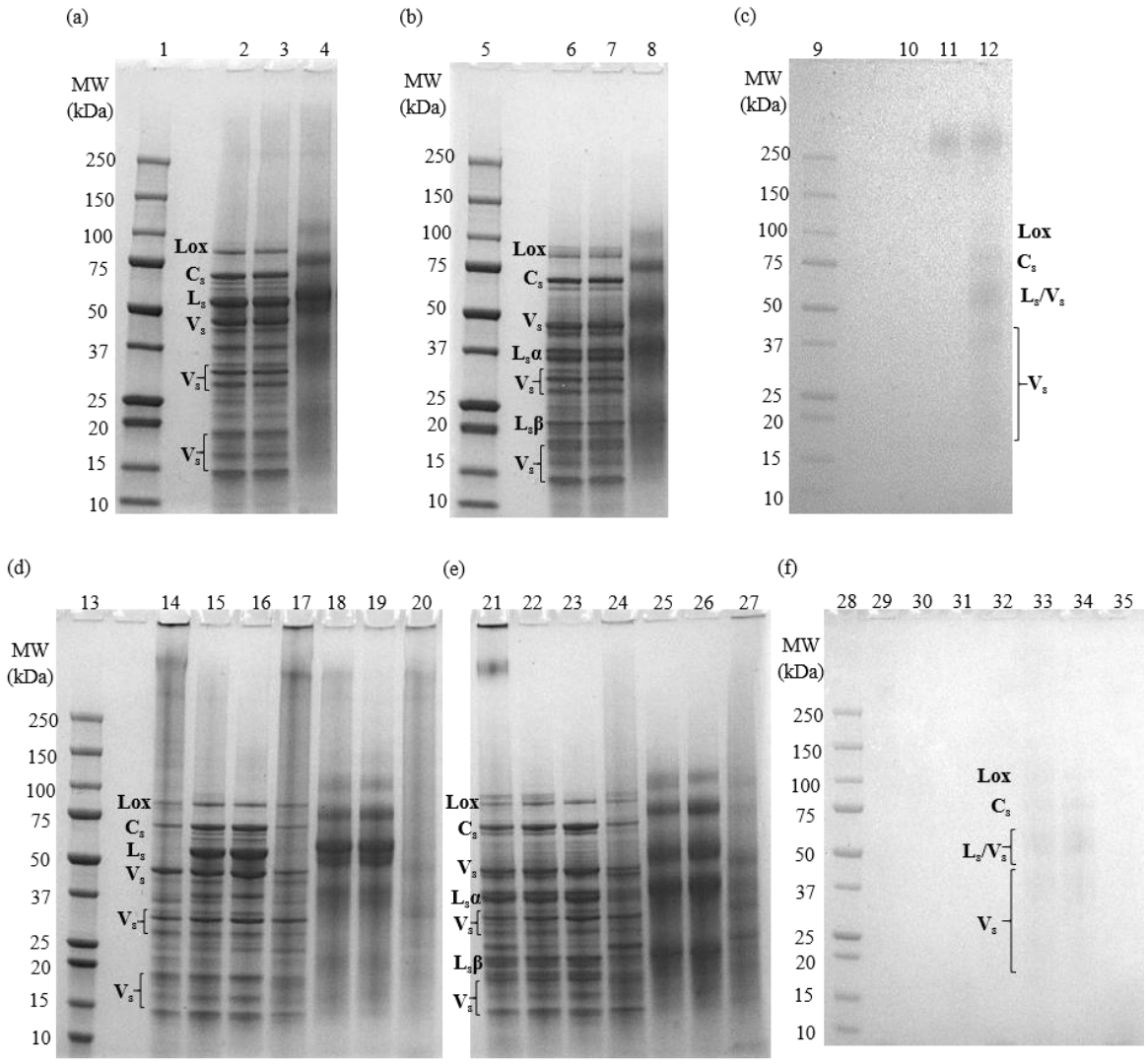

Figure 3. SDS-PAGE visualization of the protein profiles of samples under non-reducing (a, d) and reducing (b, e) conditions using Coomassie staining, and glycoprotein profiles (c, f) of samples under non-reducing conditions using periodic acid-Schiff staining. Lanes 1, 5, 9, 13, 28: Molecular weight (MW) marker; Lanes 2, 6, 10, 15, 22, 30: nPPI; Lanes 3, 7, 11: nPPI+MD before incubation; and Lanes 4, 8, 12, 18, 25, 33: PG-PP after incubation; Lanes 14, 21, 29: cPPI; Lanes 16, 23, 31: PW-PPI; Lanes 17, 24, 32: PN-PPI; Lanes 19, 26, 34: PW-PG-PP; Lanes 20, 27, 35: PN-PG-PP. Lox: lipoxygenase; C_s: subunits of convicilin; L_s subunits of legumin; V_s: subunits of vicilin; $L_s\alpha$: acidic peptides cleaved from legumin subunits; $L_s\beta$: basic peptides cleaved from legumin subunits.

3.3 Evaluation of HIC-purified protein

3.3.1 Separation of unreacted maltodextrin from protein

Separation of unreacted maltodextrin from PG-PP was needed to obtain a higher purity protein sample, as the original incubated sample was composed of ~20% protein and ~80% maltodextrin (w/w). During HIC purification, unreacted maltodextrin was eluted during the first ~35 minutes, while PW-PG-PP was collected between 35 and 60 minutes and PN-PG-PP collected between 60 and 90 minutes. Total carbohydrate content of 10-minute interval fractions of eluent was monitored (**Figure 4**). Fractions 1-3 (0-30 minutes, 2M ammonium sulfate wash) contained the majority of the recovered maltodextrin (~74%), while fractions 4-6 (30-60 minutes, DDW wash) contained ~26% of the recovered maltodextrin, and fractions 7-9 (60-90 minutes, 0.1M NaOH wash/cleaning) contained <1% (**Figure 4**). As intended, most unreacted maltodextrin was eluted with the 30 minute ammonium sulfate wash and, presumably, the first 5 minutes of the DDW wash collected in fraction 4 as protein was not detected until ~5 minutes into the DDW wash (**Figure 12**, **Appendix G**). The remaining maltodextrin recovered in fractions 4-6 was attributed to unreacted maltodextrin that was not removed during the first ~35 minutes of elution and to reacted maltodextrin chains covalently linked to proteins, confirmed by preliminary trials (data not shown). Wang & Ismail (2012) found a similar distribution of recovered dextran from PG-whey protein purified by a similar HIC method.

However, this is the first time that a purified PG-PP product has been produced by removing unreacted carbohydrates. It is vital to remove unreacted carbohydrates and produce a purified protein ingredient for a variety of reasons. From an industry perspective, removal is needed to manufacture a high purity protein ingredient that has commercial value as a healthy ingredient, since excess carbohydrates would dilute the protein content and contribute additional calories to a product with a targeted protein claim. Excess carbohydrates may also impact the protein functionality (e.g., compete with protein for water) or impart their own undesired, functionality in certain products (e.g., viscosity and body) (Kennedy et al., 2012). Importantly, the Maillard reaction has the potential to progress to the advanced stage over storage when unreacted

carbohydrates are present, due to potential temperature abuse and moisture gain (Rao, Rocca-Smith, Schoenfuss, & Labuza, 2012).

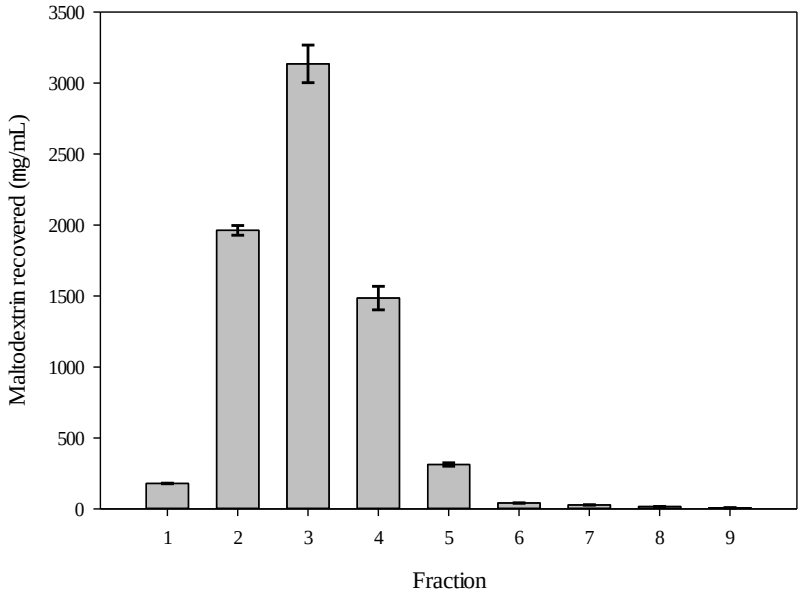

Figure 4. Maltodextrin recovered from 10-minute interval fractions collected during hydrophobic interaction chromatographic removal of unreacted maltodextrin from partially-glycated pea protein (PG-PP). Error bars represent standard error (n=2).

3.3.2 Composition of purified protein and controls

The composition of purified PG-PP, purified PPI, and reference samples (cPPI and nPPI) was determined. The total carbohydrates and ash contents of both reference samples were relatively low (**Table 3**). The slight compositional difference between nPPI and cPPI could be attributed to presumed differences in extraction protocol. The protein content, which was the major component, was similar to those reported previously for PPI (Bu et al., 2022; Cui et al., 2020; Stone et al., 2015).

Prior to scaling-up the production of purified PG-PP (PW-PG-PP) for comprehensive evaluation, small-scale purification (~1% protein (w/v) injections) produced a PW-PG-PP sample of 70.6% protein and 22.3% carbohydrate (w/w). However, the scaled-up purification of PW-PG-PP required injections with higher protein % (~3% protein (w/v) injections). The resulting PW-PG-PP had lower protein purity (56.2%) and higher carbohydrate content (40.9%) compared to the small-scale sample

(**Table 3**). The reduced protein purity and increased carbohydrate content of the scaled-up PW-PG-PP compared to the small-scale sample was most likely due to residual unreacted maltodextrin, resulting from incomplete removal. Moreover, the distribution of recovered maltodextrin from scaled-up purification (**Figure 4**) provided further proof that a relatively high amount maltodextrin continued to elute during first 10 minutes of the DDW wash (**Figure 4**; fraction 4). This observation could be attributed to the higher amount of protein and carbohydrates injected onto the column, increased by a factor of 3 (e.g., 0.4 g PG-PP injected during small-scale purification vs. 1.2 g PG-PP injected during scaled-up purification). The scaled-up purification would benefit from increasing the column volume and/or increasing the 2M ammonium sulfate wash volume to ensure a more complete removal of unreacted carbohydrates, thereby producing a PW-PG-PP sample with higher protein purity. Regardless, the protein and carbohydrate content of PW-PG-PP produced by both small-scale and scale-up purifications was similar to that of PG-whey protein (~60% and 30% (w/w), respectively) purified by a similar HIC method (Wang & Ismail, 2012).

Table 3. Protein, carbohydrate, and ash contents of commercial pea protein isolate (cPPI), native pea protein isolate (nPPI), HIC purified nPPI (water (PW-PPI) and NaOH fractions (PN-PPI)), and HIC purified partially-glycated pea protein (water (PW-PG-PP) and NaOH fractions (PN-PG-PP)).

Sample	Protein (%)*	Total carbohydrates (%)*	Ash (%)*
cPPI	79.5[d2]	6.81[b]	5.74[d]
nPPI	86.0[e]	2.84[a]	3.82[c]
PW-PPI	100.0[f]	1.47[a]	0.72[a]
PN-PPI	59.5[c]	N/A[3]	N/A
PW-PG-PP	56.2[b]	40.91[c]	1.07[b]
PN-PG-PP	50.4[a]	N/A	N/A

[1] Percent (%) composition on a wet basis; [2] Lowercase letters indicate significant differences among means (n = 2) in each column according to the Tukey-Kramer multiple means comparison test ($P < 0.05$); [3] Not available, data not collected.

Furthermore, the composition of PW-PG-PP differed from that of PN-PG-PP and that of the purified PPI fractions (PW-PPI and PN-PPI), produced as controls. PW-PG-PP had a significantly higher protein content than PN-PG-PP (**Table 3**). While carbohydrate and ash content were not assessed in PN-PG-PP due to limitations in sample size, it can be assumed that the remaining components of PN-PG-PP were primarily ash and carbohydrate. Similarly, PW-PPI also had a significantly higher protein content than PN-PPI (**Table 3**). Additionally, it is important to note that a protein conversion factor of 6.25 was routinely utilized in protein content calculations across all samples. This standard protein conversion factor did not account for the differences in protein profile following purification. As will be discussed in Section 3.3.3, the protein profiles of purified water and NaOH fractions changed upon purification by HIC (**Figures 3d** and **3e**). Therefore, the nitrogen content of each of the purified samples might have differed. Accordingly, the protein content calculated using the 6.25 conversion might not have reflected the true value, which can explain the 100% protein content of PW-PPI.

