THE USE OF SOLID-STATE FERMENTATION TO IMPROVE THE PROTEIN QUALITY, FUNCTIONALITY AND FLAVOUR OF PULSE PROTEIN ISOLATES

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ABSTRACT

The aim of this research was to investigate the effect of solid-state fermentation (SSF) by *Aspergillus oryzae* NRRL 5590 on pea (PPI) and navy bean protein isolate (NBPI) to improve the functional properties, protein quality and flavour profiles. In study 1, proximate composition, physicochemical, functionality properties, and protein quality were evaluated. It was found that degree of hydrolysis of fermented PPI after 48 h was 9.3 and for fermented NBPI after 72 h was 3.6. Regarding to proximate composition, protein content of PPI increased from 82.0 to 83.9% and protein content of NBPI (82.7%) was similar in all fermentation times. Based on surface properties results, the surface charge increased for PPI ranged from -34.1 to -38.8 mV over fermentation time. Surface hydrophobicity increased from 19.3 to 33.2 a.u. (arbitrary units) for PPI and from 11.6 to 17.7 a.u for NBPI. According to functional properties, the nitrogen solubility was found to significantly increase with fermentation time from 8.7 to 34.2 % for PPI ($p \leq 0.05$). The emulsion stability (ES) of PPI was found to decline over fermentation time (0-48 h) from 81.1 to 61.35. Whereas foaming capacity and stability increased from 155 to 175% and 33 to 69%, respectively. The solubility, ES, foaming properties were found to remain relatively constant over fermentation time (0-72 h) for NBPI. *In vitro* protein digestibility (IVPD) showed a reduction during fermentation of PPI from 88.2 to 75.2%. The methionine and cysteine were limiting amino acids for both PPI and NBPI. In summary, fermentation may be used to improve the solubility and foaming properties of PPI and WHC of NBPI for potential use as a food ingredient. In study 2, the effect of fermentation on the presence of volatile compounds in PPI and NBPI were evaluated. Gas chromatography-mass spectrometry (GC) results showed that the flavor compounds in NBPI did not change significantly with fermentation time. However, SSF enhanced new desirable flavour compounds in PPI. These findings suggest that SSF of PPI could potentially improve flavour through the masking undesirable flavour compounds. Together, SSF is an efficient method for improving the solubility, foaming properties, and flavour in PPI.

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LIST OF ABBREVIATIONS AND SYMBOLS

A. oryzae	*Aspergillus oryzae*
A. niger	*Aspergillus niger*
a.u.	Arbitrary units
ABS	Absorbance
ANOVA	Analysis of variance
ANS	8-anilino-1-naphthalenesulfonic acid
B. subtillus	*Bacillus subtillis*
CFU	Colony forming units
d.b.	Dry weight basis
DF	Dilution factor
DH	Degree of hydrolysis
DIAAS	Digestible indispensable amino acid score
DL-BAPN	Na-Benzoyl-D, L-arginine 4 nitroanilide hydrochloride
EA	Emulsion activity
ES	Emulsion stability
FAO	Food and Agriculture Organization
FC	Foaming capacity
FS	Foaming stability
GAE	Gallic acid equivalent
GRAS	Generally recognized as safe
HPLC	High-performance liquid chromatography
IVPD	*In vitro* protein digestibility
IV-PDCAAS	*In vitro* Protein Digestibility Corrected Amino Acid Score
L. brevis	*Lactobacillus brevis*
L. fermentum	*Lactobacillus fermentum*
L. plantarum	*Lactobacillus plantarum*
LAB	Lactic acid bacteria
MC	Moisture content
NBPI	Navy bean protein isolate
OHC	Oil holding capacity

PDA	Potato dextrose agar
PER	Protein efficiency ratio
pI	Isoelectric point
PPI	Pea protein isolate
R. oryzae	*Rhizopus oryzae*
R. oligosporus	*Rhizopus oligosporus*
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SmF	Submerged fermentation
SSF	Solid-state fermentation
TNBS	2,4,6-trinitrobenzenesulphonic acid
ZP	Zeta potential
"	Zeta potential
η	Dispersion viscosity
ε	Permittivity
κ	Debye length
α	Particle radius
*U*E	Electrophoretic mobility

1 INTRODUCTION

1.1 Overview

Pulses are cultivated around the world, with a major portion of them being produced and exported by Canada (Malla & Brewin, 2019). Pulses encompass the dry edible seeds of leguminous plants including peas, chickpeas, dry beans, dry broad beans, lentils, vetches, bambara beans, lupins, and other minor pulses (Venkidasamy *et al.*, 2019). These crops represent an important source of nutrition for the growing population of the world, especially for developing or underdeveloped countries, since pulse proteins are cheaper and more abundant than animal protein (Medendorp *et al*., 2022). The protein content in pulses is approximately 21%–25% (w/w) which is almost twice than that found in cereals; however, they have limiting amounts of essential amino acids such as cysteine, tryptophan, and methionine (Venkidasamy *et al*., 2019). In addition, pulses have good functional and nutritional attributes, making them attractive to the food industry. Pulses are also a good source of dietary fiber, vitamins, and minerals, such as iron, zinc, folate, and magnesium. They also contain phytochemicals, saponins and tannins which are antioxidant and anti-carcinogenic components (Wiensiger etal., 2022). Incorporating protein isolates into different foods can increase the nutritional value and add desirable functional properties to them (Boye *et al.*, 2010). These functional properties can be solubility, gelation, emulsifying ability, oil and water absorption capacity and foaming.

Furthermore, functional properties of pulse proteins contribute an important aspect in determining the competitiveness of the protein ingredient or the product in the market, as they ca impact the sensory, physical and chemical properties of a food, which includes texture and organoleptic characteristics (Singhal *et al*., 2016). Recently, there has been a growing interest by the food industry towards utilizing pulse proteins in novel products due to their nutritional value, availability, low cost, desired functional properties and beneficial health effects. Pulse protein isolates can be utilized in various food products such as non-dairy milk, beverages, cereals snack foods and bars, bakery products, baby foods, meat analogs, and nutrition supplements (Singhal *et al*., 2016).

In addition, pulses are known to have strong beany flavours which negatively affects their consumer acceptability (Roland *et al*., 2017). The off-flavour in pulses is generally associated with aldehydes, alcohols, ketones, acids, pyrazines and sulfur compounds; off-taste compounds belong to the saponins, phenolics, and alkaloids (Singh, 2017). The astringent character in pulses can be associated to the phenolics including vanillic acid, caffeic acid, ferulic acid, and p-coumaric acid, whereas ethyl esters of these phenolic compound contribute to bitterness (Hufnagel & Hofmann, 2008). Off-flavours can also be generated through the oxidation or degradation of unsaturated fatty acids by lipoxygenase and the Maillard reaction (Karolkowski *et al*., 2021). Heng *et al.* (2006) reported off-flavours also depend on the growing region and storage conditions. Flavour compounds can be reduced or eliminated using various processing techniques, such as from thermal means including extrusion, steam injection or infrared heating, or may be masked by other flavour compounds by fermentation (Heng *et al*., 2006).

The goal of this project is using solid state fermentation to improve digestibility, protein quality, and functional properties of pea and navy bean protein isolates. Additionally, applying fermentation to remove or mask off-flavour compounds from pulse protein isolates to produce a relatively bland flavour.

1.2 Objectives

The overarching aim of this research project is to apply fungal solid-state fermentation (SSF) to improve the functional attributes and protein quality of pea and navy bean protein isolates. In addition, the effect of SSF on the reduction or masking of undesirable flavor of these protein isolates at different fermentation times.

