Enhancing separation efficiency and functional properties of starch and protein from air-classified starch-rich pulse flours using wet extraction

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ABSTRACT

Starch-rich pulse flours are recognized as an underutilized and low-value by-product from air classification of pulses, mainly due to the presence of impurities (*e.g.*, proteins, dietary fibers, flavor and aroma compounds) that limits their industrial use. Therefore, this study aimed to develop an effective wet isolation method that can further separate starch and protein from starch-rich pulse flours. Alkaline extraction was performed on air-classified starch-rich faba bean and pea flours over a range of pH (8.5, 9.5, 10.5, and 11) and temperature (25, 30, 40, and 50 °C) to identify the optimum pH and temperature combinations to extract starch and protein with maximum yields and purity. The techno-functional attributes of the isolated starches and protein isolates were compared with those of commercial pea starch and protein isolate, respectively, to evaluate the effect of alkaline extraction conditions on proximate composition and functional properties of the obtained starch and protein samples. The pulse starches isolated from the optimum conditions demonstrated high purity with starch content of 95.1-97.2% and low contents of protein (≤ 0.31%) and ash (≤ 0.07%). The granular morphologies of the isolated pulse starches were generally comparable to that of commercial pea starch, but the isolated starches showed more damaged starch granules, particularly for those extracted at pH 11.0 and and 50 °C. Thermal, pasting, and gelling properties of the isolated starches were affected by the extraction conditions, with significant differences observed in gelatinization temperatures and enthalpy changes, pasting temperatures and viscosities, and gel strength. Under the optimum extraction conditions, protein isolates obtained from air-classified starch-rich pulse flours had protein purity of 77.6%-94.0% with less than 5% ash. The surface properties of protein isolates varied with extraction conditions, thereby affecting their functional properties including solubility, emulsion stability, foam capacity and stability, and water-holding capacity and oil-absorption capacity. The findings of this study highlighted the influence of extraction conditions on the purity and techno-functional attributes of the isolated starch and protein components. The comprehensive analysis provided meaningful information for the potential applications of these isolated components in various food and industrial products.

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LIST OF ABBREVIATIONS AND SYMBOLS

AACC	Approved methods of the American Association of Cereal Chemists
AEIP	Alkaline extraction/isoelectric precipitation
ANOVA	Analysis of variance
ANS	8-anilino1-naphtalensulfonic acid
CPPI	Commercial pea protein isolate
CPS	Commercial pea starch
DP	Degree of polymerization
DSC	Differential scanning calorimetry
EAI	Emulsion activity index
EC	Emulsion capacity
ES	Emulsion stability
ESI	Emulsion stability index
FC	Foaming capacity
FI	Fluorescence intensity
FIS	Isolated Faba bean starch
FIS-C1	Faba bean isolated starch under Condition 1
FIS-C2	Faba bean isolated starch under Condition 2
FPI	Faba bean protein isolate
FPI-C1	Faba bean protein isolated under Condition 1
FPI-C2	Faba bean protein isolated under Condition 2
FS	Foam stability
GOPOD	Glucose oxidase/peroxidase
NS	Not significant
OAC	Oil-absorption capacity
PIS	Isolated pea starch
PIS-C3	Pea isolated starch under Condition 3
PIS-C4	Pea isolated starch under Condition 4

PPI	Pea protein isolates
PPI-C3	Pea protein isolated under Condition 3
PPI-C4	Pea protein isolated under Condition 4
RVA	Rapid Visco Analyser
SEM	Scanning electron microscope
T_c	Conclusion temperature
T_o	Onset temperature
T_p	Peak temperature
WHC	Water-holding capacity
ΔH	Enthalpy change

1. INTRODUCTION

1.1 Overview

Pulse crops belong to the *Leguminosae* family, consisting of edible dicotyledonous seeds (Hoover, Hughes, Chung, & Liu, 2010). Pulse seeds are a rich source of starch, protein, dietary fiber, vitamins, and minerals (Roy, Boye, & Simpson, 2010). Pulses are generally consumed in a whole or split form, and they are used in preparing soups or snack foods at the household level (Sozer, Holopainen-Mantila, & Poutanen, 2017). Recent studies have focused on isolating proteins, starches, and fibers from whole pulse grains, which are useful for producing a wide variety of food products (Escobedo & Mojica, 2021). There are two common methods to fractionate pulse seeds into different ingredients: dry and wet methods (Fernando, 2021). Air classification is the mostly utilized dry method to separate finely ground pulse flours into starch-rich and protein-rich streams based on their density and particle size (Boye, Zare, & Pletch, 2010). Air classification separates the light, fine protein particles from the heavy, coarse starch granules. However, the resultant streams have poor purity (Ren, Yuan, Chigwedere, & Ai, 2021). For example, the starch-rich pulse flours from air classification have 8-20% protein, which thus limits the industrial applications of the starch-rich stream (Li et al., 2019). In addition, the pulse processing industry is interested in recovering the protein from the starch-rich flours. Therefore, the focus of this thesis research was on: (1) determining the optimal conditions to extract starch and protein from air-classified starch-rich faba bean and pea flours using a wet extraction method; (2) characterizing the chemical compositions, structures, and physicochemical properties of the isolated starch and protein.

For the first part of the study, wet extraction process was utilized to isolate starch and protein from commercial air-classified starch-rich faba bean and pea flours. Alkaline extraction followed by isoelectric precipitation was optimized to yield high-purity protein and starch isolates from starch-rich pulse flours. In the second part, the isolated starch and protein samples from the optimized conditions of alkaline extraction/isoelectric precipitation was characterized for their

proximate compositions and functional properties in comparison with those of commercial pea starch and protein isolate, respectively.

This research has advanced our knowledge on developing feasible wet methods to effectively isolate both starch and protein of high purity from air-classified starch-rich flours. The new wet extraction methods can be simply incorporated into the present industrial practices to extract high-purity starch and protein. In addition, the new information regarding the physicochemical properties of the high-purity starch and protein will be useful for their industrial utilization.

1.2 Hypotheses:

The following hypotheses were tested in the research:

- Conditions to separate and purify starch and protein in air-classified starch-rich faba bean and pea flours can be optimized in alkaline extraction/isoelectric precipitation method by testing a range of incubation temperatures and extraction pH conditions.
- The isolated pea and faba bean starches will have purity and techno-functional attributes comparable to those of commercial pea starch.
- The pea and faba bean protein isolates from the air-classified starch-rich flours will have purity and techno-functional attributes similar to those of commercial pea protein isolate.

1.3 Objectives

This study aimed at the following objectives:

- To fractionate starch and protein from air-classified starch-rich pea and faba bean flours using an alkaline extraction/isoelectric precipitation method over a range of incubation temperatures and extraction pH levels.
- To measure the purity and yields of the isolated starch and protein; to determine the optimal isolation conditions using a response optimizer method.
- To characterize the chemical compositions, structures, and physicochemical properties of the isolated pea and faba bean starches in comparison with a commercial pea starch.
- To characterize the chemical compositions and physicochemical properties of the pea and faba bean protein isolates from the air-classified starch-rich flours in comparison with a commercial pea protein isolate.

2. LITERATURE REVIEW

2.1 Importance of pulses for the agri-food industry

Pulses are dried edible seeds of leguminous crops that have been consumed for thousands of years throughout the world (Kumar & Pandey, 2020; Venkidasamy et al., 2019). The commonly consumed pulses include dry beans, dry broad beans, lentils, dry peas, pigeon peas, chickpeas, cow peas, Bambara beans, vetches, and lupins (Campos-Vega, Loarca-Piña, & Oomah, 2010; Nasir & Sidhu, 2012). Pulses are cultivated across diverse geographical regions globally, with North America, Asia, and Australia emerging as the main producers (Roy et al., 2010). Canada is one of the largest pulse producers in the world, and the main pulses cultivated in the country include dry beans, dry peas, lentils, and chickpeas (Statistics Canada., 2022). Canada stands as the largest pulse exporter in the world, which exported 7.46 million tonnes of pulses in 2022 (Bamber et al., 2024). Saskatchewan is the top pulse producer in Canada, contributing to about 80% of Canadian pulse production (Bekkering, 2014).

2.2 Chemical composition of pulses

Pulses are rich in carbohydrates, proteins, dietary fibers, vitamins, minerals, and phytochemicals (Hall, Hillen, & Garden Robinson, 2017; Singh, Singh, Shevkani, Singh, & Kaur, 2017). The starch and protein contents of most pulses are in the range of 40-50% and 15-30%, respectively (Hall et al., 2017). Due to the relatively high protein content of pulses, they are considered as a promising source of plant proteins. Pulse proteins can be classified into two major protein classes, namely globulin and albumin (Singh, 2017). Globulins, accounting for 35–72% of total protein in pulse seeds, serve as the primary storage proteins, with the remaining protein fraction primarily comprising albumins (Dahl, Foster, Tyler, & Tyler, 2012). Pulses contain high contents of amino acids such as lysine, leucine, aspartic acid, glutamic acid, and arginine, but tend to be deficient in sulfur-containing amino acids, including cysteine, methionine, and tryptophan (Tiwari & Singh, 2012). Therefore, it is recommended to consume pulses with cereals or other

foods that are rich in sulfur-containing amino acids to obtain a well-balanced essential amino acid profile (Boye et al., 2010; Venkidasamy et al., 2019).

2.3 Nutritional benefits of pulse consumption

In the past few decades, pulses have drawn considerable research attention, not only for being a sustainable plant protein source but also for their unique nutrient-dense profile (Ferreira, Vasconcelos, Gil, & Pinto, 2021). Even though pulses are mainly composed of carbohydrates, they are considered as a low-glycemic food compared to other carbohydrate-rich foods (Rizkalla, Bellisle, & Slama, 2002). The possible factors responsible for the low-glycemic effect of pulses include intact cell wall structure rich in fiber and protein and the presence of phenolic compounds, which slow down starch digestion (Brummer, Kaviani, & Tosh, 2015; Chibbar, Ambigaipalan, & Hoover, 2010; Dhital, Bhattarai, Gorham, & Gidley, 2016; Setia et al., 2019). Consumption of pulses as low-glycemic food can prevent the risks of coronary heart diseases, type-2 diabetes, obesity, and other metabolic syndrome (Ni, Jia, Ding, Wu, & Yang, 2022; Vlachos, Malisova, Lindberg, & Karaniki, 2020). Pulses are an excellent plant-protein source for vegetarians and vegans to ensure adequate intake of proteins (Havemeier, Erickson, & Slavin, 2017). The protein content of pulses is two-fold that of whole-grain cereals, and they are rich in lysine, which is usually the primary limiting amino acid of cereal grains (Mudryj, Yu, & Aukema, 2014). Cereals, such as wheat and rice, have become the staple foods of the human diet around the world, and the protein quality of those diets can be significantly improved by consuming pulses along with cereals, specially for vegetarians and vegans (Awika, 2011).

In addition to macronutrients, pulses provide good amounts of vitamins and minerals (Hall et al., 2017; Mudryj et al., 2014). They are abundant sources of vitamin A, vitamin B complex (thiamin, niacin, folate, and riboflavin), and vitamin E (Hall et al., 2017; Mudryj et al., 2014; Ofuya & Akhidue, 2005; Liu et al., 2022). Furthermore, pulses have relatively high levels of essential minerals such as potassium, magnesium, manganese, selenium, iron, and zinc, which are important for human growth and development (Singh, 2017). Pulses are rich in phenolic compounds such as phenolic acids, tannins, and flavonoids (Singh, Singh, Kaur, & Singh, 2017). These polyphenolic compounds have anti-tumoral, antiplatelet, anti-inflammatory, and anti-allergic properties that are helpful in protecting living systems against oxidative damage (Singh, 2017). Previous studies have

suggested that lentils, black beans, and red kidney beans have higher antioxidant capacity due to the higher phenolic content found in those pulses (Xu & Chang, 2008).

2.4 Food uses of pulses

It is projected that the world population will reach 9.7 billion by 2050 (Lu & Grundy, 2017). To adequately meet the rising demand for food, scientists suggest that food production needs to increase by 40% to 90% to cater the nutritional needs of the constantly growing global population (Rajpurohit & Li, 2023). While animal protein-based diets remain highly popular, the conventional animal food production system is facing the challenge of sustainability (Calicioglu, Flammini, Bracco, Bellù, & Sims, 2019; Li, 2020). Therefore, plant-based proteins, especially pulse proteins, provide a promising solution as a sustainable alternative protein source (Rajpurohit & Li, 2023). Pulses are conventionally consumed in a cooked or canned form of whole or split grains (Li et al., 2019). However, with the recent developments in the pulse processing industry and market trend toward sustainable vegan-protein sources, the agri-food industry is now focusing on fractionating pulses into their main components such as starch, protein, and fiber, which are popular ingredients that can significantly expand the use of pulses in the human diet (Allotey, Kwofie, Adewale, Lam, & Ngadi, 2022; Pelgrom, Boom, & Schutyser, 2015).

2.5 Pulse fractionation

Pulse cotyledons consist of starch granules embedded in protein and fiber matrices, and fractionation is a value-added process that involves separating and extracting those proteins, starches, and dietary fibers from pulses (Boye et al., 2010; Fernando, 2021). Generally, there are two common pulse fractionation methods: dry and wet fractionation (Hoover et al., 2010). Fractionation of pulses into starch and protein will offer functional advantages and nutritional benefits that can be customized into different food products.

2.5.1 Dry fractionation

Dry fractionation of pulses is a more sustainable way of producing protein-rich and starch-rich fractions from pulses (Rivera, Siliveru, & Li, 2022). Air classification is the most common dry fractionation method, which involves dehulling, pin milling, and air classification of starch and protein to produce starch-rich and protein-rich fractions (Assatory, Vitelli, Rajabzadeh, &

Legge, 2019; Ren et al., 2021). As the first step of dry fractionation, dried whole seeds undergo dehulling to remove the outer hull or seed coat (Fernando, 2021). This step helps to improve the nutritional quality of starch-rich and protein-rich flours by reducing the presence of anti-nutritional factors, which tend to be more concentrated in the seed coat (Kumar et al., 2022). Further, other researchers have found that dehulling of pulses can improve the techno-functional properties of the resultant fractions (Pulivarthi et al., 2023).

After dehulling, pulse grains are subjected to milling to reduce the particle size (Thakur, Scanlon, Tyler, Milani, & Paliwal, 2019). Milling releases starch granules that are entrapped in the protein/fiber matrix of pulse cotyledons (Chigwedere et al., 2018). Various milling techniques, including roller milling, pin milling, and hammer milling, can be utilized to grind pulse grains to achieve desired particle sizes for intended applications (Pulivarthi et al., 2023). For air classification, pin milling is most commonly applied, which can lead to a substantial difference in the sizes of generated starch granules (≥ 18 μm) and protein fragments (1–3 μm), enabling them to be separated through air classification (Schutyser, Pelgrom, Van der Goot, & Boom, 2015). During air classification, finely ground pulse flour and an air stream are introduced into a chamber, where centrifugal and gravitational forces act jointly to separate the flour into light, fine particles and heavy, coarse particles based on their size and density (Grasso, Lynch, Arendt, & O'Mahony, 2022). Based on the composition of these fractions, the light and fine fraction is called protein-rich flour, and the coarse and heavy fraction is called starch-rich flour (Fernando, 2021).

Some of the most promising features of air classification over wet fractionation are that it does not utilize water or chemical reagents and that it is energy efficient (Fernando, 2021; Pulivarthi et al., 2023; Schutyser et al., 2015). Therefore, the former is considered as an economical and sustainable method to process pulses into starch-rich and protein-rich streams (Skylas et al., 2024). However, there are major drawbacks in this dry method such as the poor separation efficiency, which leads to poor purity in the resultant starch-rich and protein-rich fractions (Ren et al., 2021). The poor separation efficiency of air classification is mainly due to the intricate arrangement of protein and starch within pulse cotyledons (Boye et al., 2010). In pulse cotyledons, starch granules are entrapped in cellular structure rich in protein and fiber and thus there are strong physical interactions among starch, protein, and fiber (Dhital, Bhattarai, Gorham, & Gidley, 2016; Setia et al., 2019). This intricate association makes their separation via dry fractionation a challenging task (Boye et al., 2010; Tyler, 1984). Moreover, the random air turbulence and

collisions between particles within the air classifier further intensifies the difficulty in achieving satisfactory separation of starch and protein (Pulivarthi et al., 2023).

2.5.2 Wet fractionation

Wet fractionation method has been widely used to produce commercial pulse protein isolates and concentrates over the past few decades (Berghout, Boom, & Van Der Goot, 2014). This process typically involves milling pulse seeds into pulse flours and separation of pulse starch and protein in an aqueous medium (Schutyser & van der Goot, 2011). There are several wet extraction techniques to isolate protein and starch from pulse flours such as alkali extraction/ isoelectric precipitation, acid extraction, salt extraction and ultrafiltration (Boye et al., 2010).

2.5.2.1 Alkali extraction/ isoelectric precipitation

Alkaline extraction/isoelectric precipitation (AEIP) is a common extraction technique that exploits the varying solubility of proteins at different pHs to extract high-purity protein isolates or concentrates (Lam, Can Karaca, Tyler, & Nickerson, 2018). Generally, legume proteins are highly soluble at alkaline pHs, whereas they show a minimum solubility at their isoelectric point of around pH 4-5 (Cruz-Solis, Ibarra-Herrera, Rocha-Pizaña, & Luna-Vital, 2023). In the AEIP method, pulse flour is dispersed in water, and the pH of the flour-water mixture is elevated to an alkaline level using NaOH or KOH. The mixture is then allowed to stand for a period of time with continuous stirring to facilitate protein solubilization. Henceforth, the mixture is filtered or centrifuged to remove insoluble starch and fibers, and the pH of the soluble fraction is adjusted to the isoelectric point to induce protein precipitation. The recovered protein is washed, neutralized, and dried to produce protein isolates or concentrates (Berghout et al., 2014; Boye et al., 2010; Cruz-Solis et al., 2023; Gao et al., 2020; Schutyser & van der Goot, 2011). Under the influence of alkaline conditions, disulfide bonds in the proteins break up, which results in protein dissolution (Jinjie Yang et al., 2023). During isoelectric precipitation, the solubility of proteins drops as their net charge is adjusted close to zero, which is achieved by altering the pH of the medium (Meng, Ma, & Fitzgerald, 2018). One of the challenges in this AEIP method is determining the optimum processing parameters to achieve a higher yield with satisfactory purity (Rivera et al., 2022). Critical processing parameters include flour to water ratio, extraction pH, extraction temperature, and extraction time (Russin, Arcand, & Boye, 2007). Therefore, many research studies have been

carried out to evaluate the effects of the applied extraction parameters on protein yield, purity, and techno-functional properties (Cui et al., 2020; Gao et al., 2020; Higa, Boyd, Sopiwnyk, & Nickerson, 2022).