3.3.3 Protein and glycoprotein profiles of purified protein and controls

Differences in the protein and glycoprotein profiles of the purified protein were noted compared to reference samples (nPPI and cPPI). Highly polymerized proteins were present in the cPPI reference sample, noted by excessive smearing in the upper part of the gel (MW >250 kDa) and dark banding at the top of the gel under non-reducing conditions (**Figure 3d**; Lane 14). Under reducing conditions (**Figure 3e**; Lane 21), the smearing was less apparent, but HMW, intense banding at the top of the gel remained, indicating possible covalent linkages, other than disulfide bonds, might have contributed to protein polymerization. The severe processing conditions (e.g., heat exposure, physical processing) used to produce cPPI likely induced polymerization, which could reduce its functional value (Bu et al., 2022; Shand et al., 2007). Conversely, polymerization was minimal in the nPPI reference sample, as minimal smearing was apparent in the HMW region; protein polymers were resolved under reducing conditions, indicating disulfide interactions were the primary drivers of polymerization (**Figures 3d** and **3e**; Lane 15 and 22). With a low occurrence of protein polymerization and the native globular protein structures largely intact, nPPI could have better functionality than cPPI.

The nPPI sample was further purified by HIC, with resulting PW-PPI and PN-PPI samples serving as controls to the purified PG-PP samples. The protein profile of PW-PPI was similar to that of nPPI, with all major protein subunits (convicilin, legumin (acidic and basic), and vicilin subunits) present in both samples with similar band intensity and proportion (**Figures 3d** and **3e**, Lanes 15-16 and 22-23) (Lam et al., 2018). In contrast to nPPI, however, no HMW smearing was observed in PW-PPI, as the more polymerized and hydrophobic proteins interacted longer with the HIC column, preventing elution in the water fraction during the purification. Additionally, minor bands, attributed to more hydrophobic vicilin subunits and other various proteins (primarily <50 kDa), were missing from the PW-PPI sample, but observed in the PN-PPI sample (**Figures 3d** and **3e**, Lanes 15-17 and 22-24). The PN-PPI sample also contained more polymerized protein than nPPI and PW-PPI, noted by HMW smearing and dark banding at the top of the gel under non-reducing conditions. Under reducing conditions, much of the intense staining and dark banding was no longer evident, although light smearing along the lane was still present; this indicated that the protein polymerization observed in PN-PPI was largely the result of disulfide interchange, with minor contributions from other covalent linkages (**Figure 3e**; Lane 24). As expected, the NaOH wash removed the more polymerized and hydrophobic proteins that strongly associated with the HIC media during the initial washes. The NaOH wash caused ionization and increased charge on the hydrophobic proteins, therefore, increasing their hydrophilicity and allowing them to elute (O'Connor & Cummins, 2017). Moreover, the PN-PPI sample appeared to have more polymerized protein than nPPI, which may have resulted from the relatively increased concentration of polymerized protein in PN-PPI.

Importantly, removal of unreacted maltodextrin from PG-PP produced the PW-PG-PP sample of interest, as well as PN-PG-PP for reference. The PW-PG-PP protein profile closely mirrored that of PG-PP, with all major, glycated globulin subunits present in both samples with similar band intensity and proportion (**Figures 3d** and **3e**; Lanes 18-19 and 25-26). The presence of these glycated proteins in both PG-PP and PW-PG-PP was further confirmed by glycoprotein staining (**Figure 3f**; Lanes 33-34). The similar protein and glycoprotein profiles suggested that most glycated proteins in PG-PP were water soluble, as they were hydrophilic, eluting in the water fraction. The structural and

functional characteristics of PW-PG-PP will be discussed in the following sections. Additionally, a notable loss in HMW smearing from the PG-PP sample was observed in PW-PG-PP, rather, appearing in the PN-PG-PP sample (**Figures 3d** and **3e**; Lanes 18-20 and 25-27). The polymerized and more hydrophobic proteins in the upper lane area of PG-PP interacted with the HIC media during the ammonium sulfate and water washes, only eluting once the NaOH wash interfered with these hydrophobic interactions (O'Connor & Cummins, 2017). These protein polymers were formed by both disulfide and other covalent linkages, as smearing and a HMW band at the top of the gel remained in the PN-PG-PP sample under reducing conditions (**Figure 3e**; Lane 27). PN-PG-PP clearly contained unreacted convicilin, vicilin, and legumin subunits, as well, evident by the banding observed under reducing conditions. Although, the banding corresponding to these subunits appeared more broad in PN-PG-PP than in the nPPI sample (**Figure 3e**; Lanes 22 and 27). The more broad banding and presence of smearing align with the presence of glycated proteins in PN-PG-PP, although to a lesser degree than in PW-PG-PP. It was noted that no glycoproteins were clearly evident in PN-PG-PP, however. This suggested that the majority of glycated proteins eluted in the water wash, and the concentration of glycoproteins in PN-PG-PP was probably below the threshold of the stain (**Figure 3f**; Lane 35).

HIC purification of PG-PP was successful in increasing the sample's protein content, while eliminating most of the hydrophobic, polymerized proteins and concentrating the hydrophilic, glycated proteins. Accordingly, the unique protein and glycoproteins profiles of these purified samples could have major implications on the protein structural and functional characteristics. As this study intended to investigate the ability of a novel glycation method to enhance pea protein solubility and thermal stability, while producing a purified sample, the structural characteristics impacting these properties were further investigated and discussed in the following sections.

3.4 Structural characterization of purified protein

3.4.1 Protein denaturation

Protein denaturation properties greatly influence protein functionality (Damodaran, 2017). Importantly, Maillard-induced glycation has the potential to enhance

protein thermal stability by inducing partial denaturation and increasing the thermal denaturation temperature (Wang & Ismail, 2012). Several studies have reported these changes in protein denaturation upon glycation, however, the particular effect of glycation depends on the protein type and Maillard reaction conditions (Pirestani et al., 2018; Wang & Ismail, 2012; Zha, Gao, et al., 2021; Zha et al., 2020). In this study, the denaturation state, as monitored by measuring the enthalpy of denaturation (ΔH), and thermal denaturation temperatures (T_d) of reference (cPPI and nPPI) and purified (PW-PPI and PW-PG-PP) samples were investigated to assess the impact of glycation and HIC purification processes on the protein's thermal stability.

No endothermic peaks were observed in the cPPI reference, indicating complete denaturation (**Figure 14**, **Appendix H**). As Bu et al. (2022) reported, the severe commercial processing conditions used to produce cPPI led to protein denaturation and subsequent polymerization (**Figure 3d**; Lane 14). Conversely, two endothermic peaks were observed in the nPPI sample, corresponding to vicilin and legumin proteins. The T_d of each peak and their combined ΔH aligned with the values reported by Bu et al. (2022) (**Table 4**). While the nPPI vicilin and legumin peaks slightly overlapped, two distinct peaks were clearly identifiable (**Figure 15**, **Appendix H**). However, the vicilin and legumin endothermic peaks in purified PPI and PG-PP samples were less distinct (**Figures 16-17**, **Appendix H**). PW-PP and PW-PG-PP endothermic peaks overlapped to a greater degree than nPPI peaks, ultimately appearing as one peak. Therefore, the total ΔH of nPPI, including both vicilin and legumin peaks, was determined in order to compare the denaturation state of nPPI to the HIC purified samples. Additionally, the midpoint temperature of the single (i.e., overlapping) peak was deemed the T_d for each of the purified samples.

Table 4. Denaturation temperatures and enthalpy, surface hydrophobicity, surface charge, and secondary structures of commercial pea protein isolate (cPPI), native pea protein isolate (nPPI), HIC purified nPPI (water (PW-PPI) and NaOH fractions (PN-PPI)), and HIC purified partially-glycated pea protein (water (PW-PG-PP) and NaOH fractions (PN-PG-PP)).

Samples	Denaturation Temperature and Enthalpy				Surface Properties			Secondary Structure			
					Surface Hydrophobicity	Surface Charge		ATR-FTIR			
	Denaturation Temperature			Enthalpy of Denaturation[1]		pH 3.4	pH 7.0	α Helix	β Sheet	β Turn	Random Coil
	T_d, °C			ΔH, J g-1	RFI	mV	mV	Relative Percentage			
	Vicilin	***Legumin***	***Vicilin/Legumin***[2]								
cPPI	~	~	~	~	13822^{c}	+23.0^{d}	-32.6^{c}	16.6^{a}	37.5^{a}	32.2^{b}	13.8^{c}
nPPI	82.2	90.4	N/A[3]	7.92^{a}[4]	9245^{b}	+30.1^{e}	-37.1^{d}	20.6^{b}	43.6^{b}	31.3^{b}	4.5^{a}
PW-PPI	N/A[3]	N/A	86.9*	10.54^{b}	4715^{a}	+23.3^{d}	-23.0^{b}	18.5^{ab}	47.0^{c}	28.2^{a}	6.3^{b}
PN-PPI	N/A	N/A	N/A	N/A	12140^{c}	+11.5^{b}	-21.2^{b}	N/A	N/A	N/A	N/A
PW-PG-PP	N/A	N/A	83.4	7.82^{a}	6237^{a}	+20.5^{c}	-21.5^{b}	18.4^{ab}	45.0^{b}	30.7^{ab}	5.9^{ab}
PN-PG-PP	N/A	N/A	N/A	N/A	9000^{b}	+7.0^{a}	-17.9^{a}	N/A	N/A	N/A	N/A

~ No peak of denaturation observed; [1] Total enthalpy of denaturation; [2] Unable to distinguish vicilin and legumin peaks; [3] Not applicable, data not collected; [4] Lowercase letters indicate significant differences among means (n = 3) in each column according to the Tukey-Kramer multiple means comparison test ($P < 0.05$); * Designates a significant difference between means in Vicilin/Legumin T_d as tested by a two-sample unpaired t-test ($P < 0.05$).

The ΔH of the PW-PPI endothermic peak was significantly larger than the total ΔH of the nPPI endothermic peaks (**Table 4**). The increased ΔH of PW-PPI reflected its unique protein profile resulting from HIC purification of nPPI. More energy was required to unfold PW-PPI than nPPI, as PW-PPI consisted of fewer denatured (and subsequently polymerized) proteins and a relatively higher concentration of native proteins (**Figure 3d**; Lanes 15-16). Furthermore, the T_d of the PW-PPI endothermic peak falls within the range of the vicilin and legumin peaks observed in nPPI, as expected. As more peak overlapping was observed in PW-PPI, the observed T_d of PW-PPI reflects the average T_d of these major proteins. Endothermic peak overlapping may be the result of changes in the protein profile after HIC purification and the potential presence of ammonium sulfate in PW-PPI, compared to nPPI, as protein homogeneity and the presence of stabilizing salts have previously been associated with changes in thermal transitions in pea protein isolates (Mession et al., 2013; Shand et al., 2007). While the denaturation temperature of nPPI did not increase upon purification, the removal of denatured and polymerized proteins could alter PW-PPI structural properties and favor enhanced functionality, namely solubility.

Similar to PW-PPI, only one endothermic peak was discernible in PW-PG-PP, as major endothermic peaks of partially-glycated proteins overlapped. The ΔH of PW-PG-PP was similar to that of nPPI, but significantly lower than that of PW-PPI (**Table 4**). Maillard-induced glycation is expected to partially denature proteins (Wang et al., 2013; Wang & Ismail, 2012; Zha et al., 2020), however, purification of nPPI resulted in an increased ΔH, as PW-PPI served as a control to PW-PG-PP. Therefore, a balance between protein denaturation and removal of denatured and polymerized proteins via HIC purification (**Figure 3d**; Lanes 18-19) might have maintained the ΔH of PW-PG-PP, in comparison to nPPI. In this study, the combination of glycation and HIC purification had no effect on ΔH, while others found that glycation and HIC purification of whey protein decreased its ΔH (Wang et al., 2013; Wang & Ismail, 2012). The decreased ΔH of PG-whey protein was attributed to changes in protein secondary and tertiary structure upon glycation. Moreover, the T_d of PW-PG-PP was significantly lower than that of PW-PPI, but within the range of vicilin and legumin peaks observed in nPPI (**Table 4**). Denaturation temperature differences between the purified samples could be attributed to

the differences in protein secondary structures, as observed in past studies (Pirestani et al., 2017, 2018; Wang et al., 2013). Evidently, the T_d of PW-PG-PP remained close to that of nPPI peaks, although the PW-PG-PP endothermic peak was more broad and less defined than the vicilin and legumin peaks of nPPI (**Figure 17**, **Appendix H**). While other studies have found that glycation increases T_d, the persistent T_d observed in this study may be attributed to the distinct Maillard reaction conditions, substrates, and composition of PW-PG-PP (including a unique protein, carbohydrate, and ash/ammonium sulfate content) compared to other glycated samples (Pirestani et al., 2018; Wang & Ismail, 2012).