Specific objectives include:

- To examine the effect of SSF on the degrees of hydrolysis, surface, and hydrophobicity properties of protein isolates.
- To examine the effect of SSF on the functional properties of pulse protein isolates.
- To examine the effect of SSF on the amino acid profile, *in vitro* protein digestibility and protein quality of protein isolates.
- To examine the effect of SSF on flavor compounds and aroma profile of protein isolates.

1.3 Hypotheses

The following hypotheses will be tested during this research project:

- Microbial SSF growth in conjunction with microbial metabolic activity will result in greater unraveling of pulse protein structure with fermentation time, along with increased degrees of hydrolysis.
- Hydrophobicity and surface charge will increase as fermentation time increases due to exposure of a greater number of buried reactive groups.
- Oil and water holding capacities will increase with fermentation time due to exposure of a greater number of reactive groups.
- Fermentation will result in greater foaming, emulsification, and solubility, due to release of smaller peptides.
- As fermentation time increases, protein digestibility will increase.
- Longer fermentation time will result in a greater reduction or masking of undesirable flavour compounds by newly-formed aromatic compounds.

2 LITRATURE REVIEW

2.1 Pulses

Pulses consist of the dry edible seeds from non-oilseed legumes. They represent a rich source of proteins, complex carbohydrates, vitamins, and minerals such as iron, potassium, and folate (Nosworthy *et al*., 2017). Pulses belong to the *Leguminoseae* family and commonly includes dry grains but does not include soybean (*Glycine max*) and peanuts (*Arachis hypogaea*) which are grown for producing edible oil (Becerra-Tomás *et al*., 2019; Bessada *et al*., 2019). In general, pulses for human consumption include several types of beans (*Phaseolus vulgaris* L.; *Vigna unguiculata* (L.) Walp), faba beans (*Vicia faba* L.), lima bean (*Phaseolus lunatus* L.), peas (*Pisum sativum* L.), chickpea (*Cicer arietinum* L.), lentil (*Lens culinaris Medik*), lupin (*Lupinus albus* L.), among other crops (Giusti *et al*., 2017; Dilis, & Trichopoulou, 2009).

2.2 Pulse composition

As the demand for animal proteins in the world increases, there is an increasing interest in the consumption of proteins from plant sources (Malcolmson & Han, 2019). In many developing countries, animal protein is too expensive and has environmental sustainability issues. Alternatively, pulses represent an economically and nutritionally viable source for proteins. Pulse crops are considered to be a rich source of proteins (22%-24%), carbohydrates and micronutrients, but have low amounts of fat with the exception of chickpea (5%–7%). The amount of protein in pulses is two times more than cereals. For example, the protein content is 27%–34% for faba beans (Nosworthy *et al.,* 2018) 22%–26% for peas, beans, lentils and chickpeas (Raw, 2012; Wang & Daun, 2004, 2006), and 33%–47% for lupins (Tounsi-Hammami *et al*., 2019). However, for cereal grains protein content is 7%–13% for barley, 2%–5% for rice and 9%–15% for wheat (Dahl, 2019). Pulse proteins are limiting in sulfur-containing amino acids (cysteine and methionine) and tryptophan but are rich in lysine (Zha et al., 2021). In contrast, cereals are limiting in lysine but are rich in the sulfur-containing amino acids. As such, pulses and cereals are often consumed as part of a complementary diet. Major proteins in pulses are globulin and albumin accounting for almost 70%.

and 10%–20% of the total pulse protein, respectively (Vogelsang-O'Dwyer *et al*., 2021). There are also prolamins and glutelins as minor amounts (Nasir *et al*., 2022). In addition, there are also some bioactive compounds such as polyphenols (including tannins), saponins, and lectins and enzymes inhibitors (e.g., trypsin inhibitors, chymotrypsin inhibitors and α-amylase inhibitors) (Patterson *et al*., 2017). These compounds can have negative effects on the nutritional aspects of pulses. For instance, trypsin or chymotrypsin inhibitors or polyphenols can decrease their digestibility, whereas others can affect mineral absorption (Azarpazhooh & Ahmed, 2022). Processing technologies, such as soaking, dehulling, boiling, germination and fermentation may be effectively used to reduce levels of these compounds (Patterson *et al*., 2017).

Pulses have potential advantages to human health and the alleviation of different diseases due to existence of various bioactive compounds, protein, and dietary fibers. Pulses such as chickpeas have health benefits against digestive illness, for example type 2 diabetes (T2D) and cardiovascular diseases (CVD). In addition, it has been reported previously that fermented pulse starch has potential anticarcinogenic activity (Pittaway *et al*., 2007; Venkidasamy *et al*., 2019).

2.3 Pea

One of the major pulses is pea (*Pisum sativum* L.) which has the potential to be an alternative to soy (Vidal-Valverde *et al*., 2003) because it is a non-genetically modified organism (GMO) with a low allergenicity compared to soy. However, like other plant proteins, there are challenges in utilizing pea protein as a food ingredient such as limitations in functionality and flavor issues. Pea is cultivated on over 25 million acres worldwide and is ranked fourth in global plant protein production ranking. Meanwhile, Canada is the largest producer and exporter of pea grains all over the world (Lu *et al*., 2019). The most important types of peas grown worldwide are Austrian winter pea, green pea, maple pea, marrowfat pea and yellow pea. More than 60 varieties of peas have been developed by crop scientists in Canada (Roy *et al*., 2010). Pea is a good source of protein and carbohydrate, low in fat, and contains important vitamins and minerals. It has a well-balanced amino acid profile and compared to cereal proteins, pea protein is rich in lysine, leucine and phenylalanine (Lu *et al*., 2019). For example, pea protein consists of 20-30% lysine-rich protein (Erem *et al*., 2021). But it is limiting in methionine and cysteine. Therefore, pea protein is usually consumed along with cereal grains, since they have a complementary essential amino acid

profile in that cereal proteins are generally limiting in lysine but contain higher levels of sulfur amino acids (methionine, cysteine) (Swanson, 1990).

The protein content of pea ranges from 23.1% to 30.9%, depending on cultivation conditions (Lam *et al*., 2018). The storage proteins found within the pea include the water-soluble albumins and the more abundant, salt-soluble globulins. Globulins consist primarily of vicilin (7*S*) and legumin (11*S*) proteins. In pea protein, albumin accounts for 15-25% of the total protein, whereas globulin accounts for 50-60% of the total protein. Most of the essential amino acids (e.g., tryptophan, lysine, threonine, cysteine and methionine) exist in albumin in comparison with globulin (Boye *et al*., 2010).

2.4 Navy bean

Navy bean is another type of pulse and a variety of common beans (*Phaseolus vulgaris*) which provide essential nutrition globally for a relatively low cost due to their richness in protein, carbohydrates, dietary fiber, and micronutrients. Navy beans have tremendous health benefits, including putative abilities to inhibit colon carcinogenesis, lower the risk of obesity, total cholesterol, and cardiovascular risk factors (Guldiken *et al*., 2021; Toews & Wang, 2013). Navy bean contains 54%–79% of globulin which includes a major fraction of phaseolin (7*S*) and a minor fraction of legumin (11*S*). Navy bean has a characteristic "S" (Sanilac) type phaseolin of which the three subunits correspond to the molecular weights of 45.75, 42.80, and 40.74 kD. The limiting amino acids for navy bean are usually the sulfur amino acids, methionine and cysteine (Tabtabaei *et al*., 2019).