2.5.2.2 Acid extraction

During acid extraction, the pH of the flour-water mixture is lowered to below 4 to facilitate the solubilization of pulse proteins, which is followed by isoelectric precipitation or membrane filtration to separate proteins from the insoluble components (Boye et al., 2010). Similar to alkaline extraction, the adopted extraction conditions can influence the yield and purity of the protein isolates or concentrates (Boye et al., 2010; Rahman & Lamsal, 2021). Unlike alkaline extraction, acid extraction is less popular because it tends to have a lower protein yield (Rahman & Lamsal, 2021). This is attributable to the lesser cell wall degradation and reduced protein solubility near isoelectric point as compared to alkaline extraction method (Pojić, Mišan, & Tiwari, 2018).

2.5.2.3 Salt extraction and micellization

The salt extraction process is based on the salting-in and salting-out phenomena of plant proteins (Liu et al., 2022). During salt extraction, flour is dispersed in a salt solution with a specific ionic strength to assist with the solubilization of proteins. Subsequently, insoluble matter is removed by centrifugation or filtration and the solubilized proteins in the supernatant are further extracted (Lam et al., 2018). To induce protein precipitation, the ionic strength of the supernatant is reduced by dilution, and this process is known as micellization (Shanthakumar et al., 2022). Upon dilution, solubilized proteins form low-molecular-weight aggregates and at a specific protein concentration these aggregates reassociate to form thermodynamically stable spheres called micelles (Lam et al., 2018; Shanthakumar et al., 2022). Because micelle formation requires time, the diluted salt solution is allowed to stand for a while to maximize the micellization prior to centrifugation and drying (Vogelsang-O'Dwyer et al., 2020). Salt extraction is a selective process as various proteins precipitate at distinct ionic strengths. Therefore, the type of salt and its concentration are dependent upon the characteristics of the protein that is targeted for extraction (Boye et al., 2010; Lam et al., 2018). One of the advantages of salt extraction over alkaline or acid extraction is that salt extraction does not need elevated temperatures or extreme pH levels to extract proteins (Muranyi et al., 2016).

Compared to dry fractionation, wet fractionation produces protein isolates or concentrates with higher purity (Rajpurohit & Li, 2023). However, the wet fractionation process requires more energy, water, and other resources and generates a large volume of effluent compromising environmental sustainability (Assatory et al., 2019). Also, the pH and temperature conditions used in wet fractionation may result in denaturation of native protein structure, thereby altering the protein functionality (Assatory et al., 2019; Boye et al., 2010).

2.5.2.4 Hybrid fractionation

Hybrid fractionation methods adopt elements from both dry and wet fractionation techniques, thus harnessing the strengths of each approach (Ren et al., 2021). The goal of such hybrid fractionation methods is to improve the protein and starch separation efficiency while increasing the process sustainability (Rivera et al., 2022). Recent studies have tested these hybrid methods for isolating starch and protein from different pulse varieties, and the results suggest that such hybrid fractionation methods can produce starch and protein streams possessing acceptable purity and functionality with minimal processing efforts and costs (Dumoulin, Jacquet, Malumba, Richel, & Blecker, 2021; Li et al., 2019; Pelgrom et al., 2015; Yang, Eikelboom, van der Linden, de Vries, & Venema, 2022).

2.6 Structure, functional properties and industrial uses of pulse starch

2.6.1 Granular morphology and structure of pulse starches

Present in a granular form, starch is the primary carbohydrate reserve in pulse cotyledons (Ren et al., 2021). These granules display various shapes and sizes, depending on the specific pulse variety. The majority of pulse starch granules exhibit oval or spherical shape, with average sizes ranging from 19 to 28 μm (Ma, Wang, Wang, Jane, & Du, 2017). Generally, starch granules have a smooth surface, but their surface can become scratched or damaged due to physical damage (*e.g.*, during milling) (Li et al., 2019). Starch granules primarily consist of two α-glucans: amylose and amylopectin (Bertoft, 2017). Amylose is an essentially linear molecule that comprises D-glucopyranosyl units primarily linked by α-(1,4) linkages, whereas amylopectin is a highly branched molecule that comprises α-(1,4) and α-(1,6) (around 5%) linked D-glucopyranosyl units (Kabir et al., 2022). Pulse starches generally have a higher amylose content of 30-40%, whereas cereal starches have an amylose content of 15-30% (Ren et al., 2021). The degree of

polymerization (DP) of amylose in pulse starches is approximately 1100 – 1400, which is significantly higher than that of normal corn starch (Li et al., 2019; Raghunathan, Hoover, Waduge, Liu, & Warkentin, 2017; Zhou, Hoover, & Liu, 2004).

In waxy and normal starches, amylopectin branch chains form double helices, which can further develop into lamellar crystalline structure within granules (Jane, 2006). These amylopectin branch chains can be classified into A, B1, B2, B3, and B4 chains based on their lengths and the organization in the clusters of amylopectin (Ren et al., 2021). Amylopectins of pulse starches contain smaller proportions of short branch chains of DP 6-12 but larger proportions of longer branch chains of DP 13-24 and DP 25-36 when compared with those of normal maize and tapioca starches (Li et al., 2019). In pulse starches, amylose is present in amorphous regions, interspersed and intertwined among amylopectin (Figure 2.1) (Jane, 2006). Starches from different botanical sources show different crystalline structures based on their contents and sizes of double-helical crystallites and different packing patterns (Hoover et al., 2010; Ren et al., 2021). Crystalline structures of starches can be revealed using X-ray diffraction analysis. According to Hizukuri, Kaneko, and Takeda (1983), there are three distinct crystalline patterns of native starches, namely A-, B-, and C-type. Pulse starches typically exhibit the C-type X-ray diffraction pattern, which is a mixture of A- and B-type polymorphs (Bogracheva, Morris, Ring, & Hedley, 1998). It has been suggested that B-type polymorphs are located in the center of a pulse starch granule, surrounded by A-type polymorphs in the periphery (Bogracheva, Morris, Ring, & Hedley, 1998; Wang, Yu, & Yu, 2008).

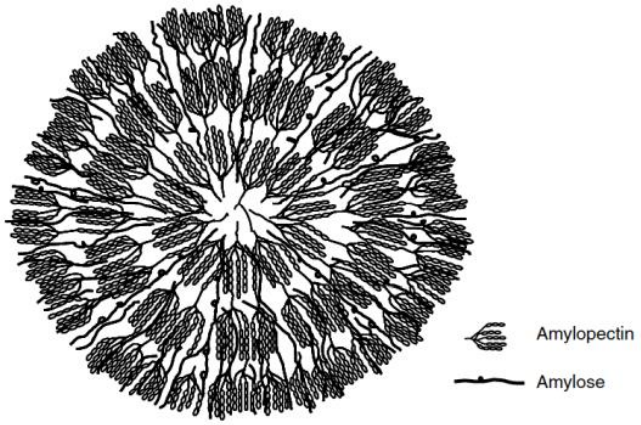

Figure 2.2 Schematic organization of a normal starch granule (Jane, 2006).

2.6.2 Functional properties of pulse starch

Gelatinization and retrogradation characteristics: When starch is subjected to heating in excess water, the starch granules undergo a phase transition from order to disorder, a process referred to as gelatinization (Ratnayake & Jackson, 2008). In the presence of heat and water, double-helical crystallites in starch granules are dissociated and the crystalline structure is gradually lost (Ren et al., 2021). This thermal transition is usually required for industrial applications of starch (Ratnayake & Jackson, 2008). Starch gelatinization can be analyzed using differential scanning calorimetry (DSC) (Ai & Jane, 2015). The method can measure starch gelatinization temperature and enthalpy change (ΔH), which reflect the thermal stability of starch double-helical crystallites, and the energy required to melt them, respectively (Soler et al., 2020). Starch gelatinization is not an instantaneous process; instead, it occurs progressively over a range of temperature. Therefore, starch gelatinization temperatures are usually reported as onset temperature (T_o), peak temperature (T_p), and conclusion temperature (T_c) (Li et al., 2019). After gelatinized starch is subjected to cooling, dissociated amylose and amylopectin chains partially re-form ordered structures, a process known as starch retrogradation (Ren et al., 2021). Starch retrogradation is associated with a series of physical changes, including viscosity increment, gel formation, water expulsion (syneresis), and rearrangement and reassociation of amylopectin branch chains (Wang, Li, Copeland, Niu, & Wang, 2015). As a result of retrogradation, the relative crystallinity of starch increases. Retrogradation process can take up to a few weeks to complete, in particular for amylopectin molecules, and there are intrinsic and extrinsic factors affecting the rate and extent of starch retrogradation (Chang, Zheng, Zhang, & Zeng, 2021). The primary extrinsic factor affecting starch retrogradation is the storage temperature of gelatinized starch (Aguirre, Osella, Carrara, Sánchez, & Buera, 2011). Starch usually shows the maximum retrogradation rate at a storage temperature close to 4°C (Ding, Zhang, Tan, Fu, & Huang, 2019). Some of the intrinsic factors of starch that affect starch retrogradation include branch-chain-length distribution of amylopectin and amylose content (Ambigaipalan, Hoover, Donner, & Liu, 2013). Pulse starches commonly exhibit a higher retrogradation due to their higher proportions of DP 13-24 branch chains and greater amylose content as compared to maize and tapioca starches (Li et al., 2019).

Pasting and gelling properties: When subjected to heating and stirring in water, starch granules gradually lose its granular structure and some amylose and small amylopectin molecules

leach out, contributing to the development of high viscosity (Ren et al., 2021). This phenomenon is known as starch pasting, a unique property of starch closely linked to gelatinization (BeMiller, 2011). Amylopectin is mainly responsible for the initial viscosity development during heating, whereas amylose restricts granular swelling (Tester & Morrison, 1990). Amylose tends to maintain the granular integrity, and upon cooling it facilitates intermolecular re-association, resulting in a higher final viscosity (Li et al., 2019). Pasting profile of starch is often studied using Rapid Visco Analyser (RVA), Brabender Amylograph, or rheometer (Ai & Jane, 2015). Pasting profile of starch depicts the dynamic viscosity development of starch as a function of heating temperature and time. Key parameters of starch pasting curve include pasting temperature, peak viscosity, breakdown viscosity, setback viscosity, and final viscosity (Ren et al., 2021). Generally, pulse starches show a lower peak viscosity and higher final viscosity compared to normal maize starch, primarily due to the higher amylose content of the former (Liu et al., 2019).

When starch paste is transferred to a mold and subjected to cooling, certain samples can form a self-standing and viscoelastic structure known as starch gel (Wang, Liu, & Ai, 2022). During the gelation process, swollen starch granules with good integrity deform each other to hold the shape of the mold and leached-out amylose molecules play a role of a glue to further strengthen the gel network (Ren et al., 2021; Wang et al., 2022). The gelling properties of starch can be studied using either a texture analyzer or a rheometer: the former typically measures the strength of a starch gel through a uniaxial compression test, while the latter is able to continuously determine rheological properties of a starch gel corresponding to heating/cooling temperature, shear stress and rate, and frequency sweep *etc.* (Ai & Jane, 2015; Wang et al., 2022). Several important intrinsic and extrinsic factors influence the gelling capability of starch, and the presence of swollen starch granules with good integrity after cooking in water is critical for the gelation of normal starches (Ren et al., 2024; Wang et al., 2022). Normal starches with a relatively high amylose content, such as pulse starches (containing 30-40% amylose), can better maintain their granular integrity during heating in water, which can thus develop firmer gels than waxy and other normal starches (Li et al., 2019).

2.6.3 Industrial applications of pulse starches

Pulse starches offer versatile and sustainable options for various industrial applications due to their unique functional properties (Li et al., 2022). Pulse starches are often used as a substitute

for wheat-based ingredients in preparing gluten free food products (Woomer & Adedeji, 2021). Moreover, these starches can act as binders, thickeners, and gelling agents in different food products such as sauces, canned foods, desserts, and processed meat products (Ren et al., 2021). These starches can be modified chemically or enzymatically to produce different food ingredients as well as food additives (Zha, Rao, & Chen, 2021). Extensive research efforts have been made to produce starch-based bioplastics as an alternative to petroleum-based plastics (Bai, Portillo-Perez, Petronilho, Gonçalves, & Martinez, 2024; Falua, Babaei-Ghazvini, & Acharya, 2024). Furthermore, these pulse starches can be used as bulking agents in pharmaceutical industry (Ratnayake & Naguleswaran, 2022).

2.7 Structure, functional properties, and industrial uses of pulse proteins

2.7.1 Structure of pulse proteins

Over recent years, there has been a notable surge in the demand for plant proteins, largely driven by population growth, dietary shifts, a growing focus on environmental sustainability, heightened health consciousness, and ethical considerations. Pulses, such as pea, lentil, faba bean, have emerged as an alternative plant protein source with protein abundance and nutritional quality (Nadeeshani, Senevirathne, Somaratne, & Bandara, 2022). The protein content in pulses can vary from 20% to 30% depending on the pulse variety, genotypes, and environmental conditions (Singh, 2017). Pulses contain most of the essential amino acids but relatively low in sulfur-containing amino acids such as methionine, cysteine, and tryptophan (Shevkani, Singh, Chen, Kaur, & Yu, 2019).

Pulse proteins are dominated by globulins and albumins with low levels of prolamins and glutelins (Bessada, Barreira, & Oliveira, 2019). Globulins are salt-soluble proteins that represent approximately 70% of the total protein in pulses (Shevkani et al., 2019). Pulse globulins can be classified into two fractions as legumins (11S) and vicilins (7S) based on the sedimentation coefficient (S = Svedberg Unit) (Nadeeshani et al., 2022). Legumins typically consist of six subunits (hexamer), each weighing around ~60 kDa, resulting in an overall molecular weight ranging from ~300 - 400 kDa of legumins (Singhal, Can Karaca, Tyler, & Nickerson, 2016). In contrast, vicilin is a trimer that is made of 3 subunits with an average molecular weight of ~150 – 200 kDa (Gravel & Doyen, 2023). Vicilin can be divided into several subcategories as β-conglycinin, γ-conglycinin, and basic 7S globulin (Bg) (Singh, Meena, Kumar, Dubey, & Hassan,

2015). Legumin typically exhibits a quaternary protein structure characterized by numerous disulfide bonds, imparting greater rigidity (Li et al., 2021). Also, legumin are rich in sulfur-containing amino acids and arginine, while vicilin has other non-sulfur containing amino acids such as isoleucine, leucine, phenylalanine, and lysine (Lam et al., 2018). There is a third type of globulin present in some pulse varieties, which is known as convicilin (Shevkani et al., 2019). Convicilin is primarily present in peas, featuring sulfur-containing amino acids along with a small carbohydrate subunit (Boye et al., 2010). Albumins are water-soluble proteins that constitute 10%–20% of the total pulse protein with an average molecular weight of ~5-80 kDa (Shevkani et al., 2019). Pulse albumins typically exhibit higher levels of cysteine and methionine compared to pulse globulins (Tripathi et al., 2021). Most of the enzymes, and antinutritional factors, such as lectins, amylase inhibitors, protease inhibitors found in pulses, are primarily classified under albumins (Lu, He, Zhang, & Bing, 2020).

2.7.2 Functional properties of pulse proteins

Solubility: Protein solubility plays a vital role in determining their associated functional properties such as emulsifying, foaming and gelation properties (Bessada et al., 2019). The solubility of pulse proteins is influenced by various intrinsic and extrinsic factors. Among them surface hydrophobicity and surface charge of proteins are crucial factors in determining protein solubility (Shevkani & Singh, 2015). Generally, an abundance of hydrophobic patches on protein surfaces tends to hinder the protein solubility, while proteins with a net charge on their surface exhibit higher solubility (Karaca, Low, & Nickerson, 2011). In addition, external factors such as pH, temperature, type, and concentration of salts present in the medium also have a significant effect on pulse protein solubility (Grossmann & McClements, 2023). Most of pulse proteins exhibit a higher solubility around acidic and alkaline pH, whereas the solubility will be minimized around their isoelectric point (~ pH 4.5) (Boye et al., 2010). At the isoelectric point (pI), proteins carry no net charge, resulting in minimal electrostatic repulsion between protein molecules (Guldiken, Saffon, Nickerson, & Ghosh, 2023). Consequently, protein-protein interactions become favorable, often leading to aggregation of proteins (Zhang, Jeganathan, Dong, Chen, & Vasanthan, 2021). The presence of salts can also influence the solubility of proteins (Tanger, Müller, Andlinger, & Kulozik, 2022). Certain salts have the ability to modify hydration layer around protein molecules by either promoting protein-water interactions or salt-water interactions

(Lam et al., 2018). When protein-water interactions are favored, proteins may undergo salt-induced solubilization. Conversely, when salt-water interactions are promoted, proteins may experience salt-induced aggregation (Tiwari & Singh, 2012). Generally, calcium salts promote protein solubility, whereas ammonium salts can hinder the solubility of proteins, depending on their ionic strength (Shevkani et al., 2019).

Karaca et al. (2011) studied the solubility of various protein isolates from pulses prepared using alkaline extraction and isoelectric precipitation. They found that pea protein isolates exhibited the lowest solubility at 61.4%, while isolates from faba bean, lentil, and chickpea had solubility values greater than 90%. More recent research by Higa, House, and Nickerson (2024) reported variations in the solubility for legume proteins prepared with alkaline extraction and isoelectric precipitation for yellow pea, green lentil, and chickpea protein isolates with solubility values of 66.30%, 57.59%, and 74.85%, respectively.

Water-holding and oil-absorption capacities: Water-holding (WHC) and oil-absorption (OAC) capacities of proteins are important to enhance the food texture, mouthfeel, and flavor of food products (Shevkani, Kaur, Kumar, & Singh, 2015). WHC can be defined as the amount of water that can be held by one gram of protein material (Boye et al., 2010). WHC of proteins plays a crucial role in developing foods that absorb water without dissolving, as seen in dishes like soup and custard (Sreerama, Sashikala, Pratape, & Singh, 2012). WHC of pulse proteins primarily stems from their hydrophilic components, including the polar and charged side chains of proteins and carbohydrates (Patil et al., 2024). These elements possess a higher affinity to water molecules, thereby influencing the WHC of proteins. WHC of pulse proteins may vary, depending on factors such as the pulse type and variety, purity of proteins, and the technique used for protein extraction. OAC of proteins plays a crucial role in developing foods such as meat substitutes and baked foods where fat contributes to the mouthfeel and flavor of the foods (Aryee, Agyei, & Udenigwe, 2018; Awuchi, Igwe, & Echeta, 2019). Similar to WHC, OAC can be defined as the amount of oil that can be absorbed by one gram of protein (Boye et al., 2010). Typically, the absorption between protein and oil occurs through physical interactions between the nonpolar side chains of amino acids and the aliphatic chains present in oils and fats (Shevkani et al., 2019). Variations in OAC are observed among proteins sourced from different pulses (Vioque, Alaiz, & Girón-Calle, 2012).

Higa et al. (2024) reported the WHC and OAC of yellow pea, green lentil, and chickpea protein isolates prepared using the abovementioned AEIP method. The WHC values were 1.72,

1.34, and 1.69 g/g, respectively, whereas the OAC of these isolates at pH 7.0 were reported to be 1.27, 0.64, and 1.05 g/g respectively. In a different study, Stone, Karalash, Tyler, Warkentin, and Nickerson (2015) found that the WHC of pea protein isolates extracted using AEIP method ranged from 2.4 to 2.6 g/g for different pea cultivars. They also reported the OAC of these protein isolates to be between 3.5 and 3.8 g/g.