DSC results from this study confirmed that HIC purification of water soluble proteins increased the ΔH of the purified sample by removing denatured and polymerized proteins with no observed impact on the denaturation temperature. This is the first study to evaluate the effect of pea protein glycation and HIC purification on protein denaturation properties. Reduction in denatured/polymerized protein content could enhance the purified protein functionality, namely solubility, for beverage applications.

3.4.2 Protein surface properties

Protein denaturation exposes hydrophobic resides that are typically buried within the interior moiety of globular proteins. Hence, unfolding of globular protein structures typically increases surface hydrophobicity, which directly impacts the manner in which protein molecules interact with other molecules and in turn, protein functionality. Despite the unfolding that typically occurs upon Maillard-induced glycation, glycation reduces protein surface hydrophobicity through the covalent linkage of hydrophilic carbohydrates (de Oliveira et al., 2016; Wang & Ismail, 2012; Zha, Gao, et al., 2021). Surface hydrophobicity has not been directly investigated much in past glycation studies, however, as partially glycated proteins are rarely purified. Additionally, the effect of HIC purification, in particular, on the structure and functionality of PG proteins is not well-researched, having only been applied to PG-whey protein in the past (Wang et al., 2013; Wang & Ismail, 2012). In HIC, proteins separate on the basis of hydrophobicity. Therefore, it is expected that less hydrophobic proteins elute with the water wash, while more hydrophobic proteins interact with the column media, eluting upon ionization

induced by an NaOH wash. As HIC is capable of removing excess carbohydrates, while purifying hydrophilic proteins, further investigation of HIC purification of glycated proteins is warranted. In this study, the effect of glycation and HIC purification on protein surface hydrophobicity were directly investigated.

The net surface hydrophobicity of nPPI was significantly lower than that of cPPI, similar to the observations of Bu et al. (2022) (**Table 4**). As discussed, cPPI was potentially exposed to severe commercial processing conditions, leading to protein unfolding and exposure of the internal, hydrophobic core, further complimenting the results of protein profiling and extent of denaturation (**Table 4**, **Figure 3d**; Lane 14). The milder extraction conditions used to prepare nPPI resulted in less denaturation, compared to cPPI, and consequently a lower surface hydrophobicity (**Table 4**). Furthermore, HIC purification of nPPI produced PW-PPI and PN-PPI samples with significantly different surface hydrophobicity. PW-PPI had nearly half of the net surface hydrophobicity of nPPI, while PN-PPI had a significantly higher surface hydrophobicity than nPPI (**Table 4**). The HIC purification process fractionated the proteins in nPPI into the more hydrophilic proteins (PW-PPI) and the more hydrophobic proteins (PN-PP), aligning with the principles of HIC (O'Connor & Cummins, 2017). These results are also in agreement with protein profiling observations that PW-PPI was less denatured and polymerized than nPPI and PN-PPI (**Table 4**, **Figure 3d**; Lanes 15-17). The PW-PPI surface hydrophobicity was even notably lower than the surface hydrophobicity of whey protein isolate, which is known for its high surface hydrophilicity and solubility (Wang & Ismail, 2012). Consequently, these results demonstrated that HIC fractionation of pea protein can produce hydrophilic and hydrophobic protein fractions, each with potentially increased value for different applications. For example, the hydrophilic fraction can be utilized for beverage applications and the hydrophobic fraction for applications that rely on protein-protein interactions, such as meat alternatives.

Glycation coupled with HIC purification resulted in significantly lower surface hydrophobicity of PW-PG-PP, compared to nPPI (**Table 4**). However, the surface hydrophobicity of PW-PG-PP was not significantly different form that of PW-PPI. Nonetheless, HIC successfully fractionated the proteins in PG-PP into hydrophilic (PW-PG-PP) and more hydrophobic (PN-PG-PP) fractions, as observed with nPPI purification.

The difference in surface hydrophobicity between water and NaOH fractions most likely resulted in the observed differences in the degree of protein polymerization (**Figure 3d**; Lanes 18-20). Higher surface hydrophobicity enhances attractive forces among protein molecules and facilitates further interactions via disulfide linkages. Accordingly, the more denatured and polymerized proteins eluted with the NaOH wash, explaining the higher surface hydrophobicity of PN-PG-PP than PW-PG-PP (**Table 4**). The surface hydrophobicity of PN-PG-PP was significantly lower than that of PN-PPI, but similar to nPPI. As discussed, PN-PG-PP may have contained partially-glycated proteins, although minimally compared to PW-PG-PP. The combination of unreacted, denatured/polymerized, and partially-glycated proteins could explain the lower surface hydrophobicity of PN-PG-PP compared to PN-PPI (**Figures 3d** and **3e**; Lanes 17, 20, 24, and 27). Glycation appeared to have reduced the surface hydrophobicity of even the more hydrophobic proteins, which could impact their functionality and provide added-value to a combined glycation and HIC purification commercial protein modification process.

Similar to surface hydrophobicity, surface change (zeta potential, ζ) is an important surface property that directly influences protein interactions and functionality. Specifically, charged residues on the protein surface cause repulsive electrostatic forces among protein molecules, enhancing protein-water interactions and consequently protein solubility (Damodaran, 2017). Glycation and HIC purification processes have the potential to alter the net surface charge of proteins by altering the intrinsic protein characteristics, such as protein profile, denaturation state, composition, and intermolecular interactions. Additionally, protein surface charge is directly dependent upon the pH, therefore, this study evaluated the surface charge of reference and purified samples under acidic and neutral conditions. Since protein solubility and thermal stability testing was performed at an acidic pH (3.4) to mimic RTD beverage conditions, the acidic ζ measurement was taken at pH 3.4, specifically.

The surface charge of nPPI and that of cPPI, at both pH levels, were similar to previously reported values (Bu et al., 2022; Ladjal-Ettoumi, Boudries, Chibane, & Romero, 2016). However, regardless of pH level, all purified samples (PW-PPI, PN-PPI, PW-PG-PPI, PN-PG-PP) had less net surface charge than nPPI (**Table 4**). In addition, the surface charge of cPPI was similar or greater than that of the purified samples, despite the

fact that cPPI was the most denatured and polymerized (**Table 4**, **Figure 3d**; Lane 14). Residual ammonium sulfate in the HIC fractionated samples might have shielded charges on the surface of the protein, ultimately reducing the observed surface charge load (Lam et al., 2018). This effect was similarly observed by Bu et al. (2022) in undialyzed, modified PPI samples containing salts, as well by Bogahawaththa et al. (2019) in PPI solutions with added sodium chloride. While fractionated samples in this study were dialyzed and had relatively low ash levels (PW-PPI and PW-PG-PP <2% ash, **Table 2**), residual ammonium sulfate would not have been detected by the dry ashing method, as it decomposes above 280ºC. The residual ammonium sulfate was likely minimal, but enough to neutralize ionizeable groups (e.g., carbonxyl and amino groups) (Zhu, Huang, & Chen, 2022).

Although charge shielding by residual salts might have occurred in all HIC fractions, NaOH fractions, specifically, had the lowest surface charges at pH 3.4 (**Table 4**). This finding is in agreement with their protein profiles, as PN-PPI and PN-PG-PP appeared to consist of more denatured and polymerized proteins than the water-fractions (**Figure 3d**; Lanes 15-20). Additionally, purified PG-PP fractions had significantly lower surface charges than their respective purified PPI counterparts at pH 3.4 (**Table 4**). The covalent linkage of maltodextrin chains in purified PG-PP fractions blocked some of the ε-amino groups of lysine residues that would otherwise be protonated at pH 3.4, thus, contributing to the lower net positive charge of purified PG-PP fractions compared to their purified PPI counterparts. Wang & Ismail (2012) similarly noted that the isoelectric point of whey protein was reduced upon glycation, due to the blockage of amino groups. Additionally, glycation might have led to a reduction in the measured ζ of the purfieid PG-PP fractions, as the matlodextrin layer at the surface of the protein might have shielded charges on the protein, as Chen, Chen, Wu, & Yu (2016) observed in peanut protein isolate-maltodextrin conjugates.

3.4.3 Protein secondary structures

To further monitor the effects of glycation and HIC purification on protein structure, the relative abundance of protein secondary structures (i.e., α-helix, β-sheet, β-turn, and random coil) was assessed in reference and purified water fractions. cPPI had

the lowest relative abundance of α-helix and β-sheet structures and the highest abundance of random coil structures (**Table 4**). The severe commercial processing conditions that cPPI was likely exposed to resulted in the denaturation at the secondary structure level, as observed by Bu et al. (2022). Produced on a lab-scale, nPPI was exposed to milder extraction and drying conditions than cPPI, retaining a significantly larger share of α-helix and β-sheet structures. Further, compared to nPPI, PW-PPI had a significantly higher relative abundance of β-sheet, with relatively small differences in β-turn and random coil structures (**Table 4**). These results could be attributed to the change in protein profile and increased enthalpy of denaturation, as denatured/polymerized proteins were not present in the PW-PPI fraction (**Table 4**, **Figure 3d**; Lanes 15-17). On the other hand, glycation coupled with HIC purification did not result in significant differences in secondary structures between nPPI and PW-PG-PP. Minimal changes in protein secondary structure could explain minimal differences in PW-PG-PP ΔH compared to that of nPPI (**Table 4**). However, others have found an increased abundance of β-sheet structures upon glycation (Li, Xue, Chen, Ding, & Wang, 2014; Pirestani et al., 2017; Wang et al., 2013). The effect of glycation on secondary structures can potentially depend on the particular substrates, Maillard reaction conditions, and purification protocol that may alter the protein profile, if purified. High thermal stability is associated with a high relative abundance of β-sheets, although other protein structural components and environmental conditions impact the ability of proteins to withstand thermal treatment, as well (Damodaran, 2017). These minimal changes in the secondary structures upon glycation and purification might not have a direct bearing on functionality, namely solubility and thermal stability.

3.5 Impact of Maillard-induced glycation and HIC-purification on protein solubility and thermal stability

Protein ingredients must have high solubility and thermal stability to incorporate well into many foods, but especially high-protein, RTD beverages. To ensure product safety, beverages undergo thermal processes, which could be detrimental to the flavor, texture, and color. Therefore, acidification might be employed to reduce the severity of the thermal treatment. However, acidification is often detrimental to the product quality

and shelf life, as proteins-protein interactions are induced upon heating at acidic conditions, near their isoelectric point. With low solubility, plant proteins have a tendency to polymerize and sediment from solution over storage (Lu et al., 2020; Vogelsang-O'Dwyer et al., 2021). A lack of beverage stability, observed through sedimentation, is undesirable to consumers both visually and sensorially. Therefore, the effect of glycation and purification on pea protein solubility and thermal stability (heating at 80^{o}C for 30 minutes) at pH 3.4 was investigated, with reference to the native and commercial counterparts.

Heating of the 5% protein solutions significantly increased the solubility of all protein samples, as Bu et al. (2022) also observed (**Table 5**). However, exposing proteins to elevated temperatures typically results in the unfolding of proteins and exposure of buried hydrophobic groups, ultimately decreasing protein solubility. As Bogahawaththa et al. (2019) found, though, the particular time/temperature and environmental conditions of different thermal treatments can uniquely impact protein solubility. Therefore, the increased protein solubility observed upon heating in this study may be related to the particular heating and environmental conditions used. Under these conditions, partial unfolding of proteins by the disruption of hydrogen bonding and electrostatic interactions might have increased the amount of exposed functional groups that interact with water (Damodaran, 2017). However, unfolded proteins at a relatively high concentration might interact with each other over time via hydrophobic and disulfide linkages. Further investigation into the effect of acidification and industrial thermal treatments on protein solubility over time is needed to assess the storage stability of these samples.