2.5 Fermentation of pulses

Fermentation is a non-thermal process which helps to partially denature and unravel proteins so that buried reactive amino acids become exposed and digestibility improves. Further, the functional attributes of these proteins can remain similar or become positively - or negatively-affected. Because of these characteristics, there is a growing interest in fermentation by industry. Fermentation can improve the digestibility of proteins in pulses by reducing levels of chymotrypsin and trypsin inhibitors, and through the production of microbial proteases which acts to open up the protein's structure making them more susceptible to digestive enzymes (Chandra-Hioe *et al*., 2016; Reyes-Moreno *et al*., 2004). Proteases also release peptides of low molecular weight which have

potential bioactive properties (Maleki & Razavi, 2021). Fermentation can improve mineral absorption due to production of organic acids, which forms soluble complexes with minerals. In addition, fermentation can extend the shelf-life of food and prevent food loss because of spoilage, especially in developing countries where freezing, canning, and refrigeration is not accessible (Tamang *et al*., 2016). In these countries, fermentation can have special benefits, since it offers both economic and practical advantages because of its low cost and ability to enrich the food substrate with nutritional value, flavor, texture, and aroma (Starzyńska-Janiszewska *et al.*, 2014). In addition, fermentation is a non heating process thus it can prevent the loss of nutrients during the heating and cooking process (Hemalatha *et al*., 2007).

2.5.1 Different types of fermentation

There are two different kinds of commercial fermentation: submerged (SmF) and solid-state (SSF) fermentation. In SmF, nutrients are available in the water to support microbial growth from species that grow better within a high moisture environment (e.g., *Lactobacillus plantarum*) (kumar *et al*., 2021). SmF does, however, have some disadvantages. For instance, SmF uses a lot of water which must ultimately be removed through drying processes; thus, a lot of waste water is generated, and has inefficient mass and heat transfer (Postigo *et al.*, 2021). In contrast, in SSF the available free water is low, where nutrients are available as insoluble substrates. Typically, fungi (*e.g*., *Aspergillus spp.*) are well suited for SSF. In recent years, much research has been conducted on SSF to produce alcohols, organic acids, flavor compounds, biologically-active substances, and other classes of compounds, with little work on its use for protein modification. SSF has some advantages including: a) application of cheap substrates, such as cereals, wheat bran, and other agricultural products/waste; b) the purification, recovery, and disposal of downstream processes are usually simpler compare to SmF; c) improving the nutritional value and produce food with a special flavor; and d) no discharged wastewater (Wang & Daun, 2004). However, SSF does have some disadvantages. For instance, the detection and control of the fermentation parameters are more difficult in SSF since cell growth, and secretion of absorbed nutrients and metabolites are not uniform throughout the solid matrix. In addition, achieving large-scale industrial production based on SSF is not easy due to low substrate utilization rates, contamination issues, complicated reactor design that are typically batch-based (and not continuous), lack of universal mass transfer and heat transfer models, and so on (Hamidi-Esfahani *et al*., 2004; Wang & Daun, 2004).

2.6 Functionality of pulses protein

Protein functionality is related to the physicochemical properties of the protein as well as processing (e.g., shear, pressure, etc.) and extrinsic (e.g., pH, temperature, presence of salts) factors. These functional attributes include water binding, solubility, thickening, emulsification, fat binding, gelation, foaming, and flavor binding (Boye *et al*., 2010).

2.6.1 Solubility

Protein solubility is based on amount of nitrogen of a protein which can be dissolved in a solution under a specific set of conditions. Solubility is a critical property because it affects other properties such as emulsification, gelation and foaming (Kinsella, 1979). Protein solubility is dependent on intrinsic factors, such as protein amino acid composition and protein amino acid sequence and, extrinsic factors, such as pH, ionic strength, temperature, pressure and the concentration of protein (Zayas, 1997). At pH values more distant from the protein's isoelectric point, electrostatic repulsive forces dominate to keep proteins in solution, where maximum solubility occurs at alkaline (pH 9.0-10.0) or acidic (pH <3.0) pH values. In contrast, near the isoelectric point, there is no net charge on the protein, so electrostatic repulsion is minimized. At this pH (e.g., pH values from 4.0-6.0), attractive interactions, such as van der Waals forces and hydrophobicity drive protein aggregation leading to a significant reduction in solubility *(*Boye *et al.*, 2010). Solubility is also influenced by temperature, where it increases with temperature up until the denaturation temperature, and then declines. At the denaturation point, proteins lose their solubility because of unfolding and exposure of buried hydrophobic groups (Carbonaro *et al*., 1997).

The presence of salts can influence solubility in both positive and negative ways. At low concentrations, salt (e.g., NaCl) can increase protein solubility as it helps to order the hydration layer around the protein to promote more protein-water interactions. However, at high concentrations of NaCl, ions can screen the electric double layer around the protein's surface to induce protein aggregation. If a salting-out salt is present (e.g., ammonium sulphate), ion-water interactions are favored, causing a disruption of the hydration layer around the protein. Here, hydrophobic groups on the protein surface become exposed, leading to protein aggregation according to their hydrophobicity. In terms of protein concentration, when the concentration of

soluble protein reaches its maximum concentration, addition of extra protein leads to aggregation (Zayas, 1997).

Kumitch *et al*. (2020a) studied pea protein-enriched flour fermented by *Aspergillus oryzae* NRRL 5590 or *Aspergillus niger* NRRL 334 to obtain nitrogen solubility at different pH values. Solubility significantly decreased over fermentation time (0–6 h) for both fungi at all pH values. The authors attributed this to proteolytic enzymes produced by fungi that hydrolyzed protein into smaller peptides and hydrophobic and hydrophilic moieties of the protein that became exposed. Hydrophilic peptides on proteins were more susceptible to proteolytic activity; therefore, unraveling of the protein led to exposure of hydrophobic moieties (Kumitch, 2020a). Prinyawiwatkul *et al.* (1997) investigated the functional properties of cowpea flour as affected by fermentation of seeds with *Rhizopus microsporus* subsp. *Oligosporus*. They reported that fungal fermentation slightly increased solubility of heat-denatured proteins at different pH values. For example, at pH 4.0, the solubility of flour increased from 4.4% to 13.4% after 24 h of fermentation. This higher solubility may have been due to activity of fungal protease which causes molecular size reduction and exposure of hydrophilic sites (Prinyawiwatkul *et al*., 1997).

2.6.2 Emulsifying properties

Emulsions are comprised of two immiscible liquids, where one phase (known as the discontinuous phase) is dispersed as small droplets into the continuous phase of the other (Yu *et al.*, 2007). Emulsifiers are used in food processing to help with the formation and stabilization of emulsions. Emulsifying properties of proteins depend on their hydrophobic-hydrophilic ratio as well as structural constraints which determine the ability of proteins to unfold and form a film around dispersed oil droplets. During emulsion formation, proteins migrate to the oil-water interface and then reorient their hydrophobic groups towards the oil phase and hydrophilic groups towards the aqueous phase to form a viscoelastic film. This film then prevents changes such as, flocculation, coalescence, creaming, and sedimentation from occurring by electrostatic repulsion (depending on the solution pH in relation to the pI) or steric hindrance. In order to evaluate the emulsifying attributes of a protein, two indices are often used: emulsifying stability (ESI) and emulsifying activity (EAI) indices. ESI evaluates the resistance of emulsion to structure changes over a period of time, whereas EAI determines the amount of emulsified oil per unit of protein (Boye *et al.*, 2010).