Emulsifying properties: An emulsion is a dispersion of two immiscible liquids where one liquid is dispersed in the form of small droplets within another liquid (Lam & Nickerson, 2013). Such emulsions can be simply classified as either water-in-oil (W/O) emulsions or oil-in-water (O/W) emulsions (Sousa, Pereira, & Matos, 2022). Other than these emulsions, there are some advanced emulsion systems with multiple phases such as W/O/W or O/W/O and nano-emulsions (Tan & McClements, 2021). Emulsions are formed by applying mechanical shear to liquid mixture using a homogenizer (Schultz, Wagner, Urban, & Ulrich, 2004). Emulsion are thermodynamically unstable systems due to the increased interfacial surface tension (Shevkani et al., 2019). Therefore, in industry, emulsifiers are used to make stable emulsions and to prevent coalescence of droplets (Ravera, Dziza, Santini, Cristofolini, & Liggieri, 2021). Such emulsifiers typically have both hydrophilic and hydrophobic moieties, enabling them to effectively position at the oil-water or water-oil interface, thus reducing interfacial tension (Bos & Van Vliet, 2001). Interestingly, pulse proteins can also act as emulsifiers to stabilize such system by decreasing interfacial tension and preventing coalescence (Karaca et al., 2011). These proteins have hydrophilic and hydrophobic moieties facilitating their orientation at the oil-water interface and subsequent formation of an elastic layer surrounding the oil droplets (Dickinson, 2010). Moreover, proteins contribute to the viscosity of the continuous phase, thereby impeding the rate of coalescence of droplets (Sikorski, 2001). The ability of proteins to stabilize emulsions stands out as an important functional attribute that helps in developing various food items such as comminuted meats, coffee whiteners, soups, and ice creams (Shevkani et al., 2019). Emulsifying properties of pulse proteins can be measured as emulsion capacity (EC) and emulsion stability (ES) (Karaca et al., 2011). Emulsion capacity defines the amount of oil that can be emulsified by a standard amount of protein under a specific set of conditions, and emulsion stability measures the stability of the same emulsion over a defined time period (Pearce & Kinsella, 1978). There are many intrinsic and extrinsic factors influencing the EC and ES of pulse proteins. Intrinsic factors that influence the EC and ES include pulse variety, composition and molecular flexibility of proteins, protein solubility, surface

hydrophobicity of proteins, and charge on protein surface, whereas temperature, pH, and ionic strength are the most influential extrinsic factors (Dickinson, 2003; McClements, 2007).

EC and ES of pea protein isolates prepared by alkali extraction-isoelectric precipitation have been investigated by Stone et al. (2015). For different pea varieties EC and ES were ranging from 187.5-193.7% and 96.7-99.9% respectively. Karaca et al. (2011) studied the EC of various protein isolates from pulses prepared using alkaline extraction and isoelectric precipitation. They found that chickpea, faba bean, lentil and pea protein isolates exhibited the EC values ranging from 477.78-520.0 g oil/g protein. In a recent study, Higa et al. (2024) studied the emulsion activity index (EAI) and emulsion stability index (ESI) of yellow pea, green lentil and chickpea protein isolates. Results revealed that the protein isolates had EAI ranging from 23.68 to 28.12 m^2/g and ESI ranging from 11.90-12.58 min.

Foaming properties: Foams are found in diverse food applications, such as cakes, ice creams, whipped toppings, meringues, and mousses (Boye et al., 2010). Foam can be defined as a two-phase system consisting of air cells separated by a thin continuous liquid layer called the lamellar phase (Zayas, 1997). Generally, foams form by incorporating air onto liquid through whipping or shaking (Hoc & Haznar-Garbacz, 2021). Similar to emulsion systems, foams are also thermodynamically unstable and thus foaming agents are used to stabilize these foams (Dinache, Pascu, & Smarandache, 2021). Pulse proteins can function as foaming agents by creating an interfacial protein film around the air bubble, which hinders coalescence (Yang et al., 2023). Foaming properties of pulse proteins are measured as foaming capacity (FC) and foam stability (FS). Foaming capacity of proteins is referred to as the percentage volume increase due to whipping, and foam stability is measured as the change in the volume of foam over a specific time period (Boye et al., 2010). Foaming properties of pulse proteins are significantly influenced by the surface charge, solubility, and size of the molecules (Shevkani et al., 2019). If proteins are highly soluble, they can diffuse faster to the air-water interface to stabilize the foam (Zayas, 1997). Moreover, external factors, such as foaming speed, pH of the medium and testing temperature, also plays a vital role in the formation of stable foams (Amagliani, Silva, Saffon, & Dombrowski, 2021).

Higa et al. (2024) studied the foam capacity (FC) and foam stability (FS) of yellow pea, green lentil, and chickpea protein isolates, finding FC values ranging from 220% to 247.8% and FS values ranging from 87.5% to 89.2%. In another study, Stone et al. (2015) analyzed the FC and

FS of pea protein isolates produced by alkali extraction and isoelectric precipitation. Their results showed that pea protein isolates had FC values of 155.0% to 183.3% and FS values of 68.0% to 69.6%, respectively.

2.7.3 Industrial applications of pulse proteins

Pulse proteins have a growing market demand due to their nutritional value, affordability, functional attributes, and health promoting effects (Vogelsang-O'Dwyer, Zannini, & Arendt, 2021). Consequently, advancements in food processing techniques have enabled the efficient production of pulse protein-rich flours, concentrates, and isolates, which can be incorporated into different food preparations and formulations (Nadeeshani et al., 2022). Pulse proteins are extensively utilized in producing meat analogues such as sausages, meatballs, Salisbury steaks as a meat replacer as well as gelling, emulsifying, texturizing, water, and fat-binding agents (Sha & Xiong, 2020). Furthermore, pulse proteins can be utilized in producing baked goods, textured vegetable proteins, pasta, noodles, and extruded snacks (Rajpurohit & Li, 2023). Pulse proteins are also utilized in non-food applications, such as the production of cosmetics, packaging materials and microencapsulation of bioactive ingredients (Nadeeshani et al., 2022).

3. EXTRACTING PROTEIN AND STARCH FROM STARCH-RICH PULSE FLOURS USING AN ALKALINE EXTRACTION/ISOELECTRIC PRECIPITATION METHOD WITH OPTIMIZATION

3.1 Abstract

Starch-rich pulse flours are recognized as an underutilized and low-value by-product from air classification of pulses, and their industrial use is limited mainly due to the presence of impurities (*e.g.*, proteins, dietary fibers, flavor and aroma compounds). Therefore, this study aimed to develop an effective wet isolation method that can further separate starch and protein from starch-rich pulse flours. Alkaline extraction was performed on air-classified starch-rich faba bean and pea flours over a range of pH (8.5, 9.5, 10.5, and 11) and temperature (25, 30, 40, and 50 °C) to identify the optimum pH and temperature combination to extract starch and protein with maximum yields and purity. The isolated faba bean and pea starches and the protein isolates extracted under different alkaline conditions had a purity of 95.0-98.6% and 79.7-93.1% respectively. This research provided valuable insights into the optimum conditions to extract starch and protein with high yields and purity for potential value-added utilization in the industry.

3.2 Introduction

Pulses are dried edible seeds of leguminous crops that have been consumed for thousands of years in different regions of the world (Kumar & Pandey, 2020; Venkidasamy et al., 2019). Common market classes of pulses include peas, lentils, faba beans, dry beans, and chickpeas (Ren et al., 2021). Pulses are known as a rich source of nutrients that are essential for human health, including carbohydrates, proteins, vitamins, minerals, and phytochemicals (*e.g.*, isoflavones, phytosterols, and saponins) (Langyan et al., 2022). Over the past few decades, pulses have received continuous research interest not only as a sustainable source of protein but also for their health benefits, such as improved digestion, better weight management, and reduced risk of chronic diseases such as coronary heart diseases and diabetes due to their low-glycemic effect (Ferreira et al., 2021).

However, their beneficial health effects, cost-effectiveness, environmental sustainability, ethical considerations, and the growing trend towards vegetarian and vegan diets have increased the demand for pulse proteins as sustainable plant-based options (Hoover et al., 2010). As a result, the pulse processing industry is focusing on fractionating pulses into their major components, such as starch, protein, and fiber. (Allotey et al., 2022; Pelgrom et al., 2015). Two common fractionation methods are applied for this purpose, namely wet fractionation and dry fractionation (Hoover et al., 2010).

In the pulse industry, wet fractionation has been widely used to produce commercial pulse protein isolates and concentrates (Berghout et al., 2014). The process in general involves wet milling of pulse seeds and separation of starch and protein in an aqueous medium (Schutyser & van der Goot, 2011). Different wet extraction techniques can be employed to isolate starch and protein from pulse flours, including alkaline extraction/isoelectric precipitation, acid extraction, salt extraction, and ultrafiltration (Boye et al., 2010). By contrast, dry fractionation usually involves dehulling and milling of pulse seeds into flours and dry separation of the flour into starch-rich and protein-rich fractions (Fernando, 2021). Air classification is one of the common dry fractionation methods, which separates starch and protein particles based on their size and density (Li et al., 2019). During air classification, finely ground pulse flour and an air stream are introduced into a chamber, where centrifugal and gravitational forces act jointly to separate the flour into light, fine particles and heavy, coarse particles based on their size and density (Grasso, Lynch, Arendt, & O'Mahony, 2022). Based on the composition of these fractions, the light and fine fraction is

called protein-rich flour, and the coarse and heavy fraction is called starch-rich flour (Fernando, 2021). In comparison with wet fractionation, dry fractionation has the advantages of less time-consuming, more environment friendly, and higher energy efficiency; however, the obtained starch-rich and protein-rich streams from dry fractionation have poorer purity (Ren et al., 2021). Moreover, dry fractionation in general results in a higher level of starch damage in the final product, whereas wet fractionation typically leads to less starch damage (< 2% damaged starch) (Li et al., 2019; Naguleswaran & Vasanthan, 2010).

In Canada, a significant portion of pulses is fractionated using dry methods to produce protein-rich flours (*i.e.*, protein concentrates), which are utilized as popular ingredients in various products in the food industry (Boye et al., 2010). However, starch-rich flour is generated as a by-product during the process, which has limited application (Fernando, 2021). In general, air-classified starch-rich pulse flours contain 60-70% starch and 8-20% protein (Li et al., 2019). The pulse industry is interested in recovering the starch and protein from the starch-rich flours, which can potentially expand industrial utilization of this by-product. A previous study has shown that alkaline extraction of proteins at pH 9.5 from air-classified starch-rich pulse flours produced starch with high purity (Li et al., 2019). However, there is a lack of knowledge regarding extracting both starch and protein simultaneously from starch-rich pulse flours. Thus, the current study aimed to identify the optimum conditions for extracting starch and protein simultaneously from air-classified starch-rich pulse flours using wet fractionation. Two representative air-classified starch-rich pulse flours from pea and faba bean were included as the raw materials in this study. Alkaline extraction was optimized for simultaneous separation of starch and protein from starch-rich flour and under four different extraction pH (8.5, 9.5, 10.5, 11.0) and temperature conditions (25, 30, 40, 50 °C). The purity and yields of the isolated starch and protein isolates were measured and the response optimizer tool was employed to identify the optimized parameters for each flour variety.

The information from this study advanced our knowledge on developing feasible wet fractionation methods to effectively isolate starch and protein with high yields and purity from air-classified starch-rich flours. The wet fractionation method with optimum conditions can be easily incorporated into the present industrial practices for simultaneous starch and protein isolation.

3.3 Materials and Methods

3.3.1 Materials

Air-classified starch-rich faba bean flour was purchased from AGT Food and Ingredients (Regina, SK, Canada) and air-classified starch-rich pea flour was provided by Parrheim Foods (Saskatoon, SK, Canada). They are representative starch-rich pulse flours from air classification in Western Canada. Total Starch Assay Kit was purchased from Megazyme Ltd. (Co. Wicklow, Ireland). All the chemicals used in the study were of reagent grade or higher purity, which were purchased from Fisher Scientific Company (Ottawa, ON, Canada) or Sigma-Aldrich Canada Co. (Oakville, ON, Canada).

3.3.2 Experimental design in alkaline extraction/isoelectric precipitation method

The starch and protein isolation were performed under alkaline extraction with two independent variables as pH and temperature. A factorial design was used to evaluate the effects of pH and temperature during the wet extraction of starch and protein from starch-rich faba bean and pea flours on the four output responses, namely the isolated starch content, isolated starch yield, isolated protein content, and isolated protein yield. Sixteen different combinations (4 pHs × 4 temperatures) were selected to study the impacts of different alkaline extraction conditions on the yields and purity of isolated starches and protein isolates from starch-rich faba bean and pea flours. The independent variables and their levels were selected based on preliminary experiments and presented in Table 3.1. The mass ratio of flour to aqueous medium during the alkaline extraction was 1:10. The 16 conditions with two independent extraction batches on each flour were performed in a random order to minimize the effects of unexpected variability on the observed responses. The details of simultaneous extraction of starch and protein from starch-rich pulse flours under the 16 conditions are described below.

Table 3.1 Independent variables and their levels for full factorial design in wet extraction of starch and protein from starch-rich faba bean and pea flours

Independent variable	Level			
	1	2	3	4
pH (x_1)	8.5	9.5	10.5	11.0
Temperature (x_2)	25 °C	30 °C	40 °C	50 °C

Air-classified starch-rich faba bean and pea flours (100.0 g) were accurately weighed and suspended in 1 L of distilled water that was preheated to a target extraction temperature. The target temperatures included 25, 30, 40, and 50 ± 0.5 °C. The flour suspension was magnetically stirred at ~300 rpm for 15 min. NaOH (1.0 M) was added dropwise to the suspension to adjust the pH to a target alkaline pH (8.5, 9.5, 10.5, and 11.0). The flour suspension was heated on a heating plate to maintain the extraction temperature as indicated above and stirred at ~300 rpm for 1 h. The highest pH and temperature were kept at 11.0 and 50°C, respectively, to prevent a noticeable level of starch gelatinization, which is a detrimental factor for subsequent starch and protein separation. The suspension was then filtered through a 60-µm nylon screen to remove insoluble fibers. The filtrate was centrifuged at 4,000 *g* for 20 min to separate the supernatant and the precipitate (Li et al., 2019). The top dark layer of the precipitate after centrifugation was carefully scraped off to remove insoluble fiber and protein residue, and the remaining starch sediment was resuspended in two-fold weight of distilled water, followed by pH adjustment to 7.0 using 1.0 M HCl. The neutralized starch sediment was recovered by centrifugation at 4,000 *g* for 20 min, followed by washing with 300 mL of distilled water twice to further purify the starch. The obtained starch sediment was finally washed with 100 mL of ethanol and filtered through a Whatman #3 filter paper, and the recovered starch cake was dried overnight in at 40 °C in a convection oven.

The protein solubilized in the supernatant during the aforementioned alkaline extraction was recovered using an isoelectric precipitation method as described by Karaca et al. (2011). The pH of the supernatant was adjusted to 4.5 (isoelectric point of the majority of pulse proteins) by adding 1.0 M HCl dropwise, followed by centrifugation at 4,500 *g* and 4 °C for 20 min to separate the protein precipitate from the aqueous supernatant. The precipitate was carefully collected and resuspended in two-fold weight of distilled water and the pH was adjusted to 7.0 using 1.0 M

NaOH. The resultant protein suspension was frozen at -30 °C for 48 h, followed by freeze-drying at -20 °C for 72 h to obtain dried protein isolate. For each condition, the extraction was conducted in two independent batches (*i.e.*, n = 2 for data presentation).

3.3.3 Proximate analysis of starch-rich flours, isolated starches, and protein isolates

Proximate analysis was performed on the faba bean and pea isolated starches and protein isolates and their corresponding starch-rich flours to determine yields and purity of the derived starch and protein. Moisture content was determined using AACC Method 44-15.02 (AACC, 2000). Starch content was quantified using Total Starch Assay Kit following AACC Method 76-13.01 (AACC, 2000). Dumas combustion method using a Nitrogen/Protein Analyzer (CN628, LECO Corporation, St. Joseph, MI, U.S.A) was employed to determine the nitrogen content of the sample, which was converted to protein content by multiplying with a conversion factor of 6.25 according to AACC Method 46-30.01 (AACC, 2000).

The yields of faba bean and pea isolated starches were calculated using the following equation:

$$\text{Yield of isolated starch (\%)} = 100\% \times \left(\frac{\text{Isolated starch, dry weight} \times \%\text{Starch of isolated starch on a dry basis}}{\text{Initial dry weight of starch} - \text{rich flour} \times \%\text{Starch of starch} - \text{rich flour on a dry basis}} \right)$$

The yields of faba bean and pea protein isolates were calculated using the following equation:

$$\text{Yield of protein isolate (\%)} = 100\% \times \left(\frac{\text{Protein isolate, dry weight} \times \%\text{Protein of protein isolate on a dry basis}}{\text{Initial dry weight of starch} - \text{rich flour} \times \%\text{Protein of starch} - \text{rich flour on a dry basis}} \right)$$

3.3.4 Statistical analysis

In Section 3.3.3, each analysis was carried out in duplicate measurements for each independent batch of the starch and protein (*i.e.*, n = 4 for data presentation); to have valid statistical analysis, the starch-rich flours were characterized in four measurements. Statistical differences among the results of the isolated starches and protein isolates were analyzed using one-way ANOVA, Tukey's test at a significance level of 0.05. Two-way ANOVA test was performed to test the significance level of pH, temperature, and their interaction (pH × Temperature) during alkaline extraction on the yields and purity of resultant isolated starches and protein isolates.

Statistical analyses were performed with SPSS statistical software (IBM Corp. 2023., Version 29.0.2.0, Armonk, NY, U.S.A.) and Minitab statistical software (Minitab, LLC., 21.1. 0 PA, U.S.A.). The optimum conditions of pH and temperature for wet extraction were analyzed using the response optimizer method built within Minitab statistical software.

3.4 Results and discussion

3.4.1 Yields and purity of isolated starches and protein isolates

The starch-rich faba bean flour contained 72.2% starch and 16.2% protein, while the starch-rich pea flour contained 75.4% starch and 9.4% protein (Tables 3.2 and 3.3). This suggested that air classification is not highly effective in generating high-purity pulse starch/protein. During alkaline extraction, pulse proteins become negatively charged and more water-soluble (Gao et al., 2020). At their isoelectric point (pI), this repulsion decreases, causing the proteins to aggregate and precipitate from the aqueous medium (Alavi, Chen, Wang, & Emam-Djomeh, 2021).

The employed alkaline extraction method produced faba bean and pea isolated starches having starch contents of 95.0-98.6% and extremely low protein concentrations of 0.18-0.44%. These results indicated that the wet extraction method used in this study effectively isolated starch with good purity from starch-rich pulse flours. The yields of isolated pea starch were lower than those of faba bean starch (80.2-87.9% *versus* 85.0-92.8%; Table 3.2 & 3.3), in accordance with the observation in the study of Li et al. (2019). The lower yields of pea starch could be attributed to the stronger interaction between starch and protein in this flour. During the alkaline extraction in Section 3.3.2, the starch was not well separated from the protein and fiber after centrifugation, and thus manual scraping was conducted to remove the top layer of protein and fiber in the sediment, resulting in the removal of a larger portion of starch.