The solubility of the reference cPPI was minimal (<15%) and the lowest of all analyzed samples under non-heated and heated conditions (**Table 5**). This low solubility is attributed to the high extent of denaturation, excessive aggregation, and hydrophobicity (**Table 4**, **Figure 3d**; **Lane 14**). nPPI was significantly more soluble than cPPI, due to nPPI being relatively less denatured and polymerized, with lower surface hydrophobicity and greater surface charge (**Table 4**, **Figure 3d**; **Lane 15**). This difference in solubility of a commercial and lab-scale PPI has also been reported in past studies (Bu et al., 2022; Shand et al., 2007).

Table 5. Solubility (%) of commercial pea protein (cPPI), native pea protein (nPPI), HIC purified nPPI (PW-PPI), and HIC purified partially-glycated pea protein (PW-PG-PP) at pH 3.4 and 5% protein concentration.

Samples	Solubility (%)	
	Non-heated	Heated (80°C for 30 min)
cPPI	8.99[a1]	13.7[a]*
nPPI	42.0[b]	61.2[b]*
PW-PPI	80.0[c]	90.7[c]*
PW-PG-PP	75.2[c]	90.5[c]*

[1] Lowercase letters indicate significant differences among means ($n \geq 3$) in each column according to the Tukey-Kramer multiple means comparison test ($P < 0.05$); * Designates a significant difference between non-heated and heated samples in each row as tested by a two-sample unpaired t-test ($P < 0.05$).

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Upon HIC purification, the produced PW-PPI had nearly twice the solubility of nPPI when non-heated and nearly 30% greater solubility when heated (**Table 5**). As mentioned earlier, hydrophilic proteins were selectively fractionated during the water wash and separated from denatured and polymerized proteins that had high surface hydrophobicity and low surface charge, thereby, increasing the abundance of more soluble proteins in PW-PPI (**Table 4**, **Figure 3d**; **Lanes 16-17**). Similarly, the solubility of PW-PG-PP was significantly higher than that of nPPI and was not statistically different than that of PW-PPI (**Table 5**). The increased solubility of these purified water fractions was attributed to their relatively low surface hydrophobicity and higher surface

charge, compared to nPPI and NaOH fractions, allowing for increased protein-water interaction (Damodaran, 2017). The similar solubility of PW-PPI and PW-PG-PP under non-heated and heated conditions is likely related to their similar structural characteristics, such as surface hydrophobicity and T_d, as both were comprised of hydrophilic proteins purified by HIC (**Table 4**). Potential improvements in protein solubility by glycation might have been masked when comparing PW-PG-PP to PW-PPI, which was comprised mostly of hydrophilic proteins. Glycation might have reduced the surface hydrophobicity of more hydrophobic proteins in nPPI, "pushing" hydrophobic nPPI proteins to elute with the hydrophilic proteins in PW-PG-PP. However, protein yields of nPPI and PG-PP purification were not monitored. Future studies should monitor the impact of glycation on protein yields in both the water and NaOH fractions of nPPI and PG-PP. An improved protein yield of PW-PG-PP, compared to PW-PPI, would add value to this glycation and purification process.

Since PW-PPI and PW-PG-PP had similar solubility, the direct effect of glycation on protein solubility was not elucidated. Previous research on pea protein glycated with gum Arabic, mono- and disaccharides, and maltodextrin confirmed improved protein solubility (Kutzli, Griener, et al., 2020; Zha, Dong, et al., 2019b, 2019a; Zha, Yang, et al., 2019; Zha et al., 2020). The enhanced solubility was linked to increased surface hydrophilicity and steric repulsion of glycated proteins upon the covalent linkage of carbohydrates. However, these past studies evaluated protein solubility in the presence of unreacted carbohydrates (i.e., without any purification) and at low protein concentrations ($\leq$ 0.25% protein in buffer or water, w/w). Excess, unreacted carbohydrates must be removed from PG-protein to directly evaluate the impact of partial glycation on proteins. For example, unreacted carbohydrates may reduce protein solubility by competing with protein to interact with water, while imparting additional, undesired functionality, such as viscosity and body (Kennedy et al., 2012; Wei et al., 2020). Furthermore, the low protein concentrations used in these studies are not relevant to high-protein beverage applications. In the US, "high" protein claims can only be made for beverages containing $\geq$4.2% available protein (w/v), based on the 50 g protein daily value recommendations (21 C.F.R. § 101.54, 2022). Hence, the low protein concentrations used in these studies may not reflect their solubility in application, since protein solubility may be impacted by

increased protein concentration, where protein molecules are in closer proximity (Bogahawaththa et al., 2019; Damodaran, 2017). Additionally, while pea protein solubility increased upon glycation in these studies, the solubility of glycated samples remained at less than 50%, much lower than that of whey protein, the most commonly used protein in beverages (Wang & Ismail, 2012).

The present study was distinct from these past studies, by preparing and characterizing a partially-glycated, purified protein sample under conditions relevant to high-protein, RTD beverage applications (at 5% protein concentration), with much higher solubility (~90% solubility), nearly the solubility of whey protein, the gold standard for high protein beverages (Wang & Ismail, 2012). Removal of unreacted carbohydrates from PG-PP allowed for a more direct analysis of protein solubility, potentially without interference from excess carbohydrates. Additionally, removal of unreacted carbohydrates produced a higher purity protein ingredient with more commercial value as a healthy ingredient, and with less potential to lose quality during storage due to the formation of advanced Maillard reaction products. Primarily, results demonstrated that glycation coupled with purification significantly improved the solubility of pea protein. With such considerable improvements in solubility, further investigation into the direct effect of glycation is needed. Combining water and NaOH fractions produced from each nPPI and PG-PP would allow for more direct analysis of glycation on protein solubility and thermal stability.

Likewise, nPPI was purified by HIC, intended as a control to purified PG-PP. Unexpectedly, results of nPPI purification established that nPPI could be fractionated by HIC to produce PW-PPI with improved solubility and thermal stability, comparable to that of whey protein (Wang & Ismail, 2012). HIC fractionation, therefore, has the potential to increase the value of native protein by producing a highly soluble ingredient, along with a less soluble fraction that could be suitable for other applications, such as meat analogues. Accordingly, research into the fractionation of pea protein by HIC is warranted, specifically further elucidation of PW-PPI and PN-PPI functionality and later, scaled-up production of both fractions.

3.6 Protein digestibility

High-protein, RTD beverages are customarily consumed for their nutritional value, ideally delivering relatively large quantities of high-quality protein. In order for protein ingredients to have value in beverage applications, they must contain highly digestible protein with a sufficient quantity of essential amino acids (Paulsen, 2009). Maillard-induced glycation has the potential to reduce the nutritional quality of proteins by blocking lysine, an essential amino acid (Nooshkam et al., 2020; Tuohy et al., 2006). In addition, glycation with bulky carbohydrates may hinder the accessibility of digestive proteases to reach bindings sites and cleave peptide bonds, thus reducing the digestibility and bioaccessibility of amino acids (Gumus, Davidov-Pardo, & McClements, 2016; Nooshkam et al., 2020). Therefore, the impact of glycation and purification on the *in vitro* protein digestibility of purified and reference samples were investigated.

All samples demonstrated high *in vitro* protein digestibility (~100%), with no differences among cPPI, nPPI, and PW-PPI, demonstrating that extraction and HIC purification processes did not significantly alter digestive enzyme accessibility (**Table 7**, **Appendix L**). While statistically significant, the slightly lower digestibility of PW-PG-PP, compared to that of the other samples, is not impactful, as the digestibility score of all samples were ≥100% (**Table 7**, **Appendix L**). Additionally, based on the amino acid analysis (**Table 6**, **Appendix L**) corrected for protein content, the lysine content of all samples was greater than the recommended reference protein amount; the ratio of lysine (mg/g protein) in samples to the recommended lysine content in reference protein (mg/g protein) (based on the recommended amino acid scoring pattern for children (6 months to 3 years)) was >1 (FAO/WHO Expert Consultation, 1991). This finding indicates that glycation and/or HIC purification did not reduce the amount of bioaccessible lysine in the samples, which remained within the required amount. Although lysine is the primary site of glycation, with ~30% of lysine groups blocked (**Table 2**), the protein-carbohydrate bond remained accessible to digestive enzymes in this study. Digestibility of PG proteins, particularly PG plant proteins, has not been extensively researched. Qu et al. (2018) and Shen & Li (2021), found that rapeseed protein-dextran conjugates and pea protein-guar gum conjugates, respectively, had reduced *in vitro* digestibility upon glycation. On the other hand, others have found that glycation and subsequent HIC purification increases

protein digestibility, due to partial unfolding of the protein at elevated incubation temperatures, which increases digestive enzyme accessibility (Wang & Ismail, 2012). Differences in glycated protein digestibility are likely caused by differences in the extent of glycation (i.e., number of saccharides attached to the protein molecule), the size of the linked saccharides, and changes in protein conformation. Additionally, the impact of lysine loss on protein quality may depend upon the protein source. For example, lysine is the reported limiting amino acid for rice, wheat, and oat proteins, while sulfur-containing methionine and cysteine are limiting for pea protein (Rutherfurd et al., 2014). Protein sources with higher native lysine content, such as pea, have the potential to mitigate protein quality loss due to lysine blockage. Due to limitations in sample size, complete amino acid analysis was not completed (i.e., cysteine, methionine, and tryptophan quantification is needed) and PDCAAS was not determined. Future studies would benefit from complete amino acid analysis and PDCAAS determination, in order to fully evaluate the effect of glycation on protein quality. A decreased PDCAAS would impact beverage formulations and nutrition labeling, with beverages requiring more protein to meet protein content claims.

Chapter 4: Conclusions, Implications, and Recommendations

A clean-label process for glycating pea protein was developed in this study, with purification to produce a functionally enhanced and valuable pea protein ingredient. Maillard-induced glycation of native pea protein with endogenous pea maltodextrin was performed and the changes in protein color, amine blockage, and protein profile were monitored. Further, removal of unreacted carbohydrates by HIC produced purified, partially-glycated (PG) samples, along with purified fractions from nPPI as controls. Finally, changes in their protein structure, solubility and thermal stability, and digestibility upon combined glycation and HIC purification were assessed.

The PSP by-product of pea protein extraction was partially hydrolyzed, with hydrolysis conditions optimized to produce a maltodextrin product (DE 15.7) with reduced mono- and disaccharide content. nPPI and the produced maltodextrin were incubated under relatively mild conditions, to initiate and control the Maillard reaction to the early stage. Incubation resulted in partially-glycated pea protein (PG-PP), with minimal browning and protein polymerization, as well as moderate free amino group loss (29.6%). The targeted Amadori products were formed, as protein-maltodextrin conjugates were successfully produced without progression of the Maillard reaction to the advanced stages. With the use of the pea maltodextrin product, the hypothesized endogenous glycation of pea protein was proven, with PG-PP potentially having clean-label appeal.

Further, this is the first study to purify a partially glycated pea protein, completed by removing the majority of unreacted carbohydrates via HIC. Removal of unreacted maltodextrin and polymerized proteins from PG-PP concentrated the protein content nearly 3 times, producing a more hydrophilic PW-PG-PP. Including a purification step is vital from an industry perspective, in order for the modified ingredient to be of high protein purity, with value as a healthy ingredient. Direct analysis of the PG-protein functionality requires removal of excess carbohydrates, as well, since carbohydrates may impact protein functionality or impart their own functionality. Importantly, removal of unreacted carbohydrates helps to reduce the potential of the Maillard reaction to progress to advanced stages over storage. Maillard-induced glycation and HIC purification resulted in a sample that has the potential for application in high-protein, RTD beverages,

having nearly the solubility of whey protein, the gold standard for beverages, under acidic conditions and at a high-protein claim concentration.

Although nPPI was purified to serve as a control to the purified PG-PP, the fractionation of nPPI by HIC produced a highly soluble and thermally stable sample, PW-PPI, with solubility similar to that of whey protein under acidic conditions and with thermal treatment. Removal of hydrophobic and polymerized proteins resulted in the concentration of a hydrophilic fraction (PW-PPI) with enhanced functionality for potential use in beverages. HIC fractionation of pea protein has value for protein ingredient manufacturers, as hydrophilic and hydrophobic protein fractions can be produced, each with potentially increased value for different applications. While the hydrophilic fraction can be used in beverage applications, the hydrophobic fraction may be utilized in applications that rely on protein-protein interactions, such as meat alternatives.