Xiao *et al.* (2015) examined the effects of fermentation with *Cordyceps militaris* SN-18 on the emulsifying properties of chickpea flours. The results showed that fermented chickpeas had higher EAI and ESI than that of non-fermented chickpea regardless of the pH. The authors attributed this to fungal proteolytic activity which exposed hydrophobic groups and changed the hydrophilic-lipophilic balance. Furthermore, enzymatic hydrolysis resulted in lower molecular weight peptides that could easily move into the oil water interface and enhance emulsifying activity (Xiao *et al*., 2015).

Kumitch *et al*. (2020a) also examined pea protein-enriched flour fermented by *A. oryzae* NRRL 5590 or *A. niger* NRRL 334 for emulsification properties at pH values of 3.0, 5.0, and 7.0. Accordingly, fermentation time, pH, and fungal strain were shown to have a statistically significant effect on EA ($p < .001$). No EA was detected at pH 5 because it was near pI (pH of 4.6) of the protein. After 6 h fermentation by *A. oryzae,* EA values at pH 3.0 and 7.0 were 35.2% and 40.5%, respectively. Also, for pea protein fermented by *A. niger* after 6 h of fermentation, at pH 3.0 and 7.0, EA values were 35.5% and 37.9%, respectively. At pH 3.0 the ES enhanced slightly from 98.5% to 99.0% for *A. oryzae* and from 97.3% to 99.4% for *A. niger;* whereas, at pH 7.0 the ES values ranged from 97.6% to 94.9% for *A. oryzae* and 94.4% to 94.8% for *A. niger*, respectively. The authors attributed EA and ES to hyphae production under the wet condition by the fungi which led to the more hydrophilic nature of the substrate. In addition, protease activity of the fungi exposed hydrophobic moieties of the proteins. Over 16 h of stirring to homogenize the solution, the proteins presumably became reoriented, and the hydrophobic moieties aggregated together to form large aggregates that fell out of solution (Kumitch *et al*., 2020a).

Imbart *et al*. (2016) studied the effect of fermentation on the emulsifying properties of cowpea protein. The authors showed that fermented cowpea protein leads to a destabilization of the emulsion because of degradation of proteins by microorganisms. In addition, the size distribution of the emulsion droplets became extended as the fermentation time increased. These changes in the droplet size distributions were due to hydrolyzing of the proteins during the fermentation process which lead to changes in their ability to migrate and re-align at the interface. Furthermore, the fermented flour samples produced emulsions with reduced viscosity compared to the control emulsion, leading to reduced stability (Imbart *et al*., 2016).

2.6.3 Foaming stability and capacity

The formation of foam is an important factor in some food applications, such as whipped toppings, mousses, beverages, and meringue cakes. Foams are defined as the entrapment of air in a liquid or solid phase to form small bubbles. Similar to emulsions, proteins migrate to the interface and re-align to position their hydrophobic moieties towards the non-polar gas phase and their hydrophilic sites towards the aqueous phase. This interfacial layer helps prevent air bubble collapse and keeps them in suspension (Makri *et al*., 2005). Proteins can act as surfactant Foam capacity (FC) and stability (FS) are the most common indices for evaluating foaming properties. FC is measured as the maximum increase in volume (%) or amount of air that can be entrapped within the unit amount of protein because of whipping whereas FS is calculated as the changes in the volume of foam over a specified period of time (0–30 min) (Boye *et al*., 2010). The rate that various proteins diffuse and adsorb at the air-water interface will define the protein's ability to lower the interfacial tension and form a foam (Martin *et al.,* 2002). In addition, characteristics such as low molecular weight, high solubility, high surface hydrophobicity, greater flexibility and ease of denaturation will favor proteins to achieve optimum FC (Murray, 2007). Denaturation helps the protein to align at the interface. The major proteins in pulses are globular which can slow surface denaturation; therefore they have low foaming ability compared to animal proteins (Kaur & Singh, 2007). Foam stabilization can increase by minimizing the electrostatic repulsion at the protein's pI, or by salt addition to screen charges (Makri *et al*., 2005).

Yu *et al.* (2007) studied the functional properties such as foaming capacity of peanut protein concentrate powders fermented by *R. oligosporus* versus unfermented defatted peanut flour. Defatted peanut flour and soy protein isolate were used as references. The authors reported that defatted peanut flour had an FC of only 6%, which is very poor. Roasting of peanuts reduced the FC to 3%; however, fermentation of roasted peanut flour enhanced the FC to 8%. This can be attributed to that fungal fermentation of the peanut flour and the hydrolytic breakdown of large protein molecules resulting in better protein functionality (Yu *et al*., 2007). Kumitch *et al*. (2020a) studied the functional properties of pea protein-enriched flour fermented by *A. oryzae* NRRL 5590 or *A. niger* NRRL 334 at various pH values. In their study, the foaming properties were negatively impacted by fermentation. FC of samples fermented by *A. oryzae* and *A. niger* had a decreasing trend over fermentation time (5 d). The authors reported that decreases in protein solubility prevented the proteins from reaching the air-water interface, thus reducing the FC. In addition, FS

decreased over the fermentation time for both strains which could be attributed to weak protein–protein interactions which are unstable and resulted in bubble/foam collapse (Kumitch *et al*., 2020a)

2.6.4 Oil holding capacity of proteins

Oil holding capacity (OHC) is defined as the ability of proteins to physically entrap oil in their matrix (Boye *et al*., 2010). The OHC influences mouthfeel, texture, and flavor retention of food products (Shevkani *et al*., 2015). Different factors can affect the OHC such as the type of fat, the fat distribution, and structure of protein's matrix including, protein pore size and strand size (Ma *et al.*, 2011). The primary locations for interaction between lipid and protein are non-polar side chains of protein molecules. Thus, OHC increases when there are greater amounts of available non-polar sites at the surface. In addition, the OHC is higher when there are more hydrophobic and insoluble proteins available to interact with lipids (Zayas, 1997).

Different studies have examined the effect of fermentation on the OHC of legume flour. Xiao *et al*. (2015) used *C. militaris* SN-18 to ferment chickpeas by SSF. The OHC values of chickpea flour increased significantly by SSF from 0.95 g/g to 1.13 g/g. This increase in OHC during fermentation was attributed to unmasking of the non-polar residues from the interior protein molecules and enhancement of available hydrophobic amino acids on the surface (Xiao *et al*., 2015).

Kumitch *et al*. (2020a) studied OHC of fermented pea protein inoculated with two fungal strains, *A. oryzae* or *A. niger*. There was a significant interaction effect on OHC between fermentation time and microorganism ($p<0.001$). OHC of the pea protein fermented by *A. oryzae* and *A. niger* increased over the fermentation time from 1.25 to 1.39 g/g and 1.18 to 2.27 g/g, respectively. The authors attributed this to fungi producing proteases which lead to hydrophobic patch exposure. The hydrophobic patches on the protein can lead to aggregation of proteins into clusters which have a space inside and are called microcapillaries. Microcapillaries can physically entrap oil (Kumitch *et al*., 2020a).

Obatolu *et al*. (2007) examined the functional properties of five flours from yam bean made after different processes, including fermentation by lactic acid bacteria. Flour from fermented yam bean had an OHC of 0.40 g/g, which was not significantly different compared to the non-processed flour (0.60 g/g). The authors concluded that the increase in OHC was related to heat denaturation

of proteins which resulted in exposure of the interior non-polar residues of the protein molecules (Obatolu *et al*., 2007).