The purity of the faba bean and pea protein isolates ranged from 85.4-93.1% and 79.7-86.5%, respectively, indicating the great potential of protein recovery from air-classified starch-rich pulse flours through alkaline extraction. With respect to protein recovery yields, the applied method resulted in yields of 62.5-71.2% for faba bean and 43.8-56.1% for pea, respectively. The markedly lower yields of pea protein isolate using the wet method could be attributed to more extensive protein extraction in pea flour than that in faba bean flour during air classification (9.4% protein for pea flour *versus* 16.2% protein for faba bean flour; Table 3.2 & 3.3). Therefore, less protein was extractable from the starch-rich pea flour. The strong interaction between protein and

starch in pea flour as described above also negatively affected the pea protein extraction. Moreover, different proportions of albumins and globulins present in the air-classified starch rich pea and faba bean flours could lead to the noted difference in their extractability too (Eze, Kwofie, Adewale, Lam, & Ngadi, 2022).

Table 3.2 Starch and protein contents of starch-rich faba bean flour, isolated starches, and protein isolates, and yields of isolated starches and protein isolates from different extraction conditions. [a]

Faba bean starch-rich flour used for starch and protein extraction			As-is weight (g)	Moisture content (%)	Dry weight (g)	Starch content (%, dry basis) [b]	Protein content (%, dry basis) [b]
			100.0	8.4 ± 0.1	91.7	72.2 ± 0.2	16.2 ± 0.1
Isolated starch and protein isolate							
Extraction condition	Extraction pH	Extraction temperature (°C)	Isolated starch, dry weight (g) [b]	Isolated starch, starch content (%, dry basis)	Isolated starch, yield (% dry basis) [b]	Isolated starch, protein content (%, dry basis)	Protein isolate, dry weight (g) [b]	Protein isolate, protein content (%, dry basis)	Protein isolate, yield (% dry basis) [b]
1	8.5	25	60.7 ± 1.4 ^{ab}	97.5 ± 0.9 ^{a}	88.2 ± 3.0 ^{a}	0.26 ± 0.03 ^{bcde}	10.2 ± 0.5 ^{a}	93.1 ± 0.9 ^{g}	63.7 ± 2.3 ^{ab}
2	8.5	30	59.4 ± 0.0 ^{ab}	98.6 ± 0.9 ^{a}	87.3 ± 1.0 ^{a}	0.25 ± 0.04 ^{bcde}	10.2 ± 0.0 ^{a}	92.7 ± 0.8 ^{fg}	63.6 ± 0.6 ^{ab}
3	8.5	40	60.1 ± 0.3 ^{ab}	97.7 ± 0.9 ^{a}	87.5 ± 1.4 ^{a}	0.24 ± 0.01 ^{bcde}	10.1 ± 0.1 ^{a}	91.5 ± 0.1 ^{efg}	62.5 ± 0.4 ^{a}
4	8.5	50	59.9 ± 1.7 ^{ab}	97.4 ± 1.7 ^{a}	86.9 ± 4.3 ^{a}	0.29 ± 0.01 ^{e}	10.3 ± 0.2 ^{a}	91.1 ± 1.1 ^{def}	62.9 ± 2.1 ^{a}
5	9.5	25	61.2 ± 0.1 ^{ab}	98.2 ± 1.1 ^{a}	89.6 ± 0.5 ^{a}	0.24 ± 0.02 ^{bcde}	10.7 ± 0.0 ^{abc}	91.0 ± 0.7 ^{def}	65.5 ± 0.7 ^{abcd}
6	9.5	30	61.9 ± 2.6 ^{ab}	97.2 ± 1.4 ^{a}	89.7 ± 2.4 ^{a}	0.25 ± 0.03 ^{bcde}	10.5 ± 0.0 ^{ab}	90.5 ± 0.5 ^{cde}	64.1 ± 0.5 ^{abc}
7	9.5	40	60.6 ± 1.4 ^{ab}	96.5 ± 1.9 ^{a}	87.1 ± 4.1 ^{a}	0.28 ± 0.06 ^{cde}	10.4 ± 0.7 ^{a}	90.6 ± 0.2 ^{cde}	63.3 ± 4.1 ^{a}
8	9.5	50	60.5 ± 1.6 ^{ab}	97.3 ± 1.2 ^{a}	87.6 ± 3.6 ^{a}	0.27 ± 0.01 ^{cde}	10.9 ± 0.2 ^{abcd}	88.8 ± 0.7 ^{bc}	65.2 ± 1.9 ^{abcd}
9	10.5	25	61.1 ± 1.7 ^{ab}	97.9 ± 0.7 ^{a}	89.2 ± 3.0 ^{a}	0.22 ± 0.03 ^{abc}	11.3 ± 0.0 ^{bcde}	89.5 ± 1.1 ^{cd}	68.4 ± 1.1 ^{abcd}
10	10.5	30	62.3 ± 0.4 ^{ab}	97.9 ± 1.2 ^{a}	90.9 ± 0.5 ^{a}	0.22 ± 0.01 ^{abcd}	11.7 ± 0.2 ^{de}	90.2 ± 0.4 ^{cde}	71.2 ± 1.1 ^{d}
11	10.5	40	57.6 ± 0.2 ^{a}	97.7 ± 1.4 ^{a}	85.0 ± 0.2 ^{a}	0.20 ± 0.01 ^{ab}	11.7 ± 0.2 ^{de}	89.4 ± 1.1 ^{cd}	70.3 ± 2.0 ^{cd}
12	10.5	50	61.2 ± 2.3 ^{ab}	97.4 ± 1.0 ^{a}	88.9 ± 2.8 ^{a}	0.23 ± 0.01 ^{abcde}	11.5 ± 0.1 ^{cde}	85.9 ± 0.6 ^{a}	66.6 ± 0.0 ^{abcd}
13	11.0	25	61.3 ± 0.9 ^{ab}	97.9 ± 0.9 ^{a}	90.7 ± 0.0 ^{a}	0.28 ± 0.01 ^{de}	11.5 ± 0.0 ^{cde}	88.9 ± 0.6 ^{bc}	68.8 ± 0.2 ^{abcd}
14	11.0	30	63.5 ± 0.0 ^{b}	96.8 ± 0.7 ^{a}	92.8 ± 0.1 ^{a}	0.18 ± 0.01 ^{a}	11.7 ± 0.1 ^{de}	88.8 ± 1.1 ^{bc}	70.0 ± 1.6 ^{bcd}
15	11.0	40	62.8 ± 0.6 ^{b}	96.2 ± 0.4 ^{a}	90.1 ± 0.6 ^{a}	0.20 ± 0.01 ^{ab}	11.7 ± 0.1 ^{de}	87.1 ± 0.2 ^{ab}	68.7 ± 0.8 ^{abcd}
16	11.0	50	60.2 ± 1.5 ^{ab}	97.4 ± 0.4 ^{a}	88.5 ± 1.7 ^{a}	0.22 ± 0.01 ^{abc}	11.9 ± 0.2 ^{e}	85.4 ± 0.6 ^{a}	68.5 ± 1.1 ^{abcd}

[a] Data are presented as average ± standard deviation (n = 4); in the same column, values with the same letter are not significantly different at $p < 0.05$.

[b] Data are presented as average ± standard deviation (n = 2); in the same column, values with the same letter are not significantly different at $p < 0.05$.

Table 3.3 Starch and protein contents of initial starch-rich pea flour, isolated starches, and protein isolates, and yields of isolated starches and protein isolates from different extraction conditions. [a]

Pea starch-rich flour used for starch and protein extraction			As-is weight (g)	Moisture content (%)	Dry weight (g)	Starch content (%, dry basis) [b]	Protein content (%, dry basis) [b]		
			100.0	7.07 ± 0.1	92.9	75.4 ± 0.6	9.4 ± 0.0		
Isolated starch and protein isolate									
Extraction condition	Extraction pH	Extraction temperature (°C)	Isolated starch, dry weight (g) [b]	Isolated starch, starch content (%, dry basis)	Isolated starch, yield (% dry basis) [b]	Isolated starch, protein content (%, dry basis)	Protein isolate, dry weight (g) [b]	Protein isolate, protein content (%, dry basis)	Protein isolate, yield (% dry basis) [b]
1	8.5	25	62.3 ± 0.5 ^{a}	96.7 ± 1.0 ^{a}	86.0 ± 0.9 ^{a}	0.29 ± 0.03 ^{a}	4.9 ± 0.6 ^{abcd}	85.3 ± 2.6 ^{ab}	47.8 ± 3.8 ^{abcd}
2	8.5	30	60.5 ± 3.2 ^{a}	95.6 ± 1.1 ^{a}	82.6 ± 4.8 ^{a}	0.27 ± 0.03 ^{a}	4.4 ± 0.1 ^{a}	86.5 ± 1.5 ^{b}	43.8 ± 0.5 ^{a}
3	8.5	40	63.7 ± 3.0 ^{a}	95.1 ± 0.6 ^{a}	86.5 ± 3.8 ^{b}	0.27 ± 0.03 ^{a}	4.7 ± 0.1 ^{ab}	85.0 ± 1.8 ^{ab}	45.8 ± 0.5 ^{ab}
4	8.5	50	61.5 ± 1.0 ^{a}	95.4 ± 1.1 ^{a}	83.8 ± 0.7 ^{a}	0.44 ± 0.06 ^{b}	4.7 ± 0.1 ^{ab}	84.9 ± 0.9 ^{ab}	45.4 ± 1.4 ^{ab}
5	9.5	25	61.7 ± 1.2 ^{a}	96.6 ± 1.9 ^{a}	85.2 ± 3.6 ^{a}	0.29 ± 0.01 ^{a}	4.7 ± 0.3 ^{abc}	86.5 ± 0.6 ^{b}	46.8 ± 3.6 ^{abc}
6	9.5	30	58.4 ± 1.2 ^{a}	96.1 ± 0.8 ^{a}	80.2 ± 2.2 ^{a}	0.27 ± 0.05 ^{a}	5.1 ± 0.0 ^{abcd}	85.4 ± 0.8 ^{ab}	49.4 ± 0.7 ^{abcde}
7	9.5	40	59.3 ± 0.2 ^{a}	97.0 ± 1.1 ^{a}	82.1 ± 1.4 ^{a}	0.31 ± 0.02 ^{a}	5.0 ± 0.0 ^{abcd}	85.3 ± 1.2 ^{ab}	48.4 ± 1.3 ^{abcd}
8	9.5	50	60.6 ± 3.5 ^{a}	96.8 ± 0.8 ^{a}	83.7 ± 5.6 ^{a}	0.32 ± 0.03 ^{ab}	5.0 ± 0.2 ^{abcd}	82.5 ± 0.6 ^{ab}	47.7 ± 0.9 ^{abc}
9	10.5	25	62.8 ± 2.9 ^{a}	96.6 ± 0.7 ^{a}	86.6 ± 3.7 ^{a}	0.26 ± 0.01 ^{a}	5.4 ± 0.0 ^{bcdef}	83.4 ± 0.3 ^{ab}	52.2 ± 0.3 ^{bcde}
10	10.5	30	63.6 ± 1.5 ^{a}	95.6 ± 1.1 ^{a}	86.7 ± 0.9 ^{a}	0.29 ± 0.03 ^{a}	5.2 ± 0.0 ^{abcde}	84.8 ± 0.2 ^{ab}	50.0 ± 0.4 ^{abcde}
11	10.5	40	61.9 ± 1.0 ^{a}	96.0 ± 1.6 ^{a}	84.8 ± 2.0 ^{a}	0.31 ± 0.02 ^{a}	5.2 ± 0.2 ^{abcde}	85.5 ± 0.8 ^{ab}	50.6 ± 0.8 ^{abcde}
12	10.5	50	59.6 ± 3.2 ^{a}	97.2 ± 0.9 ^{a}	82.6 ± 4.1 ^{a}	0.28 ± 0.01 ^{a}	5.5 ± 0.0 ^{bcdef}	82.1 ± 0.2 ^{ab}	51.5 ± 0.4 ^{bcde}
13	11.0	25	60.5 ± 1.8 ^{a}	97.3 ± 1.1 ^{a}	84.1 ± 0.8 ^{a}	0.28 ± 0.01 ^{a}	5.6 ± 0.1 ^{cdef}	83.5 ± 0.6 ^{ab}	53.5 ± 1.2 ^{cde}
14	11.0	30	62.4 ± 2.5 ^{a}	97.0 ± 1.3 ^{a}	86.3 ± 2.4 ^{a}	0.25 ± 0.00 ^{a}	5.8 ± 0.2 ^{def}	83.9 ± 0.4 ^{ab}	54.7 ± 0.9 ^{de}
15	11.0	40	61.0 ± 0.6 ^{a}	96.4 ± 1.1 ^{a}	83.9 ± 0.1 ^{a}	0.24 ± 0.02 ^{a}	6.0 ± 0.4 ^{ef}	81.5 ± 0.5 ^{ab}	55.7 ± 3.2 ^{e}
16	11.0	50	64.8 ± 0.1 ^{a}	95.0 ± 1.9 ^{a}	87.9 ± 1.9 ^{a}	0.24 ± 0.01 ^{a}	6.1 ± 0.1 ^{f}	79.7 ± 0.8 ^{a}	56.1 ± 0.3 ^{e}

[a] Data are presented as average ± standard deviation (n = 4); in the same column, values with the same letter are not significantly different at $p < 0.05$.

[b] Data are presented as average ± standard deviation (n = 2); in the same column, values with the same letter are not significantly different at $p < 0.05$.

3.4.2 Effects of independent variables on responses

The effects of pH, temperature, and their interaction (pH × Temperature) on the yields and purity of the resultant isolated starches and protein isolates are presented in Table 3.4. The pH exhibited significant impacts on the yields and protein contents of faba bean and pea protein isolates ($p < 0.05$), while the temperature only showed significant influence on the protein contents of protein isolates generated from both flours. The pH × Temperature displayed significant influence on the protein contents of faba bean protein isolates, but such influence was not observed for pea protein isolates. By contrast, the pH, temperature, and pH × Temperature did not show any significant effect on the yields and starch contents of isolated faba bean and pea starches.

The different impacts of pH and temperature on starch and protein extraction could be explained by the different molecular structures and supramolecular organizations of protein and starch. Proteins are polymers with three-dimensional structure comprising various amino acid monomers (Damodaran & Parkin, 2017). Under the strong alkaline conditions and high temperatures during extraction, proteins can partially unfold and expose non-polar and free sulfhydryl (-SH) groups, which hence affected the protein yields and contents (Cruz-Solis et al., 2023). In contrast, starch occurs as insoluble semicrystalline granules consisting of amylose and amylopectin (Ren et al., 2021). The starch in faba bean and pea flours retained the granular form under the different extraction conditions, and thus the pH and temperature did not noticeably influence the yields and contents of the isolated starches. For instance, only minor starch damage was observed in the starch sample extracted under pH 11.0 and 50 °C (4.94% damaged starch; Table 4.2, Chapter 4), suggesting that the starch could preserve the granular structure even under the harsh conditions applied in this study. Therefore, the different combinations of pH and temperature did not significantly influence the isolation of starch from the two starch-rich pulse flours.

The effects of independent variables on the responses (isolated starch and protein purity and yield) were further elucidated and analyzed using contour plots. The results contour plots showed that the yields of faba bean and pea protein increased upon higher pH and temperature (Figure 3.1 A and B); in contrast, the protein contents decreased when pH and temperature increased (Figure 3.1 C and D), which agree with the findings reported by Feyzi, Milani, and Golimovahhed (2018) and Gao et al. (2020). Higher pH and temperature increased the solubility

of non-protein compounds such as non-starch polysaccharides (Gao et al., 2020), thereby enhancing the yields but lowering the purity of faba bean and pea protein isolates.

Table 3.4 Effects of pH, temperature, and their interactions on yields and contents of isolated starches and protein isolates of pea and faba bean in wet extraction. [a]

Variable	pH	Temperature	pH × Temperature
Faba bean			
Yield of isolated starch (db)	NS	NS	NS
Starch content of isolated starch (db)	NS	NS	NS
Yield of protein isolate (db)	**	NS	NS
Protein content of protein isolate (db)	**	**	**
Pea			
Yield of isolated starch (db)	NS	NS	NS
Starch content of isolated starch (db)	NS	NS	NS
Yield of protein isolate (db)	**	NS	NS
Protein content of protein isolate (db)	**	**	NS

[a] ** $p < 0.05$; NS: not significant; db: dry basis.

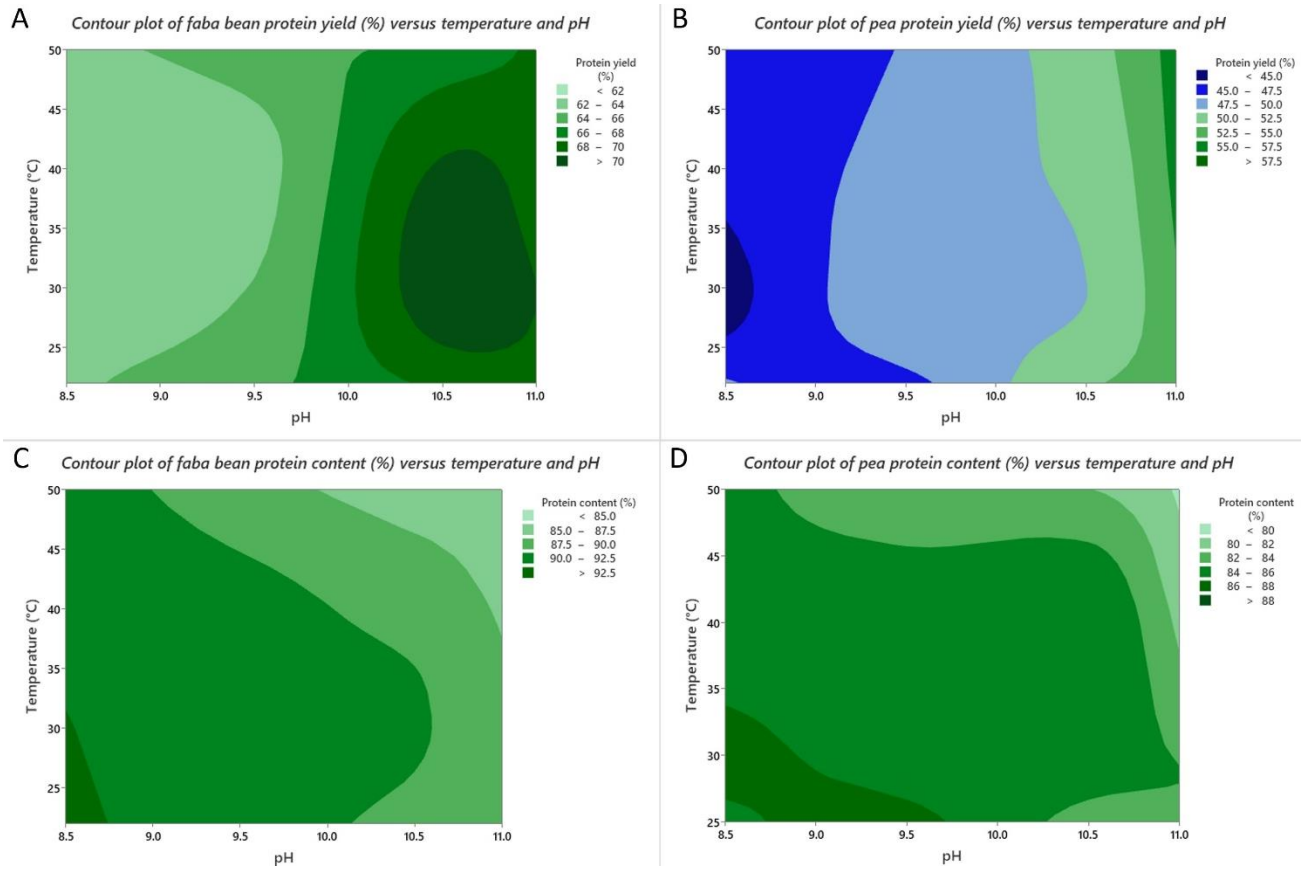

Figure 3.1 Contour plots for the effects of pH and temperature on protein yields (A and B) and protein contents (C and D) of faba bean and pea protein isolates.