While this study demonstrated that endogenous glycation coupled with HIC purification, as well as HIC purification alone, greatly improved the solubility and thermal stability of PPI, especially compared to cPPI, further investigation and method development is needed. More complete structural, functional, and nutritional characterization of purified PG-PP and fractionated PPI must be done in order to identify more potential applications for these ingredients. Specifically, more complete elucidation of PW-PG-PP, PN-PG-PP, PW-PPI, and PN-PPI structure is needed to better understand the mechanism of glycation and the impact of purification/fractionation on the functionality of each sample and fraction. Quantification of the molecular weight distribution of the fraction by size exclusion chromatography and determination of the number of carbohydrates linked to protein by multi-angle light scattering techniques should be completed. Mass spectrometry proteomic techniques, such as matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF-MS) and liquid chromatography-electrospray ionization (LC-ESI-MS), should be used to determine specific glycation sites, as well. Furthermore, as glycation and/or purification/fractionation have the potential to improve the surface activity of proteins, the emulsification and foaming properties of samples need evaluation. Complete functional analysis, also including protein gelation, viscosity, water-holding, and oil- and water-binding capacities, might

help to identify other potential food applications for purified PG-PP and fractionated PPI. Additionally, complete amino acid analysis and PDCAAS determination is needed to fully evaluate the effects of these processes on the protein nutritional quality.

Glycation and purification/fractionation method development is needed in order to eventually increase the production scale of these protein ingredients. First, maltodextrin production from PSP should be scaled-up on a pilot plant level, in order to further improve glycation and purification processes on the lab-scale. As pea maltodextrin is currently produced industrially, this process is likely to be easily scaled-up. Refinement of the glycation incubation conditions, especially modification of time and temperature conditions, are needed to reduce the incubation time, ultimately making this process more industry feasible. In particular, the Maillard reaction kinetics could be studied to optimize the incubation conditions. Reduction of the maltodextrin to protein incubation ratio would be helpful, as well, in order to reduce the amount of protein and maltodextrin separation needed. Additionally, the volume of pea protein and maltodextrin incubations, on a lab-scale and in a pilot plant, should be increased to assess the feasibility of the method and control of the Maillard reaction on a larger scale. Furthermore, efficient removal of unreacted carbohydrates needs to be pursued, possibly by increasing the ammonium sulfate wash volume. Further work should include mass balance analysis of the HIC process, assessing the yields in water and NaOH fractions produced from purified PG-PP and fractionated PPI. Managing the yields in this process would also help assess the feasibility of HIC purification. An increased yield in the PG-PP water fraction, compared to PW-PPI, would further prove the value of glycation. In addition, combination of water and NaOH fractions from HIC would also allow for more direct analysis of the effect of glycation on protein structure and functionality. Lastly, more efficient removal of residual ammonium sulfate from HIC is needed. On both lab- and industrial-scales, membrane filtration can be used to reduce the collected fraction volumes and concentrate protein by removing water and residual salt, improving the efficiency and extent of desalting. Following method development and refinement, the entire glycation and purification/fractionation processes should be scaled-up. Utilization of industrial equipment and processes (e.g., membrane filtration, pasteurization, and spray drying) can be completed to analyze the realistic effects of processing on protein

structure, functionality, and nutritional value, as well as determining the processing costs and efficiency.

Considering the use of these protein ingredients in food products, further evaluation of the protein functionality, nutritional value, and sensory qualities under different conditions and over storage is needed. As foods are typically held under different pH levels (acidic or neutral), ionic strengths, and protein concentrations, the functional properties (e.g,. solubility and thermal stability, emulsification, foaming, and gelation) of the glycated and purified/fractionated samples should be assessed over a range of pH values, ionic strengths, and protein concentrations relevant to foods. This will help determine additional, practical applications of these protein ingredients. Additionally, PDCAAS might change under different environmental conditions due to changes in protein interactions. Therefore, PDCAAS should be evaluated under the aforementioned conditions to predict potential changes in nutrition labeling. Investigation into the impact of pea protein glycation and purification/fractionation on the storage stability of these protein ingredients under various environmental conditions (e.g., pH, ionic strength, and protein concentration) and thermal treatments relevant to food and beverages, and on sensory qualities, is necessary to predict their commercial potential and potential consumer acceptability. Shelf-life investigation is needed, because holding proteins at unfavorable conditions and high concentrations, especially following thermal treatment, can result in protein unfolding and induce protein polymerization and aggregation. Protein aggregation has a destabilizing effect on beverages, in particular, leading to undesirable sedimentation of protein over storage. In general, protein polymerization might negatively impact sensory qualities, including visual, textural, and flavor qualities. The conditions that food and beverages are held at and their formulations can be optimized to prolong product shelf and limit quality reduction over storage.

Overall, this work provided foundational information and the basis for future investigation and optimization of endogenous glycation and HIC purification/fractionation processes. This work uncovered a PPI fractionation process that has the potential to increase specialty pea protein ingredient value, and paved the way for future investigation and process optimization for scaled-up production of purified PG-PP.

In particular, this work utilized glycation and HIC purification to produce pea protein with potentially added-value for application in high-protein, RTD beverages.

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Appendix A: Protein Profile of Pea Flour Subjected to *in situ* Starch Hydrolysis

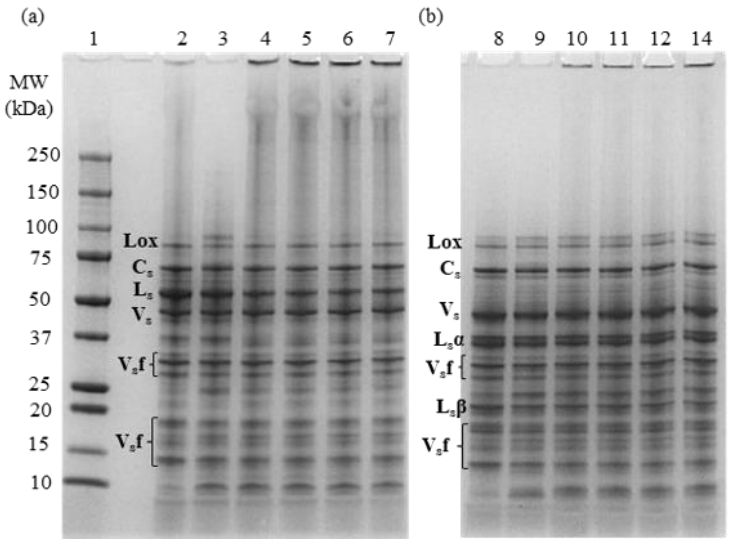

Figure 5. SDS-PAGE visualization of the protein profiles of samples (a) under nonreducing and (b) reducing conditions. Lane 1: Molecular weight (MW) marker; Lanes 2, 8: nPPI; Lanes 3, 9: Pea flour; and Lanes 4-7, 10-14: TPF from various trials with different enzyme doses and incubation times. Lox: lipoxygenase; C_s: subunits of convicilin; L_s subunits of legumin; V_s: subunits of vicilin; V_sf: fractions of vicilin subunits resulting from post-translational cleavages; $L_s\alpha$: acidic peptides cleaved from legumin subunits; $L_s\beta$: basic peptides cleaved from legumin subunit.

Appendix B: Sample Calculation for Determining Dextrose Equivalent (DE)

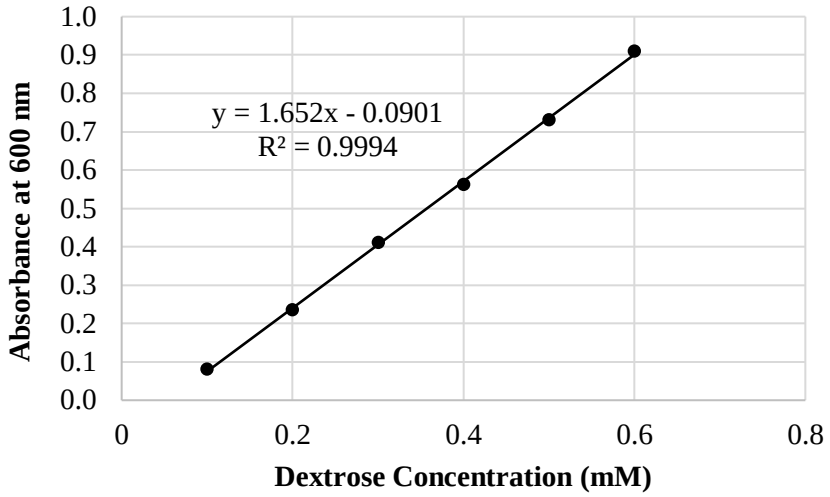

Figure 6. Dextrose standard curve used to quantify dextrose equivalent in the maltodextrin sample.

Equation 5. Apparent dextrose mass in diluted sample.

$$dextrose_{sample}\ (g) = \frac{(A_{sample} - b)}{m} * \frac{1\ mol}{1000\ mmol} * \left(Dextrose_{mm}\frac{g}{mol}\right) * 0.000045\ L\ aliquot$$

Where:

A_{sample} = Absorbance of sample at 600 nm
b = Y-intercept of the linear trendline of the dextrose standard curve
m = Slope of the linear trendline of the dextrose standard curve
$Dextrose_{mm}$ = Dextose molar mass, 180.16 g/mol

Equation 6. Sample mass in diluted sample.

$$sample\ (g) = \frac{mass\ (g)}{0.001\ L} * \frac{1}{DF} * 0.000045\ L\ aliquot$$

Where:

DF = Dilution factor, 40

Sample Calculation for DE:

*Refer to **Equations 1**, **5**, and **6**.*

Given:

Sample: Maltodextrin replication from scale-up production
Absorbance at 600 nm: 0.268
Equation of dextrose standard curve trendline: y = 1.6520x – 0.0901
Analyzed sample mass: 0.0104

$$mass\ dextrose_{sample}\ (g)$$

$$= \frac{(0.268 + 0.0901\ )}{1.6520} * \frac{1\ mol}{1000\ mmol} * 180.16 \frac{g}{mol} * 0.000045\ L\ aliquot$$

$$= 1.76\ x\ 10^{-6}\ g$$

$$sample\ (g) = \frac{0.0104\ g}{0.001\ L} * \frac{1}{40} * 0.000045\ L\ aliquot = 1.17\ x\ 10^{-5} g$$

$$Dextrose\ equivalent = \frac{1.76\ x\ 10^{-6}\ g\ dextrose_{sample}}{1.17\ x\ 10^{-5}\ g\ sample} *\ 100 = 15.0\ DE$$

Appendix C: Sample HPAEC-PAD Chromatogram and Calculations for Chain-Length Analysis

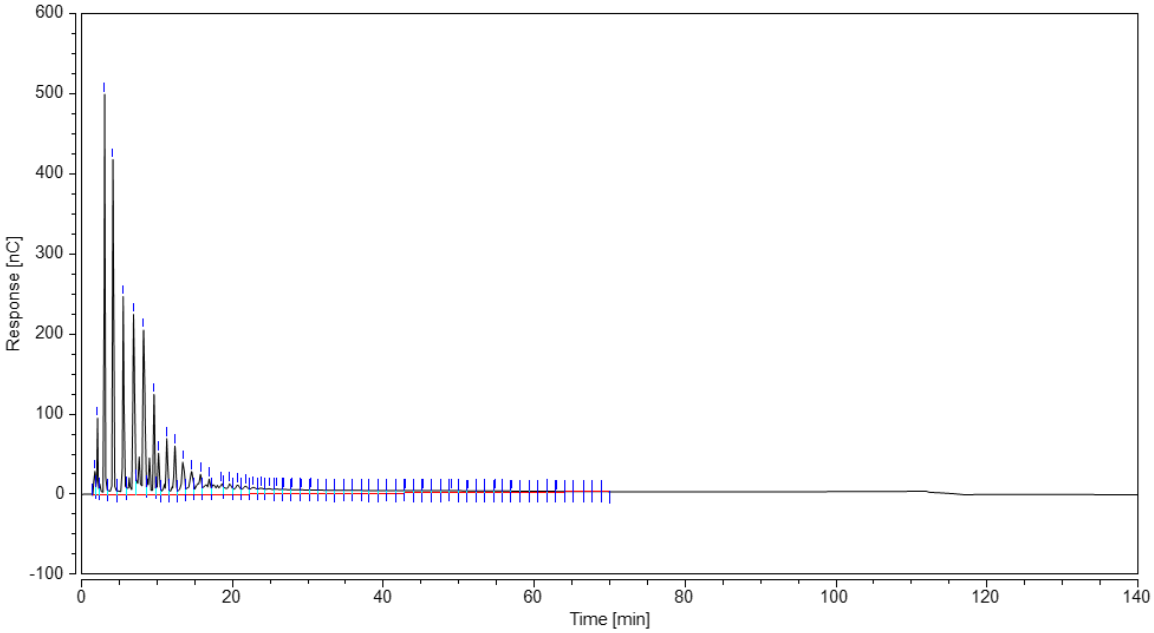

Figure 7. Sample HPAED-PAD chromatogram of one maltodextrin replicate from scale-up production, manually integrated.