2.6.5 Water hydration capacity (WHC)

Water hydration capacity (WHC) is defined as the ability of a protein to bind water within its matrix and to hold it against different forces such as pressing, centrifugation, or heating. Specifically, WHC describes the amount of water that can be absorbed per gram of protein. Various types of water relate to the WHC's of a protein. For instance, structured or vicinal water forms a monolayer around protein by interacting with the hydrophilic moieties on the protein surface. The water is strongly bound and unavailable for reactions. Multilayer water refers to absorbed water making up multiple layers around the surface of the protein, which thus becomes largely unavailable for reactions and stabilized by hydrogen bonding. WHC more relates to the free or entrapped water that can be trapped by the protein matrix or capillaries within the protein aggregates. WHC depends on the protein structure, size, amino acid composition, protein concentration and the number of exposed polar groups (Zayas, 1997).

Different studies have investigated effect of fermentation of legumes inoculated with fungi or bacteria on WHC. Xiao *et al.* (2015) examined the fermentation of chickpeas with *C. militaris SN-18*, a filamentous fungus, by SSF. The authors found that WHC of the non-fermented chickpea in this study was significantly influenced by pH, and was in the range of 2.56 to 2.85 g/g. However, for fermented chickpea, there were no significant differences in the WHC at different pH values and had higher WHC than the unfermented flour. It was reasoned that this was due to proteolytic activity during fermentation which broke peptide bonds of proteins and caused an increase in exposed polar groups (Xiao *et al*., 2015).

Kumitch *et al*. (2020b) studied WHC of fermented pea protein inoculated with *A. oryzae* or *A. niger* and found that interaction between fermentation time and fungal strain was statistically-significant ($p<0.001$). The WHC enhanced by duration of fermentation from 1.46 to 2.03 g/g and 1.60 to 1.61 g/g for *A. oryzae* and *A. niger*, respectively. The authors attributed this to the fungus protease activity which cleaved proteins into smaller peptide bonds and lead to exposure of hydrophilic sites on the protein. In addition, hyphae production by fungi resulted in more hydrophilic surface and assisted to greater binding of water (Kumitch *et al*., 2020b).

Adebowale & Maliki (2011) examined the changes in the functional properties of fermented seed flour of pigeon pea after 0, 1, 2-, 3-, 4-, and 5-d fermentation periods. The authors found that WHC decreased during fermentation from 1.42 g/g for the non-fermented flour to 1.13 g/g after 5 d of fermentation. Levels for pigeon pea were greater than obtained values for flours from other legumes such as soybean, mucuna, and lupin seed flour. Therefore, WHC depends on type of legumes and species because of differences in their intrinsic chemical compositions. In addition, WHC was improved by limited fermentation in a time-dependent manner (Adebowale & Maliki, 2011).

2.6.6 Gelation

An important index of gelling capacity is the least gelation concentration (LGC), which is the lowest concentration that is needed to produce a self-supporting gel. Lower LGC values correlate with a protein's higher gelling capacity (Boye *et al*., 2010). Although protein gels can be formed by heat, ions, and pressure, heat-induced gelation is most commonly used in the processing of different heat-set protein products. If the concentration of the protein suspension is high enough, it can form gels upon heating followed by cooling. During pumping and piping, a lower viscosity of protein suspension before heating is desirable; however, higher viscosities and gel formation after heating is desirable for some products such as sausage, meat analogs, and thickened soups. Different factors have an effect on heat-induced gelation of pulse proteins including, protein composition, structure, and pH condition (Yu *et al*., 2007).

Obatolu *et al.* (2007) studied the gelation properties of five flours from yam bean made after the grains were processed by different techniques, including fermentation by lactic acid bacteria. In this study, gelation was significantly-increased by fermentation of the beans. The authors reported the least LGC was ~14% for flour from raw yam bean, while a LGC value of 16% was observed for fermented yam beans. The LGC of legumes has been attributed to the globulin fraction and aggregation of denatured protein molecules (Obatolu *et al*., 2007). Adebowale and Maliki (2011) studied changes in the functional properties of fermented seed flour of pigeon pea for 5 days. Results showed that fermentation significantly decreased the viscosity and gelling power. Gelation power (ability to form gel) values ranged from 43.8 to 56.2%, with highest value for non-fermented seed flour and gelation power decreasing with fermentation periods. The authors reported that variation in the gelling properties can be attributed to the relative ratios of different

constituents, proteins, carbohydrates and lipids that make up the flours. In addition, interactions between such components have a significant role in the functional properties (Adebowale & Maliki, 2011).

2.7 Protein quality

Protein quality depends on the essential amino acid composition in relation to human amino acid requirements and the ability of being digested, absorbed, and utilized for metabolic functions. Usually, proteins from animal resources such as beef, chicken, pork, eggs, or dairy, are highly digestible and have enough amounts of essential amino acids for human development and growth. However, plant-based proteins are limited in one or more essential amino acids. In addition, there are more limiting factors for protein digestibility and amino acid availability in native plant protein sources (Nosworthy & House, 2017). Over the past three decades, different methods for the determining protein quality have been used (Gilani, 2012). In Canada, the protein rating system is used as the official method for determining the protein quality of a food. This system is based on application of the Protein Efficiency Ratio (PER) which is calculated through a rat feeding trial and evaluation of the weight gain per unit of consumed protein. Quality of a tested protein will be determined compared with a casein reference. The protein rating is equal to the grams of protein in a reasonable daily intake multiplied by the adjusted PER. The protein rating is then applied to regulate protein claims labels on products. If the protein rating is achieved between 20.0 and 39.9 the product will be considered as a 'source of protein'; however, any item with a protein rating of 40.0 or more is considered as an 'excellent source of protein' (Nosworthy & House, 2017).

In the United States, PER is calculated for infant formulas; however, for the other items the protein quality is evaluated by the protein digestibility corrected amino acid score (PDCAAS), which is based on the most limiting essential amino acid relative to the reference pattern, multiplied by its true digestibility (Nosworthy & House, 2017). The PDCAAS is calculated by multiplying the amino acid score by the true fecal nitrogen digestibility. The amino acid score is calculated based on the ratio of the amino acid content in the tested protein to the content of the same amino acid in a reference pattern determined by the FAO. If the calculated amino acid score is 1.00 or greater, it means there is no deficiency for that amino acid. The lowest calculated amino acid score shows the first limiting amino acid in that protein and it will be applied as the amino acid score for the calculation of PDCAAS. Protein digestibility is determined by difference between fecal protein

and the ingested nitrogen which is then expressed as percent of total nitrogen consumed. The endogenous protein in total fecal protein is also determined by feeding a protein-free diet. Thus, all the protein in the feces of the animal fed the protein-free diet is assumed as endogenous. Most food proteins, especially plant-based proteins, cannot be digested completely. The PDCAAS indicates the overall quality of a protein based on digestibility of the protein and its amino acid score. This method applies ileal digestibility rather than fecal digestibility and considers the digestibility of each amino acids instead of a single value protein (Nosworthy & House, 2017). Another method called the digestible indispensable amino acid score (DIAAS) is gaining interest internationally although it is not yet widely accepted (Rutherfurd *et al*., 2015). The DIAAS method is based on dietary protein quality measured by true ileal amino acid digestibility which is measured for each amino acid individually. DIAAS is measured at the distal end of the small intestine so that it provides a better estimation of absorbed amino acids compare to PDCAAS; therefore, in the near future, DIAAS may be used instead of PDCAAS (Rutherfurd *et al*., 2015).