3.4.3 Optimum conditions with maximum response values

Due to the negligible effect of pH and temperature on the starch yields and purity (Table 3.4), extraction conditions were optimized to maximize either content or yield of faba bean and pea protein isolates. From the response optimizer method, the optimum conditions for faba bean and pea protein extraction were determined and summarized in Table 3.5. For faba bean protein isolation, the highest protein content of 93.1% was observed at pH 8.5 and 25 °C, while the highest protein yield of 71.2% was observed at pH 10.5 and 30 °C (Table 3.2). In regard to pea protein isolation, the highest protein content of 86.5% was observed at pH 8.5 and 30 °C, whereas the highest protein yield of 56.1% was observed at pH 11.0 and 50 °C (Table 3.3). These identified optimum conditions were used in Study 2 of this thesis research.

Table 3.5 Optimum conditions for faba bean and pea protein extraction from respective starch-rich flours

Response	Condition	pH	Temperature (°C)	Experimental value (%)
Faba bean				
Protein content of protein isolate (db)	1	8.5	25	93.1 ± 0.9
Yield of protein isolate (db)	2	10.5	30	71.2 ± 1.1
Pea				
Protein content of protein isolate (db)	3	8.5	30	86.5 ± 1.5
Yield of protein isolate (db)	4	11.0	50	56.1± 0.3

3.5 Conclusions

This study developed a simple and effective wet method for isolating high-purity starch and protein simultaneously from air-classified starch-rich pulse flours. Commercial starch-rich faba bean and pea flours were subjected to alkaline extraction/isoelectric precipitation to obtain starch and protein. The extraction was performed under pHs and temperatures ranging from 8.5-11.0 and 25-50 °C, respectively, to evaluate the effects of combinations of these two important variables on starch and protein extraction. For faba bean, the starch showed extraction yield of 86.9-92.8% and purity of 96.2-98.6%, while the protein displayed extraction yield of 62.5-71.2% and purity of 85.4-93.1%. In the case of pea, the starch showed extraction yield of 80.2-87.9% and purity of 95.0-97.3%, while the protein displayed extraction yield of 43.8-56.1% and purity of 79.7-86.5%. Overall, for both pulses, the responses of protein isolation were noticeably influenced by the pH and temperature applied during extraction; however, such impacts were not observed for starch isolation. Moreover, the optimized pH and temperature combinations for extracting protein with maximum yield and purity were identified in this research. The maximum protein content and yield were observed at pH 8.5 and 25 °C and at pH 10.5 and 30 °C, respectively, for faba bean protein isolation, whereas the maximum protein content and yield were observed at pH 8.5 and 30 °C and at pH 11.0 and 30 °C, respectively, for pea protein isolation.

The obtained results provided useful information about the alkaline extraction of air-classified starch-rich flour to isolate pulse starch and protein with high purity and yields under

different pH and temperature conditions. The obtained knowledge can facilitate further innovation in processing techniques and product development. Considering the use of air-classified starch-rich flours as the raw materials in the beginning, the refined alkaline extraction process can also be accepted as a hybrid fractionation technology because it combines both dry and wet fractionation techniques to produce high-purity starch and protein ingredients with relatively low processing efforts.

3.6 Connection to next study

In this chapter, optimum alkaline extraction conditions were determined using the response optimizer method to isolate high-purity starch and protein from starch-rich pulse flours. However, there remained a lack of understanding on how the different extraction conditions (*i.e.*, pH and temperature) affect the functional properties of the resultant starch and protein. Therefore, Chapter 4 focused on the characterization of isolated starch and protein from the optimized conditions as identified in this chapter. Within the framework, particular emphasis was placed on elucidating the structural features and functional attributes of isolated faba bean and pea starches and proteins.

4. CHARACTERIZATION OF ISOLATED FABA BEAN AND PEA STARCHES AND PROTEIN ISOLATES EXTRACTED UNDER OPTIMIZED CONDITIONS

4.1 Abstract

The objective of this study was to characterize isolated faba bean and pea starches and protein isolates extracted from respective air-classified starch-rich flours under optimized conditions and to compare them with commercial pea starch and protein isolate, respectively. Isolated pulse starches demonstrated high purity with starch contents of 95.1-97.1% and low contents of protein (≤ 0.31%) and ash (≤ 0.07%). The granular morphologies of the isolated pulse starches were generally comparable to that of commercial pea starch, but the isolated starches showed more damaged starch granules, particularly for those extracted at pH 11.0. Thermal, pasting, and gelling properties of the isolated starches were affected by the extraction conditions, with significant differences observed in gelatinization temperatures and enthalpy changes, pasting temperatures and viscosities, and gel strength. Protein isolates obtained from air-classified starch-rich pulse flours had protein purity of 77.6%-94.0% and less than 5% of ash. The surface properties of protein isolates varied with extraction conditions, thereby affecting the functional properties of those proteins including solubility, emulsion stability, foam capacity and stability, and water-holding capacity and oil-absorption capacity. The findings highlighted the influence of extraction conditions on the purity and techno-functional attributes of the isolated starch and protein components from starch-rich pulse flours. The comprehensive analysis provided meaningful information for the potential applications of these isolated components in various food and industrial products.

4.2 Introduction

Recently, there are rising demands for plant proteins, which is largely driven by population growth, dietary shifts, a growing focus on environmental sustainability, heightened health consciousness, and ethical considerations (Vogelsang-O'Dwyer et al., 2021). However, there is a challenge in ensuring that plant-based proteins can offer adequate nutritional quality as animal proteins for consumers. Pulses, such as peas, lentils, faba beans, and dry beans, have emerged as alternative plant protein sources due to their protein abundance and nutritional quality (Nadeeshani et al., 2022). The protein contents of pulses are approximately two-fold that of cereal grains, and their amino acid profiles are complementary to each other (Mudryj et al., 2014). Consequently, consuming pulses along with cereals can enhance protein quality in human diets, particularly for vegans and vegetarians (Awika, 2011).

Pulses are often processed through wet or dry fractionation methods to produce protein ingredients such as protein concentrates or isolates (Schutyser & van der Goot, 2011). These versatile ingredients can be utilized in various food products such as snacks, bakery products, meat alternatives and dairy alternatives. Traditionally, protein isolates are produced through wet fractionation, providing protein contents > 80% and yields ranging from 60% to 90% (Dumoulin et al., 2021). However, wet fractionation has several drawbacks, which include: (1) high water and energy consumption and high operating costs; (2) less environmental sustainability (Assatory et al., 2019); and (3) potential compromise of protein functional properties due to harsh extraction conditions; for example, acid or alkaline pH and elevated temperatures. By contrast, dry fractionation process is a more sustainable way to produce protein-rich and starch-rich flour streams of pulses (Rivera et al., 2022). Air classification is the most common dry fractionation technique that separates finely ground pulse flours into protein-rich and starch-rich streams based on their density and particle size (Boye et al., 2010). However, the resultant streams have poor purity due to the low separation efficiency of air classification (Ren et al., 2021). For instance, starch-rich flours produced from air classification still contain relatively high protein contents (7.9-20.0%) (Ren et al., 2021). Due to the presence of protein and other impurities (*e.g.*, dietary fibers, flavor and aroma compounds), the air-classified starch-rich flour has limited applications. In addition, the residual proteins found in the starch-rich flour are considered as a loss in protein extraction, which thus gives a low protein yield in air classification overall (Dumoulin et al., 2021).

Consequently, it is meaningful to refine wet fractionation methods that can efficiently separate starch and protein components in starch-rich pulse flour, which can greatly enhance industrial utilization of this co-product from air classification of pulses. In Chapter 3, we have optimized the alkaline extraction conditions to simultaneously isolate high-purity starch and protein from air-classified starch-rich pulse flours with high yields. The maximum protein content and yield were achieved at pH 8.5 and 25 °C and at pH 10.5 and 30 °C, respectively, for protein isolation from air-classified faba bean flour; and the maximum protein content and yield were achieved at pH 8.5 and 30 °C and at pH 11.0 and 30 °C, respectively, for protein isolation from air-classified pea flour. The different conditions, however, did not exhibit significant influence on the purity and yields of isolated starches from the same air-classified pulse flours. In addition, previous studies have demonstrated that functional attributes of starch and protein are affected by their extraction conditions (Higa et al., 2024; Boye et al., 2010). It is important to study the impacts of the above extraction conditions on the functional properties of the resultant pulse starches and protein isolates. Therefore, this study aimed to: (1) analyze the proximate compositions and functional properties of starches and proteins isolated using the optimized conditions as reported in Chapter 3; and (2) compare them with those of commercial pea starch and protein isolates, respectively. The new knowledge acquired from this study can help to identify the potential applications of these obtained food ingredients.

4.3 Materials and methods

4.3.1 Materials

Air-classified starch-rich faba bean flour was purchased from AGT Food and Ingredients (Regina, SK, Canada) and air-classified starch-rich pea flour (Starlite) was obtained from Parrheim Foods (Saskatoon, SK, Canada). Commercial pea starch (CPS) and pea protein isolate (CPPI) were donated by Roquette Canada Ltd. (Winnipeg, MB, Canada). Total Starch Assay Kit and Starch Damage Assay Kit were procured from Megazyme International Ltd. (Co. Wicklow, Ireland). All the other chemicals used in the study were reagent grade and purchased from Fisher Scientific Company (Ottawa, ON, Canada) or Sigma-Aldrich Canada Co. (Oakville, ON, Canada).

4.3.2 Isolation of starch and protein from air-classified starch-rich pulse flours under optimized conditions

Starch and protein were isolated from air-classified starch-rich faba bean and pea flours according to the method as described in Section 3.3.2 under optimized alkaline extraction conditions followed by isoelectric precipitation. Extraction was carried out in three independent batches for each isolation condition as presented in Table 4.1. Techno-functional attributes of the resultant isolated pea starches (PIS) and faba bean starches (FIS) were characterized and compared with those of CPS. Similarly, techno-functional properties of the obtained faba bean protein isolates (FPI) and pea protein isolates (PPI) were determined and compared with those of CPPI.

Table 4.1 Optimum extraction conditions for faba bean and pea proteins and starches

Pulse	Extraction Condition	pH	Temperature (°C)
Faba bean	1	8.5	25
	2	10.5	30
Pea	3	8.5	30
	4	11.0	50

The starch and protein isolated from these conditions were designated as follows for ease of reference: FIS-C1 (faba bean isolated starch from Condition 1), FPI-C1 (faba bean protein isolate from Condition 1), FIS-C2 (faba bean isolated starch from Condition 2), FPI-C2 (faba bean protein isolate from Condition 2), PIS-C3 (pea isolated starch from Condition 3), PPI-C3 (pea protein isolate from Condition 3), PIS-C4 (pea isolated starch Condition 4), and PPI-C4 (pea protein isolate from Condition 4).

4.3.3 Characterization of faba bean and pea isolated starches

4.3.3.1 Proximate analysis

Proximate analysis was performed on faba bean and pea starches extracted under the optimized conditions in comparison to CPS. Moisture content was determined using AACC Method 44-15.02 (AACC, 2000). Starch content was quantified using Megazyme Total Starch

Assay Kit following AACC Method 76-13.01 (AACC, 2000). Amylose contents of the isolated starches were determined using an iodine colorimetric method with some modifications (Li, Li, Zhu, & Ai, 2021). Damaged-starch content was determined using Starch Damage Assay Kit following AACC Method 76-31.01 01 (AACC, 2000). The Dumas combustion method using a Nitrogen/Protein Analyzer (CN628, LECO Corporation, St. Joseph, MI, U.S.A) was employed to determine the nitrogen content, which was converted to protein content by multiplying with 6.25 as the conversion factor according to AACC Method 46-30.01 (AACC, 2000). Ash content was quantified using an electric muffle furnace following AACC Method 08-01.01.

4.3.3.2 Granular morphology

Granular morphology and surface structure of the isolated starches and CPS were analyzed using a field-emission scanning electron microscope (SEM, SU8010, Hitachi High Technologies Canada Inc., Rexdale, ON, Canada) under conditions of 3.0 kV and 10 μA. To prepare the sample, starch was sprinkled onto a carbon tape attached to an aluminum stub and then coated with gold using a sputtering technique. Images of the starch granules were taken at three different magnifications: 500×, 1500×, and 3500×.

4.3.3.3 Wide-angle X-ray diffraction

The isolated starches and CPS were equilibrated in a chamber with 100% relative humidity at 25°C for 24 h. Wide-angle X-ray diffraction patterns of the equilibrated samples were analyzed using Ultima IV X-ray diffractometer (Rigaku Americas Corp., Woodlands, TX, U.S.A.) operating at 40 kV and 44 mA with Cu Kα radiation ($\lambda = 1.5406$ Å). The starch powder was packed tightly in a rectangular glass cell and scanned from a diffraction angle (2θ) of 3° to 40° at a rate of 1.3°/min at room temperature. The relative crystallinity of the starch was calculated using Origin 2023b Software (OriginLab Corp., Northampton, MA, U.S.A.) according to the method described in Hayakawa, Tanaka, Nakamura, Endo, and Hoshino (1997).

4.3.3.4 Thermal properties

Gelatinization and retrogradation properties of the isolated starches and CPS were determined using a differential scanning calorimeter (DSC 8000, PerkinElmer Inc.) according to the method of (Li et al., 2019).Starch (~10 mg) was weighed into a DSC pan and hydrated with

approximately three times (v/w) of distilled water using a micro syringe. The pan was sealed and equilibrated at ambient temperature for at least 2 h, and the sample was scanned from 10 °C to 110 °C at a rate of 10 °C/min for starch gelatinization. After the DSC scanning, the sample was then stored at 4 °C for 7 days and scanned again using the same procedure to examine the melting peak of retrograded starch. Onset (T_o), peak (T_p) and conclusion (T_c) temperatures and enthalpy change (ΔH) of the thermal transitions were analyzed using Pyris software (Version 13.3.1.0014, PerkinElmer Inc.). Percentage of starch retrogradation was calculated as:

%Retrogradation = 100% × (ΔH of dissociation of retrograded starch) / (ΔH of starch gelatinization).

4.3.3.5 Pasting properties and gelling ability

Rapid Visco Analyser (RVA 4800, PerkinElmer Inc.) was used to measure the pasting properties of the isolated starches with slight modifications as described by Liu et al. (2019). Starch slurry (28.0 g total weight containing 8.0 % dry solids) was prepared and loaded to the instrument and run using the Standard Method 2 programmed in the Thermocline Software. Briefly, the suspension was equilibrated at 50 °C for 1 min, heated to 95 °C at a rate of 6 °C/min, maintained at 95 °C for 5 min, and then cooled at a rate of 6 °C/min to reach 50 °C. The rotating speed of the paddle was 960 rpm for the first 10 s to fully suspend the starch, and then it was lowered to 160 rpm for the rest of the testing period.

To prepare starch gel, the starch paste obtained from the above RVA run was poured into a plastic jar (internal diameter = 33.0 mm, height = 38.0 mm) and covered with a lid, and then stored at room temperature for 2 h. The strength of the developed starch gel was measured using a Texture Analyzer TA.XT. Plus (Texture Technologies Corp., South Hamilton, MA, U.S.A.) with Probe TA-10 (diameter = 12.7 mm). After a trigger force of 0.5 g was attained, the probe proceeded to penetrate the gel at a test speed of 0.5 mm/s and a compression depth of 10.0 mm. The maximum force during the 10.0-mm compression was defined as the strength of starch gel (Liu et al., 2019).

4.3.4 Characterization of faba bean and pea protein isolates

4.3.4.1 Proximate analysis

Proximate analysis was performed on faba bean and pea protein isolates extracted under the optimized conditions in comparison to CPPI. Moisture, protein, and ash contents were determined using the same methods as indicated in Section 4.3.3.1.

4.3.4.2 Surface charge

The surface charge (zeta potential, ζ) of the protein isolates and CPPI was determined using a Zetasizer Nano-ZS90 Analyzer (Malvern Instruments, Westborough, MA, U.S.A.) according to the method of Karaca et al. (2011). Protein solution (0.05%, w/w) was prepared to determine electrophoretic mobility. The pH of the solutions was maintained at 7.0 over 1 h by adding 0.1 M NaOH or 0.1 M HCl with continuous magnetic stirring . Electrophoretic mobility was converted to zeta potential (ζ) using Henry's equation:

$$\frac{U_E = 2\varepsilon\zeta f(\kappa\alpha)}{3\eta}$$

where ε was the permittivity of the medium (Farad/m), κα was a function of the particle radius (α in nm) and Debye length (κ in nm), and η was the viscosity (mPa.s).

4.3.4.3 Surface hydrophobicity

Surface hydrophobicity of the protein isolates and CPPI was determined in a Fluoromax-4 (Horiba Jobin Yvon Inc., Edison, NJ, USA) using the 8-anilino1-naphtalensulfonic acid (ANS)-fluorescent probe method according to the procedure of Kato and Nakai (1980). Briefly, 0.25% (w/w) stock protein solution was prepared and stirred for 1 h at pH 7.0. Dilutions of the protein solution were prepared at 0.05, 0.10, 0.15 and 0.20% from the stock solution. ANS (20 µL) was added to 1.6 mL of each protein solution and the mixture was vortexed for 10 s. The samples were incubated for 5 min in dark environment and then measured at 390 nm excitation and 470 nm emission with a slit width of 1 nm. Blank protein solution was prepared in 20 µL of water and fluorescence intensity (FI) was measured under the same conditions. Absolute intensity values were calculated by subtracting FI values of the ANS blank and protein blank from the FI of the

protein solution containing ANS. The initial slope of the plot of the FI against protein concentration was calculated by linear regression analysis and used as an index of the protein surface hydrophobicity.