Equation 7. Individual peak area carbohydrate correction.

$$Corrected\ peak\ area(nC * \text{min}) = Peak\ area\ (nC * \text{min}) * \frac{\frac{465 + (120 * 6)}{6}}{\frac{465 + (120 * DP)}{DP}}$$

Where:

DP = degree of polymerization

Equation 8. Individual peak normalized weight percentage (%).

$$Weight\ (\%) = \frac{Corrected\ peak\ area\ (nC * \text{min})}{\sum Corrected\ peak\ area\ (nC * \text{min})} * 100$$

Equation 9. Average degree of polymerization (DP).

$$Average\ DP = \frac{\sum w_i}{\sum(\frac{w_i}{DP_i})}$$

Where:

DP = degree of polymerization
w_i = normalized weight percentage
DP_i = DP of peak i

Sample Calculation for Peak Area:

*Refer to **Equations 7** and **8**.*

Given:

Sample: DP 2 peak of maltodextrin replication from scale-up production
Peak area (nC*min): 14.00
Sum of corrected peak areas (nC*min): 781.72

$$Corrected\ peak\ area(nC * \min) = Peak\ area\ (nC * \min) * \frac{\frac{465 + (120 * 6)}{6}}{\frac{465 + (120 * 2)}{2}} = 7.84\ nC * min$$

$$Weight\ (\%) = \frac{7.84\ nC * min}{781.72\ nC * min} * 100 = 1.00\%\ DP\ 2$$

Sample Calculation for Average DP:

*Refer to **Equation 9**.*

Given:

Sample: Maltodextrin from scale-up production
$\sum w_i$: 100.00%
$\sum(w_i/DP_i)$: 12.01%

$$Average\ DP = \frac{\sum w_i}{\sum(\frac{w_i}{DP_i})} = \frac{100.00\ \%}{12.01\ \%} = 8.32$$

Appendix D: Images of Incubated Samples

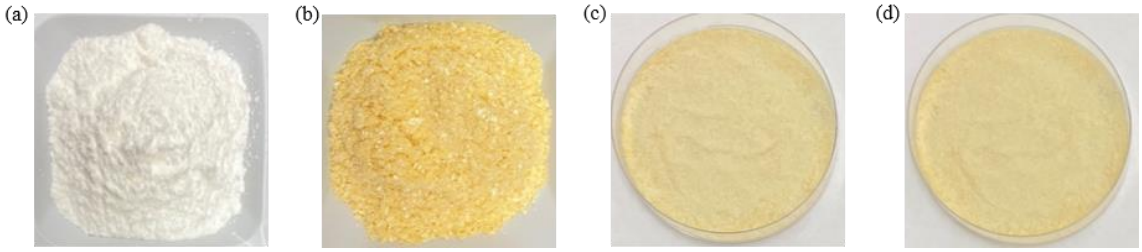

Figure 8. Maltodextrin (a), nPPI (b), nPPI+MD before incubation (c), and PG-PP after 24 hours of incubation (d).

Appendix E: Sample Calculation for Determining Free Amino Composition

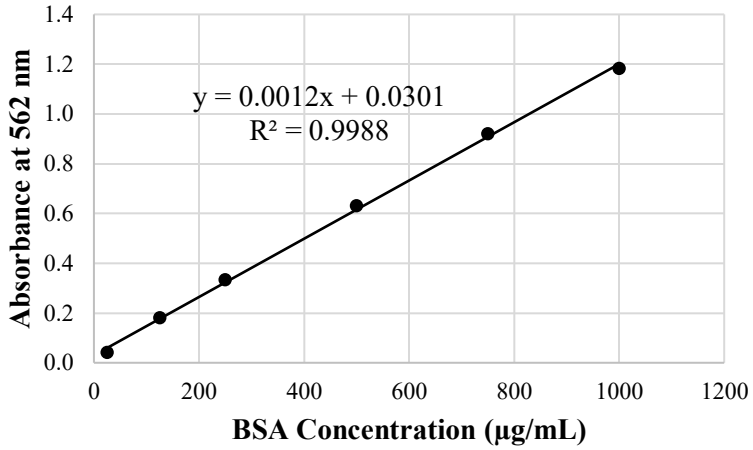

Figure 9. BSA standard curve used to quantify protein content using the BCA assay.

Equation 10. Protein content in sample.

$$Protein\ (\frac{\mu g}{mL}) = \frac{(A_{sample} - b)}{m}$$

Where:

A_{sample} = Absorbance of sample at 562 nm
b = Y-intercept of the linear trendline of the BSA standard curve
m = Slope of the linear trendline of the BSA standard curve

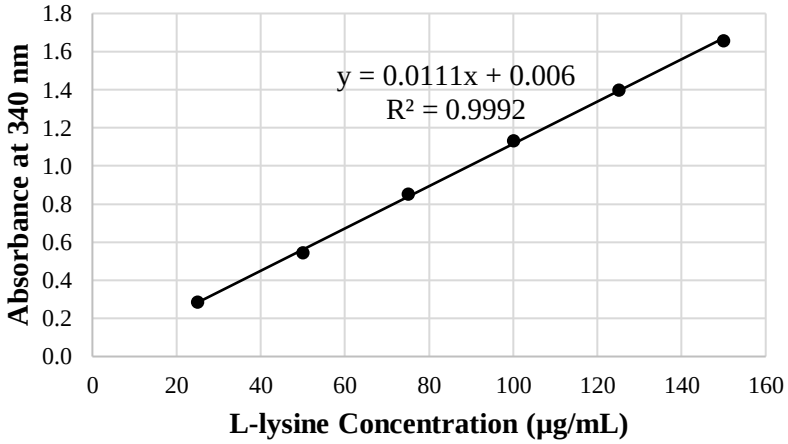

Figure 10. L-lysine standard curve used to quantify free amino groups using the OPA method.

Equation 11. Free amino groups in sample.

$$Free\ amino\ groups\ (\frac{\mu g}{mL}) = \frac{(A_{sample} - b)}{m}$$

Where:

A_{sample} = Absorbance of sample at 340 nm
b = Y-intercept of the linear trendline of the L-lysine standard curve
m = Slope of the linear trendline of the L-lysine standard curve

Sample Calculation for Percent (%) Free Amino Groups:

*Refer to **Equations 2**, **3**, **10**, and **11**.*

Given:

Sample: nPPI replicate
Absorbance at 562 nm: 0.730
Equation of BSA standard curve trendline: y = 0.0012x + 0.0301
Absorbance at 340 nm: 0.462
Equation of L-lysine standard curve trendline: y = 0.0111x – 0.0060
Average nPPI % free amino groups: 6.88%
Average PG-PP % free amino groups: 4.84%

$$Protein\ \left(\frac{\mu g}{mL}\right) = \frac{(0.730 - 0.0301)}{0.0012} = 604.7\ \frac{\mu g}{mL}$$

$$Free\ amino\ groups\ \left(\frac{\mu g}{mL}\right) = \frac{(0.462 + 0.0060)}{0.0111} = 41.1\ \frac{\mu g}{mL}$$

$$\%\ Free\ amino\ groups = \frac{41.09\ \frac{ug}{mL}}{604.7\ \frac{ug}{mL}} * \ 100 = 6.80\%\ free\ amino\ groups$$

$$\%\ Loss\ free\ amino\ groups = \frac{\%\ 6.88 - \%4.84}{\%\ 6.88} * \ 100 = 29.4\%\ loss\ free\ amino\ groups$$

Appendix F: Sample Calculation for Total Carbohydrate Content

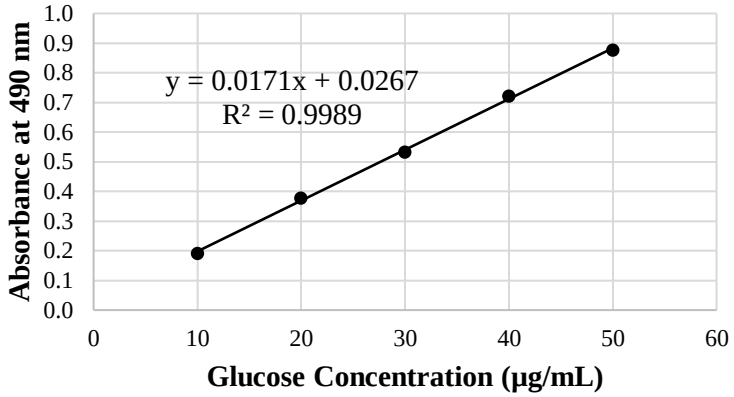

Figure 11. Glucose standard curve used to calculate total carbohydrate content following the phenol-sulfuric acid method.

Equation 12. Total carbohydrate content (glucose equivalent) in 10-minute interval fractions.

$$Total\ carbohydrates\ \left(\frac{\mu g}{mL}\right) = \frac{(A_{sample} - b)}{m} * DF$$

Where:

A_{sample} = Absorbance of sample at 490 nm
b = Y-intercept of the linear trendline of the glucose standard curve
m = Slope of the linear trendline of the glucose standard curve

Sample Calculation for total carbohydrate content:

*Refer to **Equation 12**.*

Given:

Sample: Replicate of Fraction 1 (collected from 0-10 minutes) of scaled-up PG-PP purification example
Absorbance at 490 nm: 0.6346
Dilution factor: 5
Equation of glucose standard curve trendline: y = 0.0171x + 0.0267

$$Total\ carbohydrates\ \left(\frac{\mu g}{mL}\right) = \frac{(0.6346 - 0.0267)}{0.0171} * 5 = 177.5\ \frac{\mu g}{mL}$$

Appendix G: Sample Protein Purification Chromatograms

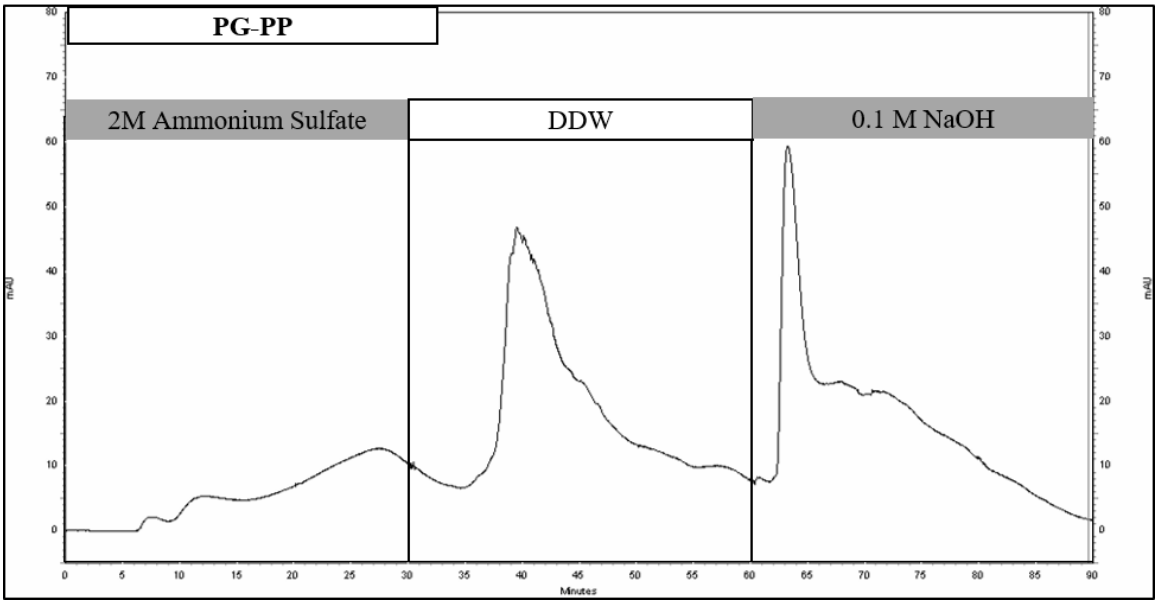

Figure 12. Sample chromatogram of scaled-up PG-PP purification (3% protein (w/v), 8 mL injection).