There are advantages and disadvantages for different methods of protein quality determination including the PER, PDCAAS, and DIAAS methods. The PER method has two main advantages compared to PDCAAS and DIAAS. The first advantage is its simple calculation of gained weight and protein intake. The second one is that PER shows the effect of the protein source on growth; whereas, PDCAAS and DIAAS indicate the digestibility of the protein source as well as amino acid utilization. PER also has some disadvantages compared to other methods. For instance, PER values of casein can yield different results from different research groups, and this difference can affect the PER value of the tested protein (McLaughlan & Campbell, 1969). Another issue related to PER is that in this method all the proteins are assumed to be used for growth in the body not for maintenance or other metabolic processes. This potential issue presents the application of PER as an indicator of protein quality on a state of growth and not maintenance. Furthermore, another issue regarding PER is that the sulfur amino acid requirements of rats is much more than for humans. Therefore, the PER of any product limiting in those amino acids, such as certain pulses, will not be equal to the quality of the same product when consumed by humans. The PER approach has been modified to account for some of these issues, but during the 1980s a new assay for protein quality was developed. This assay was based on comparative amino acid content relative to human requirements that also corrected for the protein digestibility (Gilani, 2012).

The PDCAAS method is widely accepted and used; however, this method has also undergone criticism. Some concerns about PDCAAS include the truncation of PDCAAS values, calculation using fecal digestibility, and changes in bioavailability of amino acids (Nosworthy *et al.*, 2017). Truncation of the PDCAAS value means that no protein can have a higher score than the reference protein provided by the FAO, even if it has potentially higher quality; however, PER values can be greater than for casein. The application of fecal digestibility in PDCAAS is less invasive, but it is not as accurate as the ileal sampling. The amino acid concentrations at the terminal ileum are different compared to those found in the feces due to interaction with the colonocytes and the microflora of the large intestine. In addition, bioavailability of amino acids is not considered in this method (Nosworthy & House, 2017). The European Union does not use PER, PDCAAS, or DIAAS for its regulation of protein content claims. If a food product supplies 12% of its total energy by protein it is considered as a 'source of protein' and if the protein supply 20% of total energy, then that product is considered as 'high in protein' (European Commission, 2017).

In many studies, fermentation was reported to significantly improve the protein quality for cereal and legume samples. Cuevas-Rodriguez *et al*. (2006) investigated nutritional properties of tempeh flour made from maize. The solid-state fermentation process by *Rhizopus oligosporus* improved PER from 1.78 to 2.10, calculated PER from 1.43 to 1.74, and PDCAAS from 0.55 to 0.83. The *in vitro* protein digestibility was also improved by the SSF process from 72.2 to 83.2%. The authors explained that it could be attributed to elimination of antinutritional factors such as phytic acid during fermentation and protein denaturation during the cooking step, which resulted in proteins being more vulnerable to enzyme's activity (Cuevas-Rodriguez *et al.*, 2006).

Kumitch *et al*. (2020a) studied the effect of SSF using *A. niger* or *A. oryzae* on the changes to levels of enzyme inhibitors, *in vitro* protein digestibility, and protein quality of an air-classified pea protein-enriched flour. SSF improved the digestibility; however, protein quality was not improved. *In vitro* protein digestibility increased over the fermentation time from 74.8% to 80.9% and 74.9% to 79.4% for *A. oryzae* and *A. niger* samples, respectively. The authors attributed the increase *in vitro* protein digestibility to the decrease of trypsin inhibitory activity through SSF and also decreases in pH caused by fermentation leading to increased endogenous protease activity and more readily digestible polypeptides by hydrolysis of larger proteins. However, the overall protein quality as measured by the *in vitro* protein digestibility corrected amino acid score decreased from 66.7% to 63.5% and 69.3% to 59.0% for *A. oryzae* and *A. niger*, respectively after 6 hours. The

authors concluded that fermentation was an effective processing method to increase digestibility; however, limiting amino acids were decreased by fungal utilization and also the protein quality of fermented samples decreased (Kumitch *et al*., 2020b).

3 MATERIALS AND METHODS

3.1 Material

Pea protein isolate was supplied by AGT Food and Ingredients Inc. (Regina, SK, Canada) for this research. Navy beans (Cultivar: Nautica) were harvested in the 2018 crop year and were obtained from Hensall Co-op Ltd. (Hensall, ON). The fungal strain (*Aspergillus oryzae* NRRL 5590) used in this study was supplied by Agriculture Research Service, US Department of Agriculture (Peoria, IL, United States). All chemicals were purchased from Sigma-Aldrich Co. (Oakville, ON, Canada) and were of reagent grade.

3.2 Methods

3.2.1 Preparation of navy bean protein isolate

The navy bean seeds were milled by a Cyclone Sample Mill fitted with a 1 mm screen to obtain navy bean flour. To defat the flour before protein extraction, flour was added to hexane (1:3, w/v) and mixed for 40 min using a magnetic stir plate at 500 rpm. Then the defatted flour was filtered using a Whatman #1 filter paper suction. After that, flour was dried in a fume hood for 18 h and stored at 4°C.

3.2.2 Alkali extraction–isoelectric precipitation

The method described by Stone *et al*. (2015) was used to produce the alkali extraction–isoelectric precipitated (AE-IP) navy bean protein isolate. In brief, 200 g of defatted navy bean flour was dispersed in water (1:10, w/v), adjusted to an alkaline pH (pH 9.5) using 1.0 M NaOH, and was stirred for 1 h at 500 rpm at room temperature (21–23 °C). The mixture was then centrifuged at 4500 × g for 15 min at 4 °C, and the supernatant was collected and adjusted to the isoelectric pH using 1.0 N HCl. The precipitated protein was collected by centrifugation again at 4500 ×g for 15 min at 4 °C. The pellet was collected and stored at -30 °C and then freeze dried using a Labcono Free Zone 6 freeze drier.

3.2.3 Fermentation

(*a) Microorganism and culture conditions:* The fungus *A. oryzae* (NRRL 5590) was cultivated and maintained in a potato dextrose agar (PDA). The culture was incubated at 30°C for 7 d under aerobic condition. A spore suspension of the strain was prepared in the peptone water by aseptically transferring a colony from previous cultivated microorganism petri dish using a loop into 8 mL of peptone water. Then, the sample was vortexed for approximately 30 s to 1 min. A 100 µL aliquot of the resulting spore suspension was pipette onto the PDA plate, followed by spreading the spores over the agar by a spreader. During the 7-d incubation period the plates were kept at 4°C. The strain was sub-cultured every 2 months. The selected fungus in this study is as generally recognized as safe (GRAS) therefore it can be used for food applications (Jung *et al*., 2005).

(b) Spore suspension preparation: Spor*es* were first cultivated on PDA agar plates then the spores from a single agar plate were suspended in 10 mL of deionized water. A hemocytometer was used to directly determine the concentration of spores by microscopy counting. Accordingly, *A. oryzae* spores were standardized to a concentration of 10^7 spores per gram of substrate prior to be used in fermentation tests.