4.3.4.4 Solubility

Protein solubility of the protein isolates and CPPI was determined by dispersing 0.2 g of protein in ~ 19 mL of milli-Q water. The pH of the protein solution was adjusted to 7.0 using 0.1M NaOH or HCl and it was stirred at 500 rpm for 1 h. The weights of the solutions were adjusted to 20.0 g and then centrifuged at 4180 *g* for 10 min. After the supernatant was collected, the protein content was determined using a micro-Kjeldahl digestion and distillation unit (RapidStill, Labconco, Kansas City, MO, USA). A factor of 6.25 was used to convert nitrogen content to protein content. The protein solubility was calculated using the following equation (Morr et al., 1985):

$$\text{Protein solubility (\%)} = \left(\frac{\text{Total protein suspension weight} \times \text{\% Protein in supernatant}}{\text{Sample weight} \times \text{\% Protein in sample}}\right) \times 100\%$$

4.3.4.5 Emulsion stability

Emulsion stability (ES) of the protein isolates and CPPI was determined according to the method described in the study of Stone et al. (2015) with minor modifications. Protein suspensions (2%, w/w) were prepared, and the pH were adjusted to 7.0 using 0.1M NaOH or 0.1M HCl and maintained over 1 h with constant stirring at 500 rpm. To prepare the emulsions, 5 g of each protein suspension were added to 5 g of canola oil, and then the mixture was homogenized at speed three for 3 min using an IKA homogenizer (T 10 basic ULTRA-TURRAX® with an S10 N dispersion tool). The prepared emulsions were immediately transferred to 10-mL volumetric cylinders (inner diameter = 10.80 mm; height = 100.24 mm), and they were stored at room temperature for 24 h. The stability of the emulsions was measured by observing the volume of the aqueous/serum layer before emulsification and volume of the aqueous/serum layer after 24 h of storage. ES was calculated as:

$$\text{ES\%} = \frac{\text{Volume of the aqueous phase before emulsification} - \text{Volume of the aqueous phase after 24 h of storage}}{\text{Volume of the aqueous phase before emulsification}} \times 100\%$$

4.3.4.6 Foaming capacity and stability

Foaming capacity (FC) and stability (FS) of the protein isolates and CPPI were determined according to the method as described by Stone, Nosworthy, Chiremba, House, and Nickerson (2019) with minor modifications. Protein suspensions (1%, w/w) were prepared, and the pH was adjusted to 7.0 by adding 0.1 M NaOH or 0.1M HCl and maintained over 1 h with constant stirring at 500 rpm. After that, 15 mL of the prepared suspension was transferred to a 400-mL beaker and homogenized at speed three using an IKA homogenizer (T 10 basic ULTRA-TURRAX® with an S10 N dispersion tool) for 5 min by placing the homogenizing probe slightly below the air-water interface. After homogenization, the formed foam was immediately transferred to a 50-mL graduated cylinder and initial foam volume was recorded. After 30 min, the volume of the remaining foam was recorded, and FC and FS were calculated as:

$$\text{FC\%} = \frac{\text{Volume of foam}}{\text{Initial sample volume}} \times 100\%$$

$$\text{FS\%} = \frac{\text{Volume of foam after 30 min}}{\text{Initial volume of foam}} \times 100\%$$

4.3.4.7 Water-holding capacity

Water-holding capacity (WHC) of the protein isolates and CPPI was determined by suspending 0.5 g of protein isolate in 5.0 g of water in a 50-mL screw-cap centrifuge tube. Samples were vortexed for 10 s every 5 min for a total of 30 min and then centrifuged at 4000 *g* for 15 min. The supernatant was carefully decanted, and the remaining pellet was weighed. WHC was calculated by dividing the weight gained by the isolate by the original sample weight (Stone et al., 2015).

4.3.4.8 Oil-absorption capacity

Oil-absorption capacity (OAC) of the protein isolates and CPPI was determined by suspending 0.5 g of protein isolate in 5.0 g of canola oil in a 50-mL screw-cap centrifuge tube. Samples were vortexed for 10 s every 5 min for a total of 30 min and then centrifuged at 4000 *g* for 15 min. The supernatant was carefully decanted, and the remaining pellet was weighed. OAC

was calculated by dividing the weight gained by the isolate by the original sample weight (Stone et al., 2015).

4.3.5 Statistical analysis

The proximate analyses were carried out in one replicate on each batch of isolated starch (*i.e.*, n = 3 for data presentation); for valid statistical analysis, CPS was also analyzed in triplicate. The analyses in Section 4.3.3 were performed in triplicate for each sample (n = 3) except for the wide-angle X-ray diffraction (one replicate for each sample). The analyses in Section 4.3.4 were analyzed in six replicates for each sample (n = 6). Statistical differences of the collected data were analyzed using SPSS statistical software (IBM Corp. 2023., Version 29.0.2.0, Armonk, NY, U.S.A.) with one-way ANOVA, Tukey's multiple comparison test at a significance level of 0.05.

4.4 Results and discussion

4.4.1 Characterization of faba bean and pea isolated starches

4.4.1.1 Proximate composition

Table 4.2 Chemical composition of faba bean isolated starch (FIS), pea isolated starch (PIS), and commercial pea starch (CPS)[a]

	Starch (%, dry basis)	Damaged starch (%, dry basis)	Amylose (%, dry basis)	Protein (%, dry basis)	Ash (%, dry basis)
FIS-C1	96.7 ± 0.2 bc	0.92 ± 0.02 a	35.6 ± 0.2 b	0.24 ± 0.01 b	0.07 ± 0.01 a
FIS-C2	97.1 ± 0.1 c	1.00 ± 0.03 ab	34.0 ± 0.2 a	0.20 ± 0.01 a	0.05 ± 0.01 a
PIS-C3	95.6 ± 0.4 ab	1.32 ± 0.02 b	37.7 ± 0.3 c	0.31 ± 0.02 c	0.06 ± 0.01 a
PIS-C4	95.1 ± 0.5 a	4.94 ± 0.28 c	36.2 ± 0.1 b	0.24 ± 0.00 b	0.07 ± 0.00 a
CPS	97.2 ± 0.6 c	0.71 ± 0.01 a	37.4 ± 0.5 c	0.37 ± 0.01 d	0.11 ± 0.01 b

[a] Values are presented as average ± standard deviation (n = 3); in the same column, the numbers with the same letter are not significantly different at $p < 0.05$.

Under the optimum conditions, the isolated faba bean and pea starches contained 95.1-97.1% starch, 0.20-0.31% protein, and 0.05-0.07% ash (db, Table 4.2), respectively, which were comparable to those of CPS. This observation suggested that the applied wet extraction method

was effective to generate pulse starches with high purity from air-classified starch-rich flours for further modifications and uses. Similar results have been observed by Li et al. (2019) where wet isolated pea, lentil and faba bean starches had 94.8–97.9% starch, 0.1–0.2% protein, and 0.01–0.03% ash. The authors also reported that the isolated pulse starches possessed similar starch and protein contents and lower ash contents in comparison with CPS.

Damaged-starch contents of the isolated pea and faba bean starches were higher than that of CPS (0.92–4.94% *versus* 0.71%, db). For C1-3, the damaged starch could result from those presented in the respective original air-classified starch-rich pulse flours (Li et al., 2019). Prior to air classification, the pulse flour was pin milled, and thus the applied strong mechanical force led to a high level of starch damage. A portion of these the damaged granules remained in the isolated starch samples after the wet isolation. However, under C4 condition, the resultant pea starch contained the highest level of damaged starch among the four samples, which could be attributed to the harsh extraction condition (*i.e.*, pH 11.0 and 50 °C) causing partial starch gelatinization. The amylose contents of FIS were slightly lower than that of CPS (34.0-35.6% *versus* 37.4%, db), whereas PIS comprised comparable amylose to CPS (36.2-37.7% *versus* 37.4%, db).

4.4.1.2 Granular morphology

SEM images revealed that the granular morphologies of FIS and PIS (Figure 4.1) were largely comparable to that of CPS. The granules of isolated pulse starch and CPS displayed oval, kidney or irregular shape, which were similar to the results in previous literature (Hoover et al., 2010; Li et al., 2019). Moreover, no fiber and protein residues were observed in the isolated pulse starches, in agreement with the high purity of these samples (Table 4.2). Interestingly, damaged starch granules with cracks and scratches on the surface (marked by up-arrows) and partially gelatinized starch granules (marked by triangles) were found in PIS-C4, consistent with its highest damaged-starch content (Table 4.2). This observation could be attributed to the elevated pH and temperature conditions used for the extraction of this starch sample. When starch is subjected to prolonged alkaline treatment with elevated temperature, it can undergo particle gelatinization to elevate the level of starch damage (Ai & Jane, 2015).

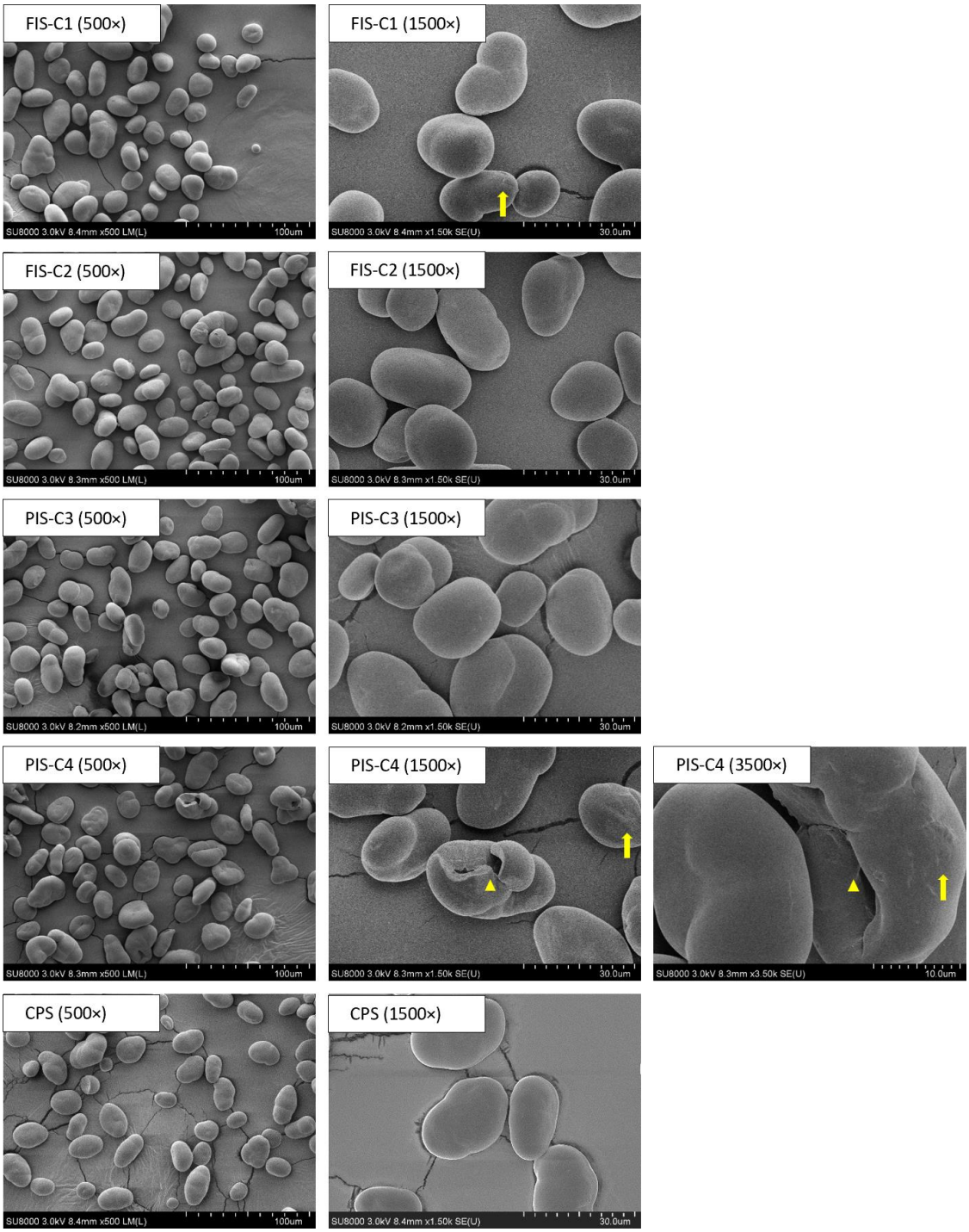

Figure 4.1 Scanning electron microscopy (SEM) images of faba bean isolated starch (FIS), pea isolated starch (PIS), and commercial pea starch (CPS). The magnification at which the image was taken is given in parentheses. Triangles mark partially gelatinized starch granules, up-arrows mark cracks and scratches on the surface of starch granules.

4.4.1.3 Wide-angle X-ray diffraction

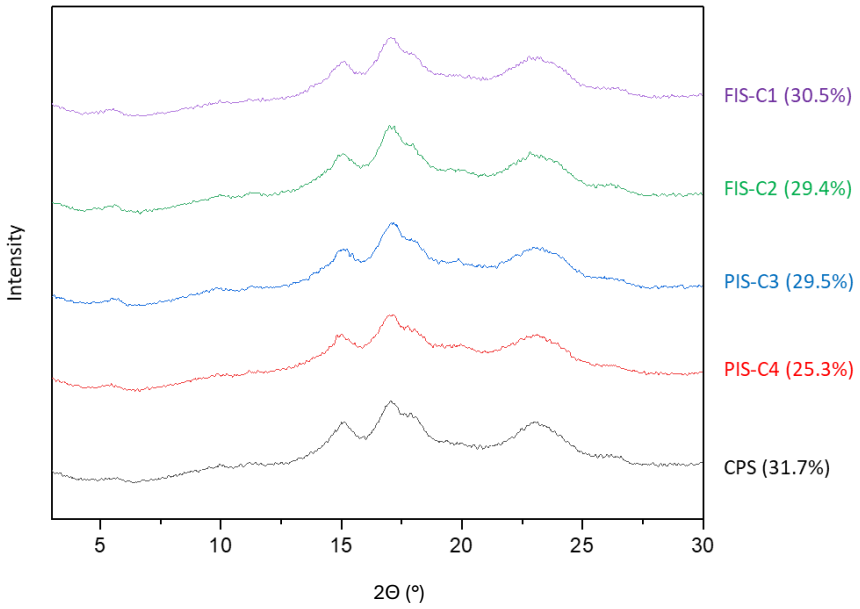

Figure 4.2 Wide angle X-ray diffraction patterns of faba bean isolated starch (FIS), pea isolated starch (PIS), and commercial pea starch (CPS). Relative crystallinity is given on the right side of the figure.

Wide-angle X-ray diffraction analysis indicated that FIS, PIS, and CPS all exhibited the C-type pattern (Figure 4.2), which is a mixture of A- and B-type polymorphs (Hoover et al., 2010). Relative crystallinity of the isolated starches ranged from 25.3-30.5%, which were slightly higher than the previously reported values. Chung et al. (2008) reported 24.4–25.5% relative crystallinity for PIS, whereas Hoover et al. (2010) reported relative crystallinity of 20.2–21.9% for FIS. However, the relative crystallinity values of FIS and PIS were lower than that of CPS (25.3-30.5% *versus* 31.7%). PIS-C4 exhibited the lowest relative crystallinity among the four samples, consistent with its highest degree of starch damage (Table 4.2).

4.4.1.4 Thermal properties

In general, FIS-C1, FIS-C2, and PIS-C3 displayed comparable starch gelatinization properties. The three samples showed gelatinization temperatures and ΔH ranging from 59.2-76.9

°C and 14.4-15.7 J/g, respectively (Table 4.3). In contrast, PIS-C4 exhibited higher gelatinization temperatures but lower ΔH than the other three isolated starches. This difference could be attributed to the higher level of damaged starch (Table 4.2) and lower relative crystallinity of PIS-C4 (Figure 4.2). Under the C4 condition, double helices in starch granules with less thermal stability were gelatinized due to the high pH and temperature (Table 4.3), and thus only double helices with stronger thermal stability remained, resulting in higher gelatinization temperatures but smaller ΔH of PIS-C4 (Ratnayake & Jackson, 2008). CPS showed the highest gelatinization temperatures and ΔH among all the tested samples, likely due to the pea variety and wet isolation method used for producing CPS.

Despite all the differences in their gelatinization properties, the retrogradation properties of the FIS, PIS, and CPS were largely comparable, which could be attributable to their similar amylose contents (Table 4.2) and branch-chain-length distributions of amylopectin as reported by Li et al. (2019). The percentages of retrogradation of the isolated starch samples were not significantly different from that of the CPS. Moreover, for each sample, the melting peak of the retrograded starch exhibited lower melting temperatures and smaller ΔH than the corresponding starch gelatinization peak. The observation was ascribed to that substantially higher energy is required to melt native double-helical crystallites during gelatinization than the melting of re-formed double-helical crystallites from retrogradation (Biliaderis, 2009).

Table 4.3 Gelatinization and retrogradation properties of faba bean isolated starch (FIS), pea isolated starch (PIS), and commercial pea starch (CPS). [a]

	Gelatinization of starch [b]				Melting of retrograded starch [b]				Retrogradation (%) [c]
	T_o (°C)	T_p (°C)	T_c (°C)	ΔH (J/g)	T_o (°C)	T_p (°C)	T_c (°C)	ΔH (J/g)	
FIS-C1	59.2 ± 0.7 a	67.1 ± 0.4 b	76.9 ± 0.6 b	14.7 ± 0.5 bc	47.3 ± 1.8 a	59.8 ± 0.5 ab	71.1 ± 0.6 ab	7.8 ± 0.9 a	53.2 ± 7.0 a
FIS-C2	59.7 ± 0.6 a	67.1 ± 0.3 b	76.7 ± 0.3 b	15.7 ± 0.0 c	49.6 ± 0.3 a	60.0 ± 0.6 b	71.8 ± 0.4 ab	7.3 ± 0.4 a	46.4 ± 2.4 a
PIS-C3	60.3 ± 0.3 a	66.2 ± 0.3 a	74.0 ± 0.7 a	14.4 ± 0.2 b	42.8 ± 0.3 a	58.4 ± 0.7 a	72.3 ± 0.6 ab	8.2 ± 0.8 a	57.2 ± 5.0 a
PIS-C4	68.0 ± 0.3 b	71.1 ± 0.4 c	77.2 ± 0.2 b	12.5 ± 0.4 a	48.8 ± 0.6 a	59.3 ± 0.5 ab	70.9 ± 0.5 a	7.1 ± 0.1 a	57.1 ± 2.6 a
CPS	67.0 ± 0.1 b	72.4 ± 0.1 d	78.9 ± 0.3 c	17.8 ± 0.8 d	42.7 ± 7.6 a	60.4 ± 0.6 b	72.6 ± 0.8 b	8.3 ± 0.4 a	46.5 ± 1.6 a

[a] Values are presented as average ± standard deviation (n = 3); in the same column, the numbers with the same letter are not significantly different at $p < 0.05$.

[b] T_o: onset temperature, T_p: peak temperature, T_c: conclusion temperature, ΔH: enthalpy change.

[c] %Retrogradation = 100% × (ΔH of dissociation of retrograded starch) / (ΔH of starch gelatinization).