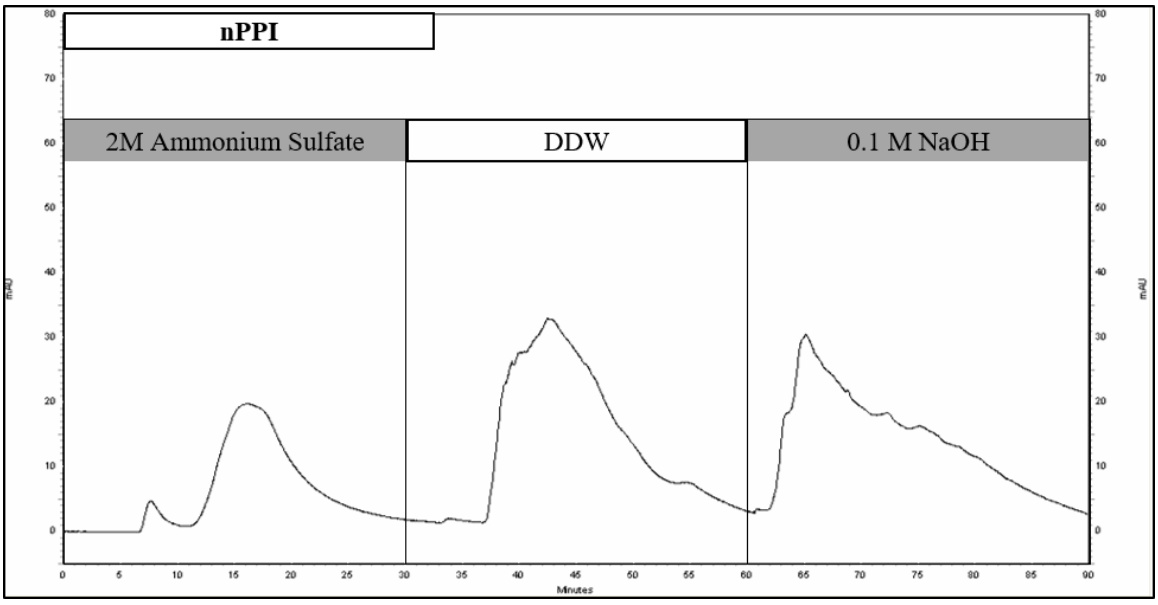

Figure 13. Sample chromatogram of nPPI purification (3% protein (w/v), 8 mL injection).

Appendix H: Sample Differential Scanning Calorimetry Thermograms

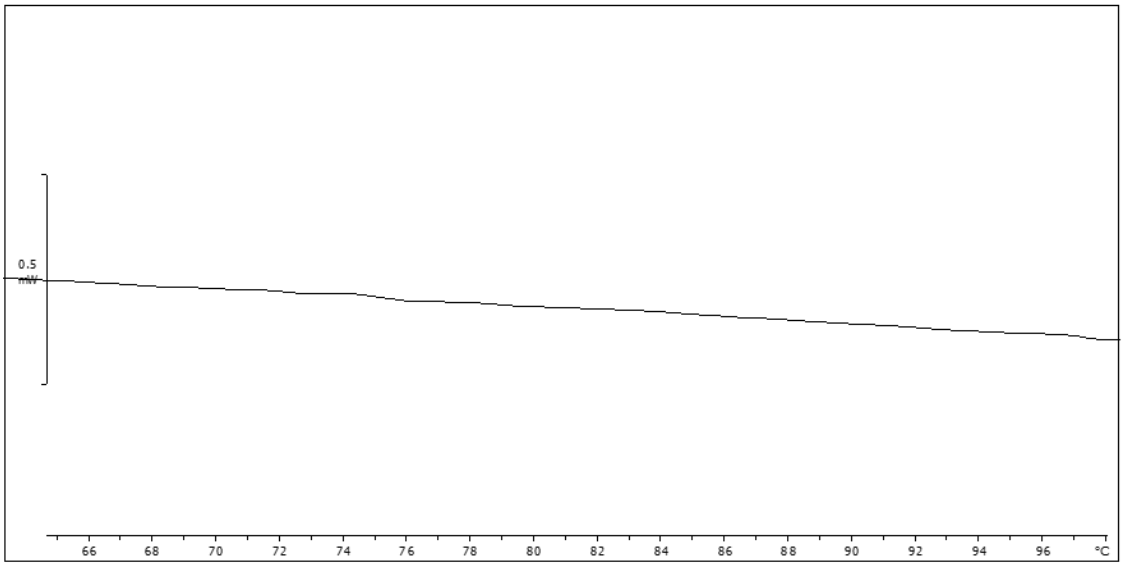

Figure 14. Sample thermogram of commercial pea protein isolate (cPPI) heated at 5ºC/min near protein denaturation peak(s).

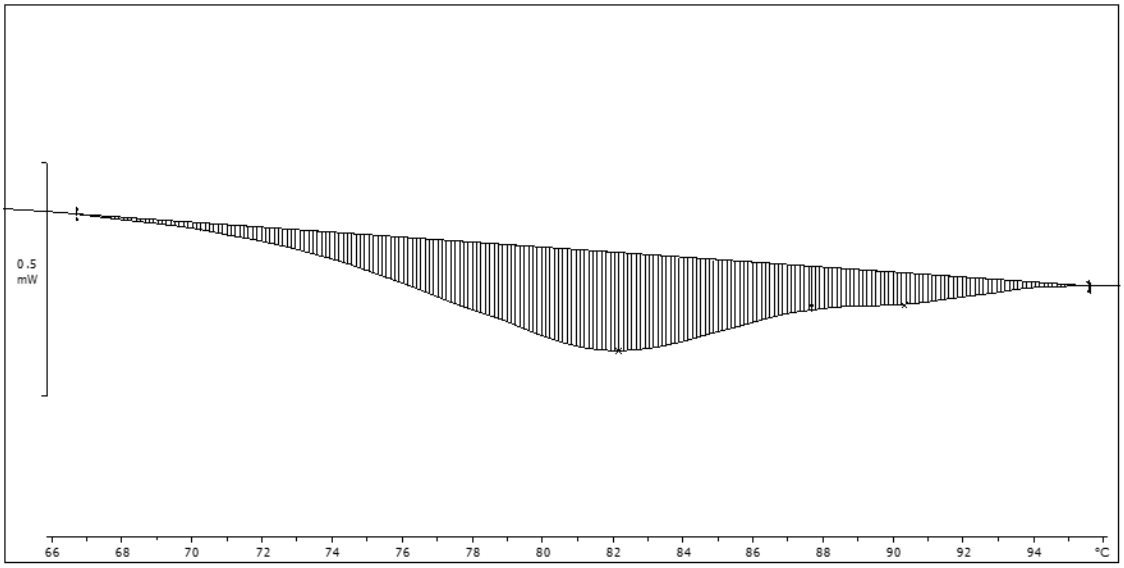

Figure 15. Sample thermogram of native pea protein isolate (nPPI) heated at 5ºC/min near protein denaturation peak(s).

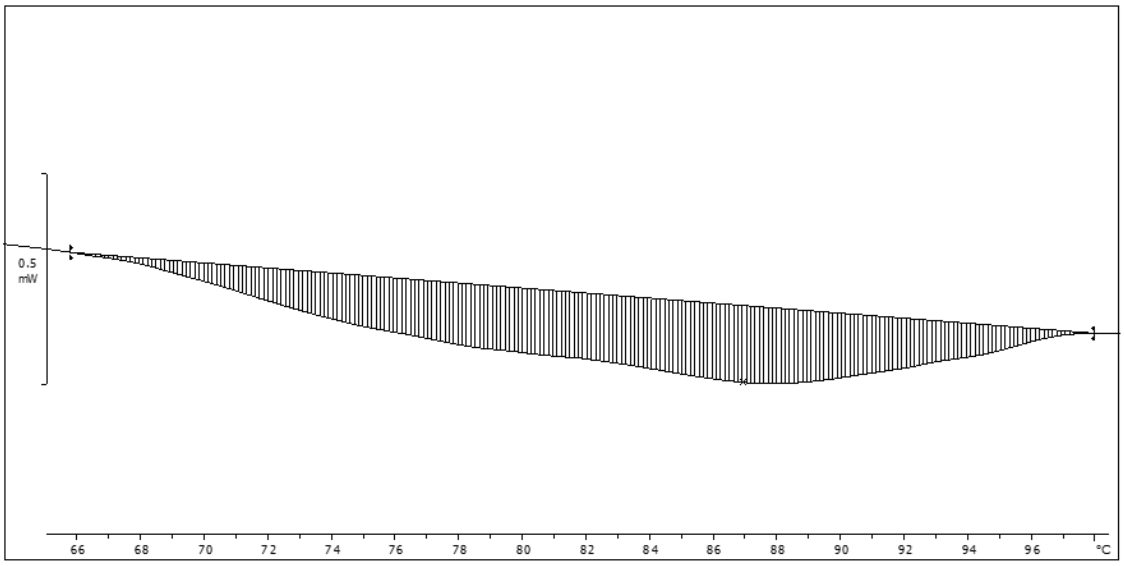

Figure 16. Sample thermogram of HIC purified nPPI (PW-PPI) heated at 5ºC/min near protein denaturation peak(s).

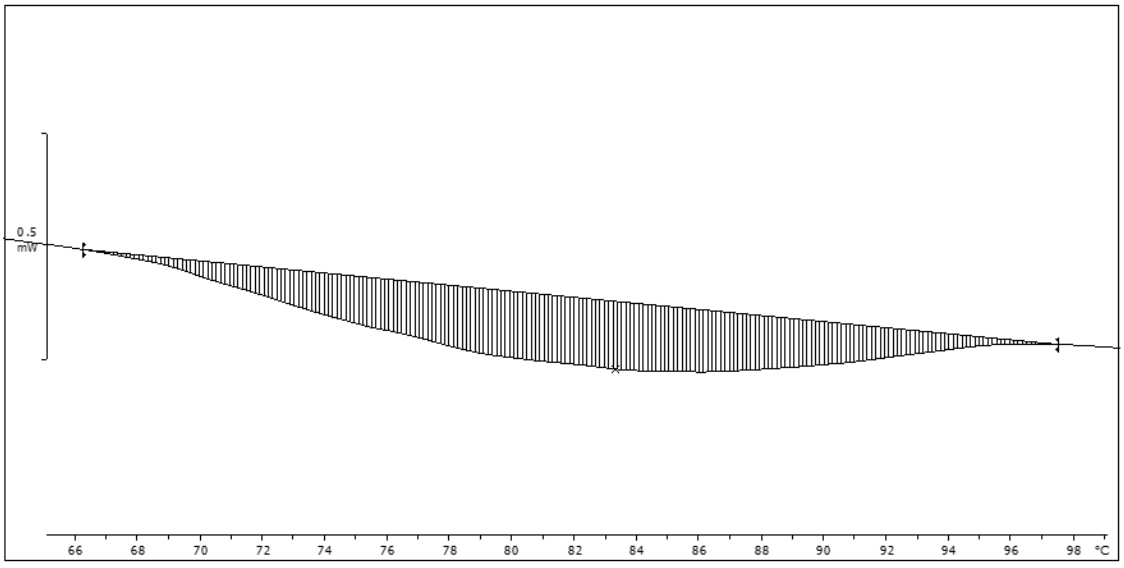

Figure 17. Sample thermogram of HIC purified partially-glycated pea protein (PW-PG-PP) heated at 5ºC/min near protein denaturation peak(s).

Appendix I: Sample Calculation for Determining Surface Hydrophobicity Index

Equation 13. Net RFI.

$$Net\ RFI = RFI_{final} - RFI_{initial}$$

Equation 14. Final RFI of sample.

$$RFI_{final} = E_{sample.final} - E_{blank.final}$$

Where:

$E_{sample.final}$ = fluorescence emission of protein sample after ANS probe is added
$E_{blank.final}$ = fluorescence emission of buffer blank after ANS probe is added

Equation 15. Initial RFI of sample.

$$RFI_{initial} = E_{sample.initial} - E_{blank.initial}$$

Where:

$E_{sample.initial}$ = fluorescence emission of protein sample before ANS probe is added
$E_{blank.initial}$ = fluorescence emission of buffer blank before ANS probe is added

Sample Calculation for surface hydrophobicity index:

*Refer to **Equations 13**, **14** and **15**.*

Given:

Sample: nPPI replicate at 0.050% protein (w/v)

$$RFI_{final} = 508.0 - 28.0 = 480.0$$

$$RFI_{initial} = 15.0 - 24.3 = 9.3$$

$$Net\ RFI = 480.0 + 9.3 = 489.3$$

Net RFI values for all protein solution concentrations (0.050%, 0.025%, 0.020%, 0.015%, 0.010%, and 0.005%) are plotted against protein concentration (**Figure 18**).