(c) Solid-state fermentation: PPI and NBPI were fermented with *A. oryzae* as described by Kumitch *et al.* (2020a). A 1 mL aliquot of the spore suspension for every 1 gram of substrate (10^7 spore/g substrate) was added to 50 g of each of the protein isolates. In order to determine required water to add in addition to the spores, the moisture content was calculated based on Eq.3.1, below:

$$M_n = \left(\frac{w_w - w_d}{w_w}\right) \times 100 \qquad \text{(Eq.3.1)}$$

where, M_n is the moisture content (%) of material of n, W_w is the weight of the wet sample, and W_d is the weight of the dry sample. The moisture of the substrate was adjusted to approximately 50%. A Kitchen Aid mixer was used to mix the sample at high speed after which the mixed 'dough' was spread evenly, at a thickness of approximately 2 cm, onto a container and incubated at 30°C in an Isotemp incubator for fermentation process. Fermentation was conducted 3 times and for each time a separate spore suspension was used to have triplicate batches. Degree of hydrolysis, functionality, proximate composition, protein quality, and flavor measurements of the samples were tested at times 0, 12, 24, 36, and 48 h for PPI and at 0, 12, 24, 36, 48, and 72 h for NBPI. Approximately 80 g of fermented PPI and NBPI samples were taken from each batch at defined intervals. The samples

then were deactivated at 80°C in a water bath for 10 min. Samples were then frozen at –20°C, followed by oven drying for 24 h at 50 °C and grinded to powder. The resultant dried powder stored at 4°C temperature in closed containers prior to other tests.

3.2.4 pH determination

The pH measurements were conducted according to Adinarayana *et al.* (2004). A 1 g of fermented PPI and NBPI samples were removed at each time point (0, 12, 24, 36, and 48 h for PPI and 0, 12, 24, 36, 48, and 72 h for NBPI) and mixed with 10 mL distilled water. The solutions were stirred for 20 min before pH was measured using an Orion 3-Star benchtop pH meter (Thermo Scientific, Waltham, MA, USA).

3.2.5 Determination of degree of hydrolysis

A 1% (w/w) protein solution of protein isolates individually (at each time point) was prepared in sodium phosphate buffer 10 mM with pH = 7.8. The protein solutions were transferred to a hot water bath (VWR Scientific Products, Radnor, PA, USA) at 80°C and maintaining for 10 min to inactivate all active enzymes and vegetative microorganism. After removing from water bath, the solutions were kept at 4°C and stirred over night to promote protein rehydration, and solubilization. The following day, the solutions were brought back to room temperature (20-24°C; ~1 h). The pH was adjusted to 7.8, once at room temperature. Further analyzes were conducted based on the method proposed by Adler-Nissen (1979). This method is based on the color change due to reaction between protein and picrylsulfonic acid to obtain N-trinitrophenyl-protein derivatives. Samples were removed to plastic cuvettes to determine the molecular absorption by a Genesys 10S UV-VIS spectrophometer (Thermo Scientific, Madison, WI, USA) at 340 nm. Data was presented as the mean ± one standard deviation from duplicate measurements, on the triplicate batches (n = 3). A standard curve was made using a 1.5 mM leucine solution.

To determine the total acid hydrolysis as part of the degree of hydrolysis calculation the method applied by Adler-Nissen (1979) and Jung *et al.* (2005) was used. About 24 mg of fermented protein isolates were transferred to a screw cap Pyrex tube containing 15 mL of 6.0 N HCl. The O_2-free nitrogen gas was then used to purge the tubes, followed by incubation at 110^{o}C for 20 h in a forced air oven. Then the pH of samples was adjusted to 7.8 with 2 M NaOH and filtered through

Whatman Grade 3 filter paper. Then, 250 µL aliquot of sample was added to 2.0 mL of 1% SDS solution in buffer followed by the addition of 250 µL to 2.0 mL of 5 mM buffer. The sample blank was prepared by adjusting a solution of 6.0 N NaOH or HCl to obtain a pH of 7.8. Measurements were conducted in triplicate and analyzed by the mentioned method. Total acid hydrolysis and degree of hydrolysis were calculated using Eqs.3.2 and 3.3 below:

$$h = (h_t - h_c) \times \mathrm{Df} \quad \text{(Eq.3.2)}$$

$$\%DH = \left(\frac{h}{h_t}\right) \times 100 \quad \text{(Eq.3.3)}$$

where h is the yield of hydrolysis equivalents of α-NH_2-glycine equivalents, h_t is the mM concentration of α-NH_2-glycine equivalents, h_c is the mM concentration of α-NH_2-glycine equivalent at time 0 of fermentation and DF is the dilution factor. Measurements were conducted in duplicate, on the triplicate batches (n =3), and were presented as the mean ± standard deviation.

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

SDS-PAGE was conducted following the method described by Laemmli (1970) in order to determine the intensity of protein hydrolysis and protein size change. The samples were loaded to the gel (12% separating gel at pH 8.6 and 4% stacking gel at pH 6.8). A suspension of 1% (w/w) of protein samples was stirred overnight. About 50 µL of solution was mixed with 50 µL of 2× SDS-PAGE sample buffer (20 mM Tris-HCl at pH 7.6, 10% SDS solution, 50% (v/v) glycerol, and 0.01% bromophenol blue, 2% β-mercaptoethanol) and was heated for 10 min at 85°C. After centrifugation at 7,500×g for 10 min, the gels were stained with 0.25% Coomassie blue stain for 1 h, and then de-stained with de-ionized water overnight. The protein bands were digitally-imaged to determine the molecular weight using ImageJ (National Institutes of Health Bethesda, MD, USA). The sum of pixel intensity in each section represented the volume for each protein band.

3.2.7 Physicochemical properties

(a) Proximate composition: Standard methods of Association of Official Analytical Chemists (AOAC) methods 923.03 (crude ash), 920.87 (crude lipid), 984.13A (crude protein; %N ×6.25), and 925.10 (moisture) were employed for determining the proximate composition of fermented PPI and NBPI (AOAC, 2005). Crude protein, ash, lipid, and moisture were expressed

on a dry weight basis (d.b.). Data was presented as the mean ± standard deviation from duplicate measurements on the triplicate batches (n = 3).

(b) Surface charge: Fermented PPI and NBPI were mixed with deionized water to obtain concentration of 0.05% (w/w) and pH was adjusted to 7.0 using 0.1 M HCl or NaOH. The suspension was stirred for 1 h via a mechanical stirrer. To determine the surface charge (or zeta potential) the method proposed by Can Karaca *et al.* (2011) was applied using a Zetasizer Nano (Malvern Instruments, Westborough, MA, USA) to measure the electrophoretic mobility. Henry's equation (Eq.4) was used to determine the zeta-potential (ζ; units: mV) from the electrophoretic mobility (U_E) using. The Henry's equation is as below:

$$U_E = 2\varepsilon.\zeta.f(\kappa\alpha)/3\eta \qquad \text{(Eq.3.4)}$$

where ε is the permittivity, $f(\kappa\alpha)$ is a function related to the Debye length (κ) and the ratio of particle radius (α), and η is the dispersion viscosity. Data was presented as the mean ± one standard deviation from duplicate measurements on each of the triplicate batches (n = 3).

c. Surface hydrophobicity. To determine the surface hydrophobicity, the modified method proposed by Kato & Nakai (1980) was used applying fluorescent probe, 8-anilino-1-naphthalenesulfonic acid (ANS). Fermented PPI and NBPI were mixed with deionized water to obtain concentration of 0.05% (w/w) and pH was adjusted to 7.0 using 0.1 M HCl or NaOH. The suspension was stirred for 1 h via a mechanical stirrer. The stock samples were then diluted to final protein concentration of 0.005, 0.010, 0.015, and 0.020% (w/w). Then, 1.6 mL of each protein isolate was mixed with 20 μL of an 8 mM ANS solution (in deionized water at pH value of 7.0) and vortexed for 10s. After vortexing, the mixture was kept in the dark for 5 min, and then the fluorescence intensity was determined using a FluoroMax-4 spectrophotometer (Horiba Jobin Yvon Inc., Edison, NJ, USA). A slit width of 1 nm was used, and the emission and excitation wavelengths were 470 and 390 nm, respectively. For sample blanks, 20 μL of deionized water with adjusted pH to 7.0 was used instead of the ANS probe. The initial slope of a plot of the fluorescence intensity (differences between protein solution with probe and same protein solution without the probe) versus protein isolate was used as an index of surface hydrophobicity. Analyzes were conducted using linear regression and all intensity data were arbitrarily divided by 10,000 before

statistical analysis and graphing. Data was presented as the mean ± one standard deviation from duplicate measurements on the triplicate batches (n = 3).