4.4.1.5 Pasting properties

Table 4.4 Pasting properties of faba bean isolated starch (FIS), pea isolated starch (PIS), and commercial pea starch (CPS) [a]

	Peak viscosity (cP)	Trough viscosity (cP)	Breakdown viscosity (cP)	Final viscosity (cP)	Setback viscosity (cP)	Pasting temperature (°C)
FIS-C1	1229.3 ± 8.0 b	1193 ± 10.4 b	36.0 ± 4.0 b	2312.7 ± 22.5 b	1119.3 ± 26.9 c	72.4 ± 0.0 a
FIS-C2	1387.0 ± 6.0 c	1328.3 ± 3.5 c	58.7 ± 2.5 c	2576.0 ± 1.7 c	1247.7 ± 5.1 c	71.8 ± 0.5 a
PIS-C3	1084.3 ± 48.0 a	1062.7 ± 37.1 a	21.7 ± 11.1 b	1757.7 ± 84.2 a	695.0 ± 48.7 b	73.0 ± 0.5 a
PIS-C4	1225.7 ± 28.6 b	1225.7 ± 29.0 b	0.0 ± 1.7 a	1625.7 ± 72.0 a	400.0 ± 49.0 a	80.0 ± 0.7 c
CPS	1446.0 ± 27.4 c	1366.3 ± 28.0 c	79.7 ± 3.5 d	2691.3 ± 110.9 c	1325.0 ± 126.9 d	76.0 ± 0.0 b

[a] Values are presented as average ± standard deviation (n = 3); in the same column, the numbers with the same letter are not significantly different at $p < 0.05$.

Pasting temperatures of the tested starches were in an ascending order of FIS-C1, FIS-C2, PIS-C3 < CPS < PIS-C4 (Figure 4.3 and Table 4.4), largely consistent with the rank order of their T_o (Table 4.3). All the FIS and PIS showed lower pasting viscosities than CPS, which was likely due to the greater granular rigidity and integrity of the latter. In contrast, the granules of the starches extracted in this study might have slightly weaker granular structure due to the alkaline condition applied in fractionation. When heat and shear were applied during pasting determination, the weakened granules could not swell to the maximum extent, thus exhibiting less pasting viscosity than CPS (Nor Nadiha, Fazilah, Bhat, & Karim, 2010).

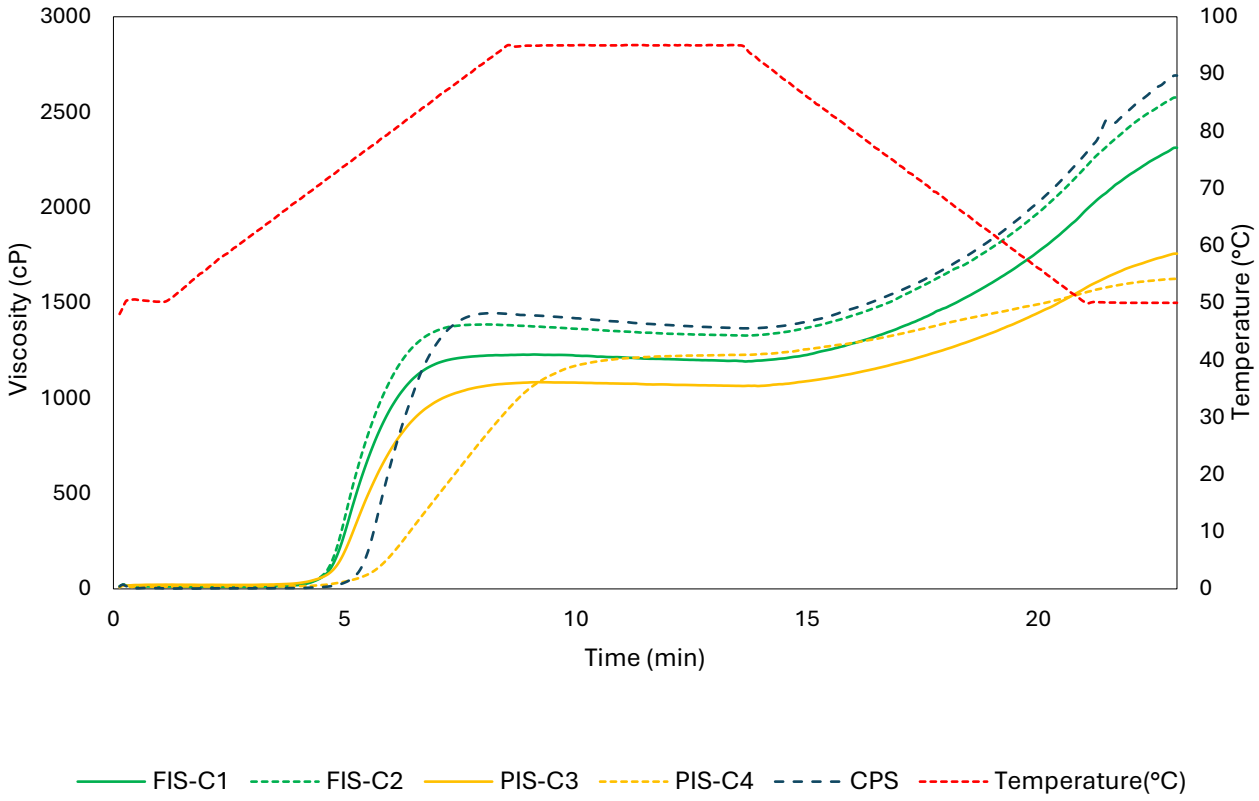

Figure 4.3 Pasting properties faba bean isolated starch (FIS), pea isolated starch (PIS), and commercial pea starch (CPS). Pasting profiles were measured using a Rapid Visco Analyser (RVA). Starch suspension (28.0 g total weight) with 8 % starch (w/w, db) was used for the determination.

Breakdown viscosity reflects the stability of starch against shear forces during heating. PIS-C4 exhibited negligible breakdown viscosity, indicating that the starch was more stable under shear and less likely to lose viscosity when subjected to heating and shearing. The study of Sinhmar et al. (2023) suggested that alkaline extraction strengthened the starch granules and potentially prevented their physical breakdown. Further studies are required to confirm the specific impact of alkaline extraction on the molecular structure of starch.

Upon cooling, notable difference in final viscosities was observed between FIS and PIS. FIS exhibited significantly higher final viscosities (2312.7 and 2576.0 cP for C1 and C2, respectively) than PIS (1757.7 and 1625.7 cP for C3 and C4, respectively), in accordance with the findings of Li et al. (2022) and Li et al. (2019). The results were attributed to stronger physical entanglement between faba bean starch molecules (Li et al., 2022). In comparison with PIS, CPS

showed higher final viscosity, likely due to different pea variety and wet isolation method used by the company to extract CPS.

4.4.1.6 Gelling ability

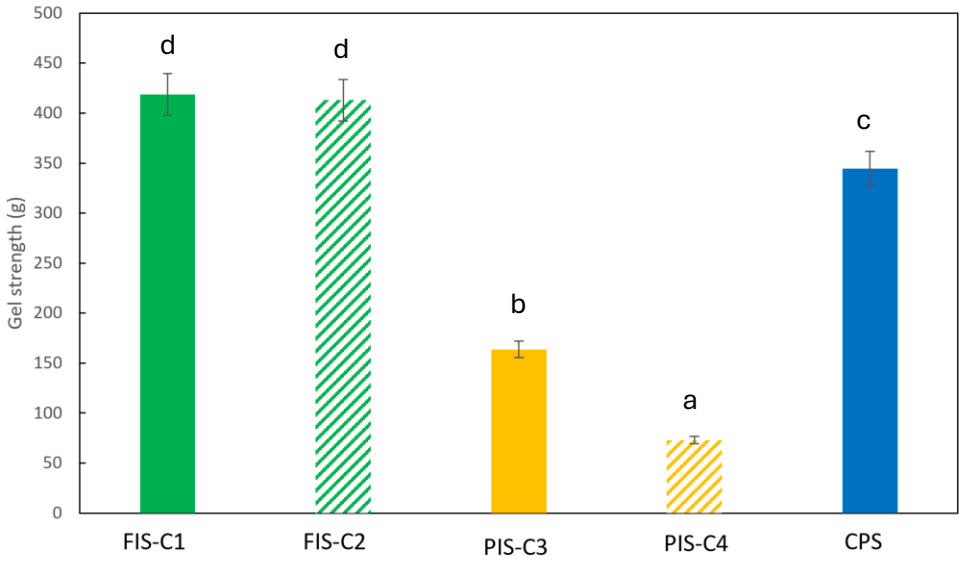

Figure 4.4 Gel hardness of faba bean isolated starch (FIS), pea isolated starch (PIS), and commercial pea starch (CPS). Data are presented as average ± standard deviation (n = 3); data with the same letter are not significantly different at $p < 0.05$.

The gel strength of PIS was notably lower than that of the CPS (72.9-163.7 g *versus* 344.4 g, Figure 4.4), corresponding well with the results of their final viscosities (Figure 4.3 and Table 4.4). This difference could be due to the use of a different pea variety for commercial starch production and variations in the wet isolation method employed to extract CPS. The extreme alkaline conditions might compromise structural integrity of the starch granules (El Halal, Kringel, Zavareze, & Dias, 2019), resulting in reduced gel strength after cooking, which was more obvious for PIS-C4. By contrast, faba bean starch showed stronger gelling capability in comparison with CPS, in good agreement with previous studies (Li et al., 2022; Li et al., 2019). The authors suggested that faba bean starch can better preserve their granular integrity during cooking, and thus form stronger gel. The extraction conditions of C1 and C2 did not significantly influence the gelling ability of the two faba bean starch samples.

4.4.2 Characterization of faba bean and pea protein isolates

4.4.2.1 Proximate composition

The proximate composition of the faba bean and pea protein isolates is given in Table 4.5. The protein isolates consisted of 77.6-94.0% protein and 4.21-5.02% ash, largely comparable with those of the commercial PPI (84.7% of protein and 4.91% of ash). The protein content was reported to be the lowest in PPI-C4, likely due to the extreme pH and temperature conditions used in this alkaline extraction process. Strong alkaline conditions and higher temperatures could enhance the solubility and extractability of non-protein compounds such as polysaccharides, thereby increasing the overall yield but reducing the protein purity (Feyzi, Varidi, Zare, & Varidi, 2015). This observation aligns with the findings of Feyzi et al. (2018), in which the protein yields from grass pea increased but the protein contents decreased as the extraction pH increased from 6.25 to 10.

Table 4.5 Chemical composition of faba bean protein isolate (FPI), pea protein isolate (PPI), and commercial pea protein isolate (CPPI)[a]

	Protein content (%, dry basis)	Ash content (%, dry basis)
FPI-C1	94.0 ± 0.1 e	5.02 ± 0.04 c
FPI-C2	89.2 ± 0.0 d	4.86 ± 0.07 c
PPI-C3	87.6 ± 0.8 c	4.62 ± 0.07 b
PPI-C4	77.6 ± 0.5 a	4.21 ± 0.11 a
CPPI	84.7 ± 0.2 b	4.91 ± 0.05 c

[a] Values are presented as average ± standard deviation (n = 3); in the same column, the numbers with the same letter are not significantly different at $p < 0.05$.

4.4.2.2 Surface properties

Surface charge (expressed as "zeta potential") and surface hydrophobicity are two important surface properties that determine the interactions between protein molecules and thus the functionality of the protein ingredient. Zeta potential and surface hydrophobicity of faba bean and pea protein isolates are shown in Table 4.6. The surface charge results revealed that all pulse protein isolates had negative values at pH 7.0, indicating that they all carried a net negative charge. Zeta potential values of faba bean and pea protein isolates ranged between −31.0 to −47.1 mV. Similar results have been reported in a previous study, where green lentil, Kabuli chickpea and

yellow pea protein concentrates showed a net negative charge, with zeta potential values ranging from −32.10 to −42.72 mV at pH 7.0 (Higa et al., 2022). A comparison of zeta potential of protein isolates from the same pulse variety but extracted using different conditions revealed that extraction pH and temperature had a significant impact on the surface charge of the extracted proteins in the prior study. The reported results indicated that proteins isolated from elevated pH and temperature conditions exhibited an increased surface charge. This increase may be attributed to differences in the ionization of groups on the protein surface and the dissociation of globulins into their subunits during different alkaline extraction conditions (Gueguen, Chevalier, And, & Schaeffer, 1988).

The CCPI showed the lowest zeta potential of −28.2 mV, likely due to variations in protein extraction conditions. In the industry, protein isolates are typically dried using spray drying, which can induce protein aggregation, resulting a decrease in the absolute value of zeta potential (Yang et al., 2022). Conversely, laboratory-scale protein isolate production often uses freeze drying, which results in less protein aggregation than CCPI.

Table 4.6 Zeta potential and surface hydrophobicity of faba bean protein isolate (FPI), pea protein isolate (PPI), and commercial pea protein isolate (CPPI) at pH 7.0 [a]

	Zeta potential (mV)	Surface hydrophobicity (a.u.)
FPI-C1	-31.0 ± 0.3 d	37.4 ± 0.5 a
FPI-C2	-38.0 ± 1.0 b	40.3 ± 1.3 b
PPI-C3	-34.7 ± 1.4 c	41.0 ± 1.1 b
PPI-C4	-47.1 ± 1.6 a	67.3 ± 0.7 c
CPPI	-28.2 ± 0.9 e	113.6 ± 0.7 d

[a] Values are presented as average ± standard deviation (n = 6); in the same column, the numbers with the same letter are not significantly different at $p < 0.05$.

Surface hydrophobicity results indicate the presence of hydrophobic moieties on the protein surface, which are influenced by the amino acid composition, protein conformation, and folding or unfolding of protein (Shen et al., 2022). The surface hydrophobicity values of isolated pulse proteins ranged from 37.4-67.3 a.u. (arbitrary units), and the faba bean and pea proteins

isolated from elevated pHs and temperatures (*i.e.*, FPI-C2 and PPI-C4) showed a significantly higher surface hydrophobicity than FPI-C1 and PPI-C3 from milder conditions. This could be attributed to greater degree of denaturation and unfolding of the protein molecules caused by the more rigorous extraction conditions of C2 and C4, which exposed more hydrophobic moieties to the protein surface. However, the CPPI had the highest surface hydrophobicity of 113.6 a.u., which could be attributed to the different processing conditions applied for this commercial sample. In commercial-scale processing, protein isolates are commonly generated through spray drying, a method that facilitates intensive thermal denaturation of proteins that may result in exposing more hydrophobic regions. This observation agrees with Feyzi et al. (2018), where spray-dried grass pea proteins had higher surface hydrophobicity than freeze-dried samples. However, in another study researchers have reported that freeze-dried cowpea and Bambara bean proteins exhibited higher hydrophobicity than spray-dried samples (Mune & Sogi, 2016).

4.4.2.3 Functional properties

The functional properties of the alkaline extracted faba bean and pea protein isolates at pH 7.0 are presented in Table 4.7. The obtained results revealed that the alkaline extraction conditions significantly influenced ($p < 0.05$) the water solubility of the isolated proteins. FPI-C1 exhibited the highest solubility of 99.0% among all the samples. PPI-C3 also demonstrated notable solubility at 86.5%. The higher solubility of these proteins suggested that they were extracted in a more native state, with a lower degree of denaturation (Fuhrmeister & Meuser, 2003). Overall, the proteins isolated under elevated pH and temperature conditions (FPI-C2 and PPI-C4) had lower solubility in comparison to FPI-C1 and PPI-C3 from milder conditions. This could be due to the higher surface hydrophobicity of FPI-C2 and PPI-C4 (Table 4.6), which promoted aggregation and folding of proteins to show a lower solubility (Higa et al., 2024). Similar results have been reported by Gao et al. (2020), where the solubility of pea protein isolates at pH 7.0 decreased from 93.6% to 80.2% as the alkaline extraction pH increased from 8.5 to 9.5.

In this study, all the prepared pulse protein isolates exhibited superior solubility as compared to CPPI, a finding that aligns with previous research (Stone et al., 2015). For instance, other researchers reported that freeze-dried pea protein isolates obtained via alkaline extraction/isoelectric precipitation approach demonstrated approximately 64% solubility, whereas the commercial pea protein isolate (Propulse, Nutri-Pea Limited, Portage le Prairie, MB, Canada)

showed significantly lower solubility of 5% at pH 7.0. Laboratory-scale protein isolates generally exhibit higher solubility compared to those produced at the commercial scale because the former group is typically freeze dried (Section 3.3.2), but the latter group undergoes harsher drying methods such as spray drying or vacuum drying (Shen, Tang, & Li, 2021).

Table 4.7 Functional properties of faba bean protein isolate (FPI), pea protein isolate (PPI), and commercial pea protein isolate (CPPI) at pH 7.0 [a]

	Solubility (%)	Emulsion stability (%)	Foam capacity (%)	Foam stability (%)	WHC (g/g)	OAC (g/g)
FPI-C1	99.0 ± 1.3 e	45.7 ± 2.3 b	58.9 ± 6.6 a	79.9 ± 8.1 b	N.D.	2.88 ± 0.20 b
FPI-C2	76.5 ± 3.4 c	46.3 ± 0.8 b	127.8 ± 6.6 d	66.9 ± 2.8 a	1.51 ± 0.05 a	3.24 ± 0.09 c
PPI-C3	86.5 ± 3.6 d	50.3 ± 2.0 c	72.2 ± 6.6 b	68.5 ± 4.2 a	N.D.	2.71 ± 0.20 b
PPI-C4	56.9 ± 1.7 b	95.7 ± 0.8 d	91.7 ± 6.6 c	92.3 ± 4.4 c	7.17 ± 0.05 c	3.40 ± 0.16 c
CPPI	43.9 ± 0.6 a	42.0 ± 1.3 a	134.4 ± 5.0 d	67.8 ± 3.1 a	1.90 ± 0.03 b	1.55 ± 0.03 a

[a] Values are presented as average ± standard deviation (n = 6); in the same column, the numbers with the same letter are not significantly different at $p < 0.05$.
N.D. = Not determinable due to the high solubility of protein isolate.

Dispersed systems, such as emulsions, exhibit thermodynamic instability due to several physicochemical driving forces, including gravitational separation, flocculation, coalescence, and Ostwald ripening (Ladjal-Ettoumi, Boudries, Chibane, & Romero, 2016). The emulsion stability (ES) of faba bean and pea proteins to stabilize canola oil in water at pH 7 upon storage for 24 h is shown in Table 4.7. There was no significant difference in the ES of FPI-C1 and FPI-C2, whereas PPI-C4 exhibited a significantly higher ES (95.7%) compared to PPI-C3. This difference could be explained by the notably high surface hydrophobicity observed in PPI-C4. The surface hydrophobicity significantly affects the ability of proteins to adsorb onto the oil-water interface, which is closely related to its emulsifying properties (Damodaran, 2005). Under C4 conditions of elevated pH and temperature, the structure of proteins was altered through unfolding to expose more hydrophobic moieties to the surface (Zhang et al., 2018), which had better adsorption onto the oil-water interface. This could result in the formation of a more stable emulsion with greater resistance to coalescence (Lima, Stephani, Perrone, & de Carvalho, 2023).

Previous research has demonstrated that denaturation or modification of protein structures can expose the hidden sulfhydryl groups, which can form new intra- and intermolecular disulfide bonds. The formation of new disulfide bonds also contributes to the stability of emulsions (Wu, Hua, Lin, & Xiao, 2011). Conversely, Joshi, Adhikari, Aldred, Panozzo, and Kasapis (2011) found that increasing sulfide bonds reduced the emulsion stability as it increased the amount of interfacial bonding and led to coalescence. This indicated that bond forming on the interface between two droplets could negatively impact emulsion stability, while interfacial bonding was crucial for improving the viscoelastic properties of the interface and thus emulsion stability (Lam & Nickerson, 2013).