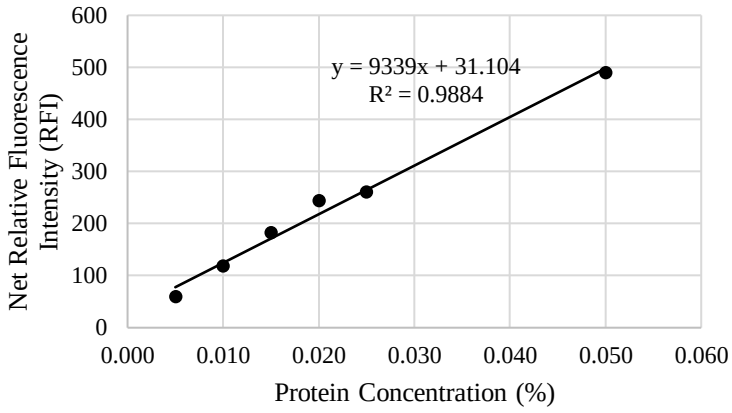

Figure 18. Net Relative Fluorescence Intensity (RFI) plotted against protein concentration (%) for nPPI replicate, to determine surface hydrophobicity index.

The slope of the linear trendline in **Figure 18** is the surface hydrophobicity index (9339).

The final value for surface hydrophobicity index is the average of three replicates.

Appendix J: Sample Spectra for Determining Protein Secondary Structure

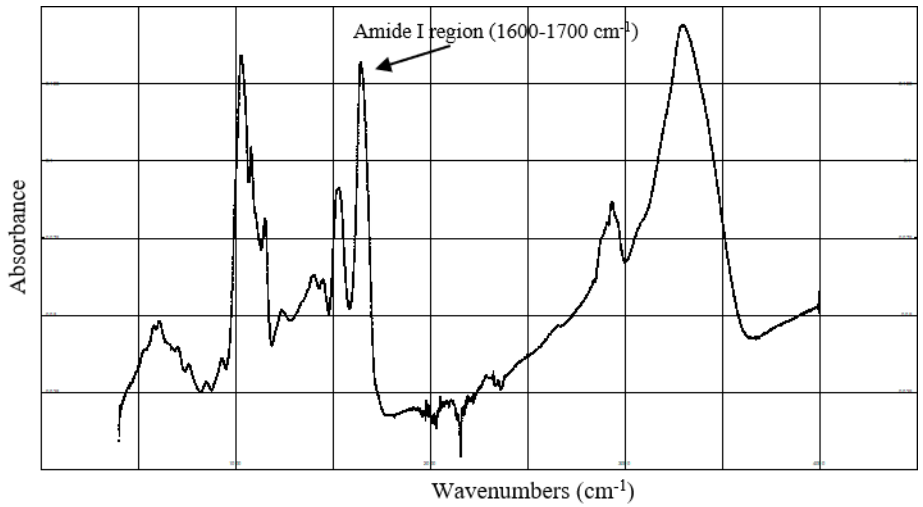

Figure 19. Original ATR-FTIR spectrum of nPPI.

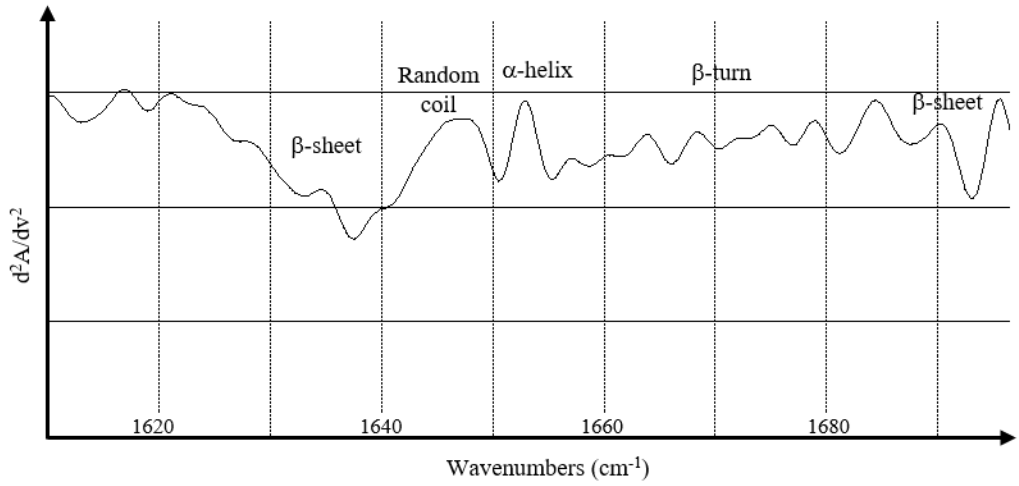

Figure 20. Second derivative spectrum of nPPI.

The original ATR-FTIR spectrum of nPPI is shown in **Figure 19**. The second derivative of Amide I band (1600 cm^{-1} – 1700 cm^{-1}) (**Figure 20**) was obtained by PeakFit v.4.12 to identify α-helix, β-sheet, β-turn, and random coil structures, according to the approximate wavenumber (cm^{-1}) ranges of 1648-1660, 1612-1641 and 1684-1694, 1662-1684, and 1640-1650, respectively.

Appendix K: Sample Calculation for Determining Protein Solubility

Sample Calculation for protein solubility:

*Refer to **Equation 4**.*

Given:

Sample: nPPI replicate (5% protein (w/v) at pH 3.4, non-heated)

$$\%\ solubility = \frac{2.27\%\ protein_{supernatant}}{5.29\%\ protein_{initial}} * 100 = 42.91\%$$

Appendix L: Amino Acid Composition and Protein Digestibility

Table 6. Partial amino acid compositions (weight percentage (%), wet basis) of commercial pea protein (cPPI), native pea protein (nPPI), HIC purified nPPI (PW-PPI), and HIC purified partially-glycated pea protein (PW-PG-PP).

	Weight percentage (%) amino acid in sample			
Amino Acid	cPPI	nPPI	PW-PPI	PW-PG-PP
L-HydroxyProline	0.00	0.00	0.00	0.14
L-Aspartic acid	8.81	8.93	10.32	7.45
L-Threonine	2.66	2.50	2.39	1.81
L-Serine	3.66	3.42	3.81	2.74
L-Glutamic acid	13.28	14.15	17.62	12.64
L-Proline	3.22	3.14	3.39	2.54
L-Glycine	2.63	2.63	2.63	1.95
L-Alanine	2.89	2.89	2.78	2.11
L-Valine	3.64	3.91	3.93	2.90
L-Isoleucine	3.53	3.76	3.96	2.90
L-Leucine	6.09	6.37	7.13	5.18
L-Tyrosine	3.05	2.98	3.06	2.10
L-Phenylalanine	4.24	4.21	4.53	3.32
L-Lysine	5.65	5.93	6.37	4.26
L-Histidine	1.91	2.01	2.14	1.58
L-Arginine	6.78	7.20	8.65	6.12

Table 7. *In vitro* protein digestibility (%) of commercial pea protein (cPPI), native pea protein (nPPI), HIC purified pea protein isolate (PW-PPI), and HIC purified partially-glycated pea protein (PW-PG-PP).

Sample	*In vitro* Protein Digestibility (%)
cPPI	108.5^{b1}
nPPI	109.4^{b}
PW-PPI	110.2^{b}
PW-PG-PP	101.7^{a}

[1] Lowercase letters indicate significant differences among means (n = 2) in each column according to the Tukey-Kramer multiple means comparison test ($P < 0.05$).

Appendix M: ANOVA Tables

Table 8. Analysis of variance on the effect of hydrolysis time (Trial 1) on maltodextrin dextrose equivalent.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
Maltodextrin produced in Trial 1	Hydrolysis time	5	659.927	1300.879	5.70e-16
	Error	12	0.507		

Table 9. Analysis of variance on the effect of 10-minute hydrolysis conditions on maltodextrin dextrose equivalent.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
Maltodextrin produced by 10-minute hydrolysis	10-minute hydrolysis conditions	4	78.546	101.010	1.59e-12
	Error	19	0.778		

Table 10. Analysis of variance on the effect of removal of small sugars (Trial 2) on maltodextrin dextrose equivalent.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
Maltodextrin produced in Trial 2	Small Sugar Removal method	2	60.547	50.386	1.45e-06
	Error	12	1.202		

Table 11. Analysis of variance on the effect of sample type on lightness (L*) of samples.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
nPPI, nPPI+MD, PG-PP	Sample Type	2	19.524	66.068	1.85e-08
	Error	16	0.296		

Table 12. Analysis of variance on the effect of sample type on red and green (a*) color of samples.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
nPPI, nPPI+MD, PG-PP	Sample Type	2	2.888	243.396	1.05e-12
	Error	16	0.012		

Table 13. Analysis of variance on the effect of sample type on yellow and blue (b*) color of samples.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
nPPI, nPPI+MD, PG-PP	Sample type	2	26.218	37.778	8.70e-07
	Error	16	0.694		

Table 14. Analysis of variance on the effect of sample type on percent free amino groups of samples.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
nPPI, nPPI+MD, PG-PP	Sample type	2	6.765	766.503	1.55e-12
	Error	11	0.009		

Table 15. Analysis of variance on the effect of sample type on protein content of samples.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
cPPI, nPPI, PW-PPI, PN-PPI, PW-PG-PP, PN-PG-PP	Sample type	5	774.481	3129.215	3.70e-10
	Error	6	0.248		

Table 16. Analysis of variance on the effect of sample type on total carbohydrate content of samples.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
cPPI, nPPI, PW-PPI, PW-PG-PP	Sample type	3	1053.430	1695.803	1.49e-11
	Error	8	0.621		

Table 17. Analysis of variance on the effect of sample type on ash content of samples.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
cPPI, nPPI, PW-PPI, PW-PG-PP	Sample type	3	11.316	2555.459	5.10e-07
	Error	4	0.004		

Table 18. Analysis of variance on the effect of sample type on total enthalpy of denaturation of samples.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
nPPI, PW-PPI, PW-PG-PP	Sample type	2	7.127	179.121	4.47e-06
	Error	6	0.040		

Table 19. Analysis of variance on the effect of sample type on surface charge at pH 3.4 of samples.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
cPPI, nPPI, PW-PPI, PN-PPI, PW-PG-PP, PN-PG-PP	Sample type	5	214.999	492.701	1.89e-13
	Error	12	0.436		

Table 20. Analysis of variance on the effect of sample type on surface charge at pH 7.0 of samples.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
cPPI, nPPI, PW-PPI, PN-PPI, PW-PG-PP, PN-PG-PP	Sample type	5	169.549	215.174	2.60e-11
	Error	12	0.788		

Table 21. Analysis of variance on the effect of sample type on surface hydrophobicity of samples.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
cPPI, nPPI, PW-PPI, PN-PPI, PW-PG-PP, PN-PG-PP	Sample type	5	3.54e07	58.853	5.04e-08
	Error	12	6.01e05		

Table 22. Analysis of variance on the effect of sample type on the relative percentage of α-helix structures on IR spectra.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
cPPI, nPPI, PW-PPI, PW-PG-PP	Sample type	3	8.158	5.217	2.75e-02
	Error	8	1.564		

Table 23. Analysis of variance on the effect of sample type on the relative percentage of β-sheet structures on IR spectra.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
cPPI, nPPI, PW-PPI, PW-PG-PP	Sample type	3	50.717	105.480	9.01e-07
	Error	8	0.481		

Table 24. Analysis of variance on the effect of sample type on the relative percentage of β-turn structures on IR spectra.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
cPPI, nPPI, PW-PPI, PW-PG-PP	Sample type	3	8.744	7.380	1.08e-02
	Error	8	1.185		

Table 25. Analysis of variance on the effect of sample type on the relative percentage of random coil structures on IR spectra.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
cPPI, nPPI, PW-PPI, PW-PG-PP	Sample type	3	52.249	122.711	4.99e-05
	Error	8	0.426		

Table 26. Analysis of variance on the effect of sample type on protein solubility of non-heated samples.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
cPPI, nPPI, PW-PPI, PW-PG-PP	Sample type	3	3266.852	831.895	2.56e-10
	Error	8	3.927		

Table 27. Analysis of variance on the effect of sample type on protein solubility of non-heated samples.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
cPPI, nPPI, PW-PPI, PW-PG-PP	Sample type	3	3951.140	759.208	3.69e-10
	Error	8	5.204		

Table 28. Analysis of variance on the effect of sample type on protein digestibility of samples.

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
cPPI, nPPI, PW-PPI, PW-PG-PP	Sample type	3	30.001	11.366	1.99e-02
	Error	4	2.640		