Foaming properties of the faba bean and pea protein isolates in comparison to CPPI are shown in Table 4.7. Results indicated that protein isolation conditions had a significant impact on their foam capacity (FC) and foam stability (FS). The highest FC of 127.8% was observed in FPI-C2, whereas FPI-C1 had the lowest FC among all the isolated protein samples. This trend was also observed in pea protein isolates, where PPI-C3 had a FC of 72.2% and the PPI-C4 had a significantly higher FC of 91.7%. The results could be attributed to the higher surface hydrophobicity of FPI-C2 and PPI-C4, which facilitated the initial accommodation of hydrophobic groups onto the air-water interface to support the formation of the film surrounding the air bubbles (Higa et al., 2024). FC has been reported to be positively correlated with the surface hydrophobicity, and it is influenced not only by the hydrophobic interactions but also by the interactions and properties of unfolded proteins at the interface (Damodaran, 2005). Similar results have been reported by Cui et al. (2020), who found that increasing the alkaline extraction pH from 8.5 to 10.0 improved FC from ~150% to ~200% in protein isolates extracted from different pea cultivars. The authors noted that the increase in extraction pH reduced surface tension, indicating that pea protein isolates (PPI) extracted at higher pH had enhanced ability to lower the air-water surface tension, thereby increasing FC.

The analysis of foam stability (FS) revealed that FPI-C1 has superior FS compared to FPI-C2. This difference can be attributed to the protein content of the samples, with FPI-C1 having a higher protein content of 94% compared to 89.2% of FPI-C2. Previous studies have shown that higher protein content can enhance foam stability by forming a stable viscoelastic film around air bubbles (Higa et al., 2024; Ma et al., 2022). However, this trend was not observed in pea protein isolates. PPI-C4, with a protein content of 77.6%, exhibited the highest foam stability (FS) of

92.3%, while PPI-C3, with a higher protein content of 87.6%, showed an FS of 68.5%. This discrepancy could be due to the presence of more non-protein compounds, such as polysaccharides, in PPI-C4. These compounds might form thick, adsorbed viscoelastic layers at the air-water interface and increase the viscosity of the bulk phase, thereby providing long-term stability (Amagliani et al., 2021). In a recent study it was reported that increasing the alkaline extraction pH from 8.5 to 10.0 resulted in greater FS (Cui et al., 2020).

Water-holding capacity (WHC), defined as the amount of water that 1 g of sample can hold, is shown in Table 4.7. The WHC of FPI-C1 and PPI-C3 could not be determined due to their exceptionally high solubility of 99.0% and 86.5%, respectively. The protein molecules could lack the flexibility needed to form protein aggregates that can physically entrap water molecules (Shi & Nickerson, 2022). For the other three samples, their WHC values were in a rank order of FPI-C2 < CPPI < PPI-C4. The reported WHC for FPI-C2 and CPPI aligns well with previously published data. Shi and Nickerson (2022) have reported that faba bean protein isolate had a WHC of 1.87 g/g whereas the pea protein isolate had a WHC of 1.93 g/g. However, the WHC of PPI-C4 was significantly higher than values reported in prior studies. This could be attributed to changes in the protein structure resulting from rigorous alkaline extraction and the presence of non-protein compounds, such as polysaccharides, that can easily bind water (Boye et al., 2010).

Oil-absorption capacity (OAC) of the faba bean and pea protein isolates is shown in Table 4.7. FPI-C1 and FPI-C2 had OAC of 2.88 g/g and 3.24 g/g, respectively, and PPI-C3 and PPI-C4 had OAC of 2.71 g/g and 3.40 g/g, respectively. The results revealed that extraction conditions significantly impacted the OAC of protein isolates. Moreover the surface hydrophobicity of protein isolate also plays an important role in determining its oil absorption capacity (OAC). It is suggested that proteins with higher levels of hydrophobic moieties on their surface can facilitate stronger interactions with oil via hydrophobic interactions, thus giving great OAC (Lam et al., 2018). However, despite CPPI having the highest surface hydrophobicity of 113.6 a.u., it had the lowest OAC of 1.55 g/g among the five samples. This indicated that OAC could be influenced not only by surface hydrophobicity but also by the processing method, structure, and the protein groups available to interact with oil (Stone et al., 2015).

4.5 Conclusions

In conclusion, the second study successfully characterized the isolated faba bean and pea starch and protein components under optimized conditions and compared them with commercial counterparts. The isolated starches and proteins demonstrated similarly high purity as compared to that of commercial pea starch and protein isolate, respectively, indicating the effectiveness of the developed fractionation method. However, the damaged-starch contents were higher in the isolated starches compared to commercial pea starch, likely due to the applied harsher extraction process in this research. The granular morphologies of the isolated pulse starches were comparable with commercial pea starch, but PIS-C4 showed more damaged starch granules. The gelatinization and retrogradation properties of the isolated starches were affected by the extraction conditions, with main differences observed in gelatinization temperatures and enthalpy changes. The pasting properties showed variations among the isolated starches, with FIS exhibiting higher peak and final viscosities than PIS. The gelling properties of the five starch samples exhibited a descending order of FIS-C1, FIS-C2 > CPS > PIS-C3 > PIS-C4.

The comprehensive analysis of the techno-functional attributes of proteins revealed the obvious impacts of extraction conditions on the surface and functional properties of protein isolates. The surface properties of protein isolates varied with extraction pHs and temperatures, which then affected their functional attributes such as solubility, emulsion stability, foam capacity and stability, and water-holding and oil-absorption capacities. Overall, the study concluded that extraction conditions significantly influenced the purity and functional attributes of the isolated starches and proteins. These findings highlight the importance of optimizing extraction pH and temperature for the purity and techno-functional attributes of the isolated starch and protein ingredients, which will determine their final industrial applications.

5. GENERAL DISCUSSION

This thesis project demonstrated the great potential of using air-classified starch-rich pulse flours to extract high-purity isolated starches and proteins. This process can help the pulse processing industry add value and expand markets for these by-products. In Canada, a large volume of pulse starches from fractionation is available as starch-rich flours through air classification. Therefore, in Study 1, two of the most common starch-rich pulse flours, faba bean and pea, were selected for isolating high-purity starches and proteins. These commercially sourced starch-rich faba bean and pea flours contained 72.2% and 75.4% starch and 16.2% and 9.4% protein, respectively, on a dry basis (Tables 3.2 and 3.3).

Alkaline extraction followed by isoelectric precipitation process was performed on air-classified starch-rich faba bean and pea flours over a range of pH (8.5, 9.5, 10.5, and 11) and temperature (25, 30, 40, and 50 °C) to identify the optimum pH and temperature combination to extract starch and protein with maximum yields and purity. The employed alkaline extraction methods produced faba bean and pea isolated starches having exceptional purity of 95.0-98.6% with minimal protein impurities of 0.18-0.44%. Moreover, the yields of isolated faba bean and pea starches were 85.0-92.8% and 80.2-87.9% respectively. The purity of the faba bean and pea protein isolates ranged from 85.4-93.1 and 79.7-86.5%, respectively. This indicated the great potential for protein recovery through alkaline extraction of air-classified starch-rich pulse flours. With respect to protein extraction and recovery, the applied alkaline extraction/isoelectric precipitation method led to protein isolate yields of 62.5-71.2% and 43.8-56.1% for faba bean and pea, respectively. The markedly lower yields of protein isolate from starch-rich pea flour could be attributed to the more extensive protein fractionation in pea flour than faba bean flour during air classification (9.4% *versus* 16.2% protein of the two original flours as presented in Tables 3.2 and 3.3.

The study investigated the effects of pH, temperature, and their interactions (pH × Temperature) on the yields and contents of isolated starches and the yields and contents of protein isolates of faba bean and pea during alkaline extraction (Table 3.4). Statistical analysis indicated

that pH had a significant impact on protein isolate yields and contents, while temperature influenced protein contents. Interaction effects were significant only for faba bean protein isolates. The pH, temperature, and their interaction did not significantly affect starch yields and contents.

The effects of pH and temperature on the yields and contents of protein isolates from faba bean and pea were further elucidated and analyzed by developing contour plots (Figure 3.1). These contour plots of faba bean and pea protein yields largely suggested an increase of response over increasing pH and temperature. In contrast, the protein purity of faba bean protein isolates and pea protein isolates decreased over increasing pH and temperature.

Since the different pHs and temperatures did not show significant impact on the isolated starch yields and contents (Table 3.4), extraction conditions were optimized based on maximizing either the content or yield of faba bean and pea protein isolates. The response optimizer method was used to determine the optimum conditions for faba bean and pea protein extraction (Table 3.5). The highest protein content of 93.1% was observed at pH 8.5 and 25 °C, and the highest protein yield of 71.2% was observed at pH 10.5 and 30 °C for faba bean protein isolation; the highest protein content of 86.5% was observed at pH 8.5 and 30 °C, and the highest protein yield of 56.1% was observed at pH 11.0 and 30 °C for pea protein isolation.

Under the optimum conditions, isolated faba bean and pea starches demonstrated high purity, containing 95.1-97.1% starch and impurities of ≤ 0.31% protein and ≤ 0.07% ash(Table 4.2). These isolated starches had starch and protein contents comparable to CPS, which consisted of 97.2% starch, 0.37% protein, and 0.11% ash. This observation suggested that the applied wet extraction method was effective to generate pulse starches of a suitable purity from air-classified starch-rich flours for further modifications and uses. The damaged-starch contents of the isolated pea and faba bean starches (0.92–4.94%) were significantly higher than that of CPS (0.71%). For conditions C1-3, the high levels of damaged starch could be from those of original air-classified starch-rich pulse flours (1.9-3.0%) as reported before (Li et al., 2019). However, when the condition was changed from C3 to C4 for isolating pea starch, the damaged starch content was increased from 1.32% to 4.94%, which could be attributed to the harsher extraction condition, pH 11.0 and 50 °C, responsible for partial starch gelatinization. Amylose contents of FIS and PIS (34.0-37.7%) were comparable or slightly lower than that of CPS (37.4%).

Scanning electron microscopy revealed that the granular morphologies of FIS and PIS (Figure 4.1) were largely similar to that of CPS. The isolated pulse starch granules and CPS

displayed oval, kidney or irregular shapes, which were similar to those of starch granules from other pulse crops as described in previous literature (Hoover et al., 2010; Li et al., 2019). Interestingly, PIS-C4 showed some damaged starch granules with cracks and scratches on the surface, consistent with the highest damaged-starch content of this sample (4.94%) as described above. Wide-angle X-ray diffraction analysis indicated that all FIS, PIS, and CPS exhibited the C-type pattern, which aligns closely with findings from prior studies (Hoover et al., 2010; Li et al., 2019). PIS-C4 showed the lowest percentage crystallinity, resulting from its highest degree of starch damage.

The gelatinization temperatures and enthalpy changes (ΔH) were mostly comparable in FIS-C1, FIS-C2, and PIS-C3, with gelatinization temperatures and ΔH ranging from 59.2-76.9 °C and 14.4-15.7 J/g, respectively. However, PIS-C4 exhibited higher gelatinization temperatures (68.0-77.2 °C) and lower ΔH (12.5 J/g) compared to the other three isolated starches. Despite all the differences in their gelatinization properties, the retrogradation properties of the FIS, PIS, and CPS were generally comparable, which could be attributable to their similar amylose contents (Table 4.2) and branch-chain-length distributions of amylopectin as reported by Li et al. (2019).

Pasting temperatures of the FIS, PIS, and CPS were in an order of FIS-C1, FIS-C2, PIS-C3 < CPS < PIS-C4 (Figure 4.3 and Table 4.4), which is largely consistent with the rank order of their T_o. All FIS and PIS showed lower peak viscosities than CPS, which was likely due to the greater granule rigidity and integrity of the latter. In contrast, the isolated starch granules may have a slightly weak structure due to alkaline treatment. Upon cooling a notable difference in final viscosities were observed between FIS and PIS. FIS exhibited significantly higher final viscosities (2312.7 and 2576.0 cP for C1 and C2 conditions, respectively) than PIS (1757.7 and 1625.7 cP for C3 and C4 conditions, respectively). This trend aligns with findings reported by Li et al. (2022) and Li et al. (2019), where faba bean starch consistently demonstrated higher final viscosities. The gel strength of PIS – 163.7 and 72.9 g for C3 and C4 conditions, respectively – was notably lower than that of the CPS at 344.4 g. However, the two isolated faba bean starches showed an exceptionally strong gelling capability with gel strength of 413.0 and 418.5 g. The greater gel strength of the faba bean starches than the pea starches agrees well with previous studies, which could be due to the ability of faba bean starches to better preserve their granular integrity during pasting (Li et al., 2022; Li et al., 2019).

To examine the proximate composition of the faba bean and pea protein isolates, their protein contents and the ash contents were quantitated (Table 4.5). The obtained protein isolates showed 77.6-94.0% protein () and 4.21-5.02% ash, which are largely consistent with CPPI (84.7% of protein and 4.91% of ash). The surface property analysis revealed that all pulse protein isolates had negative values at pH 7.0, indicating that they carried a net negative charge. Zeta potential values of the faba bean and pea protein isolates ranged between −31.0 and −47.1 mV. The surface hydrophobicity values of isolated pulse proteins were ranging from 37.4-67.3 a.u. (arbitrary units) and the faba bean and pea proteins isolated from elevated pHs and temperatures (FPI-C2 and PPI-C4) showed a significantly higher surface hydrophobicity than FPI-C1 and PPI-C3 from more benign conditions. This might be attributed to greater denaturation and unfolding of the proteins caused by the more rigorous extraction conditions, which exposed more hydrophobic moieties to the protein surface.

The analysis of functional properties of faba bean and pea protein isolates demonstrated that extraction conditions had significant effects on the functionality of proteins. FPI-C1 exhibited the highest solubility of 99.0% among all samples. PPI-C3 also demonstrated notable solubility of 86.5%. The higher solubility of these proteins suggested that they were extracted in a more native, rather than denatured state (Fuhrmeister & Meuser, 2003). Overall, the proteins isolated under elevated pH and temperature conditions (*i.e.*, FPI-C2 and PPI-C4) had lower solubility than the counterparts isolated under milder conditions (*i.e.*, FPI-C1 and PPI-C3).

The emulsion stability (ES) of faba bean and pea proteins to stabilize canola oil in water at pH 7 upon storage for 24 h showed that there was no significant difference in ES of FPI-C1 and FPI-C2 but PPI-C4 exhibited a significantly higher ES (95.7%) than PPI-C3. The comparison of the foaming properties of the faba bean and pea protein isolates with CPPI revealed that protein isolation conditions exhibited significant influence on their foaming capacity (FC) as well as foam stability (FS). The highest FC of 127.8% was observed for FPI-C2, whereas FPI-C1 had the lowest FC of 58.9% among all the isolated protein samples. The water-holding capacity (WHC) of FPI-C1 and PPI-C3 could not be determined due to their exceptionally high water solubility. In contrast, PPI-C4 exhibited a WHC significantly higher than previously reported values. This could be due to structural alterations in the protein caused by extensive alkaline extraction, as well as the presence of non-protein substances such as soluble fibers, which readily bind water (Boye et al., 2010).

FPI-C1 and FPI-C2 had oil-absorption capacity (OAC) of 2.88 and 3.24 g/g, respectively, while PPI-C3 and PPI-C4 had OAC of 2.71 and 3.40 g/g, respectively. The results revealed that the extraction conditions, rather than pulse varieties, had more impact on OAC of protein isolates.

6. GENERAL CONCLUSIONS AND FUTURE STUDIES

This study aimed to develop a simple and effective wet isolation method that could further separate high-purity starch and protein from starch-rich pulse flours. Commercial starch-rich faba bean and pea flours from air classification were subjected to alkaline extraction to obtain high-purity starch and protein. The alkaline extraction was performed over a range of pH levels (8.5, 9.5, 10.5, and 11.0) and temperatures (25, 30, 40, and 50°C) to evaluate the effects of these two important variables on starch and protein extraction, and the responses were optimized to identify the optimum pH and temperature combinations to extract starch and protein with maximum yield and purity. The isolated pulse starches had extraction yields of 80.2-92.8% and purity of 95.0-98.6% for faba bean and pea, respectively; the pulse protein isolates showed extraction yields of 43.8-71.2% and purity of 79.7-93.1% under the tested conditions. The pH level showed significant ($p < 0.05$) influence on the yields and purity of protein isolates from faba bean and pea using the alkaline extraction method, and the temperature only displayed significant ($p < 0.05$) impact on the purity of protein isolates. Conversely, both pH and temperature did not exhibit a significant ($p > 0.05$) effect on the yields and purity of isolated starches.

The second part of the study focused on characterizing the isolated faba bean and pea starch and protein components from optimized conditions and compared them with commercial counterparts. The isolated starches and proteins demonstrated high purity and starch and protein contents similar to those of the commercial samples, indicating the effectiveness of the fractionation method. However, the damaged-starch contents were higher in the isolated starches compared to that of commercial pea starch, probably due to the more rigorous extraction conditions. The granular morphologies of the isolated pulse starches were comparable with that of commercial pea starch, but PIS-C4 showed more damaged granules. The gelatinization and retrogradation properties of the isolated starches were affected by the extraction conditions, with main differences observed in gelatinization temperatures and enthalpy changes. The pasting properties showed variations among the isolated starches, with FIS exhibiting higher peak and

final viscosities than the isolated pea starches. The gelling properties of the starches showed an order of FIS-C1, FIS-C2 > CPS > PIS-C3 > PIS-C4.

The characterization of the techno-functional attributes of protein isolates revealed the significant influence of extraction conditions on their surface and functional properties. The surface properties of protein isolates varied with extraction pHs and temperatures, which consequently displayed effects on solubility, emulsion stability, foam capacity and stability, as well as water-holding and oil-absorption capacities. Overall, the study concluded that extraction conditions not only significantly influenced the yields and purity of protein isolates but also effectively altered the functional attributes of the isolated starches and proteins. The new findings will be meaningful for extracting starch and protein components from air-classified starch-rich pulse flours for value-added utilization in the industry.

Future research can be focused on scaling up the optimized extraction method from laboratory scale to pilot or industry scale and assessing the economic feasibility and reproducibility in larger-scale production settings. This step is crucial for potential industrial adoption of the developed isolation process. This will also facilitate the integration of the new method into existing fractionation processes for pulses. Moreover, future research can be performed to investigate the nutritional quality of the obtained starch and protein ingredients, which will be also important for their end uses.

Beyond isolation, the focus can be extended toward enhancing the functional and nutritional attributes of the isolated starches and proteins. This can involve exploring advanced modification techniques such as chemical derivatization, enzymatic treatments, and physical treatments. Additional research can be performed to understand the impacts of different modification methods on the structural characteristics, physicochemical properties, and nutritional profiles of the new starch and protein ingredients. It will be valuable to evaluate the performance of native and modified starch and protein ingredients in different food and industrial products.

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