3.2.8 Functionality

(a) Solubility: Solubility measurements were conducted based on a method used by Stone *et al*. (2015) with some modifications. Fermented PPI and NBPI samples were dispersed in distilled water to achieve 1% w/w solution and pH was adjusted with 1 M NaOH using a pH meter to achieve pH 7.0. The solution was then mixed for 1 h at room temperature (21°C-23°C) and then was centrifuged for 4180 × g for 10 min using a centrifuge (Eppendorf, Model 5804R, Mississauga, ON, Canada).

A 5.0 g of supernatant was transferred into a digestion flask and used to determine the amount of protein (N × 6.25) using micro-Kjeldahl digestion and distillation units (Labconco Corp., Kansas City, MO, USA). Nitrogen solubility was determined based on Eq.3.5, below:

(Eq.3.5)

$$\text{Nitrogen solubility (\%)} = \frac{\text{Nitrogen in the suspension}}{\text{Original amount of nitrogen in used fermented samples}} \times 100$$

Measurements were conducted in duplicate, for each of the triplicate batches (n=3) and were reported as the mean ± standard deviation.

(b) Water Hydration capacity (WHC) and oil holding capacity (OHC): Water hydration capacity was determined using the method described by Kumitch *et al.* (2020b). In brief, 0.5 g of fermented protein isolates weighed into a pre-weighed 10-mL graduated centrifuged tube and 5 mL of distilled water was added to that. The mixture was mixed for 10 s every 5 min for 30 min and centrifuged (Eppendorf, Model 5810R, Mississauga, ON, Canada) at 1000 x *g* for 15 min. The supernatant was then decanted, and the tubes were re-weighted. OHC was measured with a similar method by replacing the deionized water with canola oil. The WHC and OHC were expressed as difference between the weight of the wet protein isolate and dry protein isolate relative to the dry protein isolate weight (0.5 g). The measurements were conducted in duplicate on each of the triplicate batches (n = 3) and reported as the mean ± standard deviation.

(c) Emulsion stability: The emulsion stability (ES) of fermented PPI and NBPI were determined according to (Kaur & Singh, 2005). For ES, 1% w/w protein solution in deionised water

was prepared. The pH was adjusted 7.0 using 0.1 M HCl or NaOH and then the suspension was mixed using a mechanical stirrer for 1 h at room temperature (21°C-23°C). After that, 5 g of solution and then 5 g of canola oil were weighted into a 50 mL plastic centrifuge tube. Then, samples were homogenized for 3 min in a homogenizer (Omni International, Marietta, GA, USA) at speed 5 at the oil-water interface. Immediately following homogenization, the emulsion was transferred into a 10 mL graduated cylinder. The emulsion was left to separate for 30 min and the volume of the aqueous layer at bottom of graduated cylinder was recorded. % ES was calculated by using Eq.3.6 below:

Emulsifying stability (%) $(V_B-V_A)/V_B \times 100$ (Eq.3.6)

V_B is the volume of aqueous layer before emulsification (5 mL) and V_A is the volume of aqueous layer after 30 min of drainage. The measurements were conducted in duplicate on each of the triplicate batches (n = 3) and reported as the mean ± standard deviation.

(d) Foaming capacity (FC) and stability (FS): FC and FS were determined according to method proposed by Liu *et al.* (2010). The PPI and NBPI samples individually were dispersed in the de-ionized water to achieve 1% (w/w) solution. The pH of the solution was adjusted to pH 7.0 using 0.1 M HCl or NaOH and stirring at approximately 500 rpm for 1 h at room temperature (21°C-23°C). Then, a 15 mL aliquot was transferred to a 400 mL beaker and homogenized by Omni Macro Homogenizer (Omni International, Marietta, GA, USA) equipped with a 20-mm saw tooth probe at 7,200 rpm for 5 min. The samples were then transferred to a 100-mL graduated cylinder. The foam volume was measured at 0 min and 30 min. FC and FS were calculated using the Eqs.3.7 and 3.8 below:

$$FC = \frac{V_{F0}}{V_{sample}} \times 100 \quad \text{(Eq.3.7)}$$

$$FS = \frac{V_{F30}}{V_{F0}} \times 100 \quad \text{(Eq.3.8)}$$

where V_{F0} is the foam volume at the time 0 min, V_{sample} is the initial volume of sample used (15 mL), and V_{F30} is the volume of the foam after passing 30 min. The measurements were done in duplicate on each of the triplicate batches (n = 3) and reported as the mean± standard deviation.

3.2.9 Protein quality

(a) Amino acid profile: Amino acid composition in fermented PPI and NBPI were determined by Pico-Tag MT amino acid analysis system (Waters Corporation, Milford, MA, USA) and high-performance liquid chromatography (HPLC) for 15 amino acids as described by Bidlingmeyer *et al.* (1987), done at the University of Manitoba (Winnipeg, MB). About 20 mg of each sample was added to 20×150 mm screw cap Pyrex tubes. Then, 15 mL of 6 N HCl was added to tubes and then flushed with N_2 and incubated at 110^{o}C for 20 h to complete the acid hydrolysis. After acid hydrolysis, each amino acid contain was determined by HPLC using the Pico-Tag amino acid analysis system (Waters Corporation, Milford, MA). All the amino acids were determined based on AOAC Official Method 982.30, excepting tryptophan, methionine, and cysteine. The AOAC method 988.15 (1995) was used with slight modification to determine tryptophan. The samples were hydrolyzed in 10 M NaOH at 110^{o}C for 16 h (Hugli & Moore, 1972). After hydrolysis, determination of tryptophan was achieved using HPLC by reverse phase liquid chromatography with UV detection. The AOAC method 985.28 (1995) and ion-exchange chromatography with slight modification was applied to determine the amount of sulfur-containing amino acids (i.e., cysteine and methionine). Oxidation of cysteine and methionine was conducted by cold performic acid and incubated overnight at 4^{o}C for the duration of the reaction. The oxidization of the sulphur-containing amino acids was conducted by performic acid and the hydrolysis was conducted by 6 M HCl at 110^{o}C for 18 h. The reference protein standard recommended by FAO was applied in order to determine the amino acid score as the ratio of the individual amino acids in 1 g of fermented pulse samples (amino acid, mg/g protein): histidine, 19; isoleucine, 28; leucine, 66; lysine, 58; methionine + cysteine, 25; phenylalanine + tyrosine, 63; threonine, 34; tryptophan, 11; valine, 35 (FAO, 1991). The limiting amino acid was determined by the lowest ratio.

(b) In vitro *protein digestibility (IVPD):* The method used by Kumitch *et al.* (2020a) was used to determine *in vitro* protein digestibility (IVPD). A multi-enzyme system consisting of 31 mg of chymotrypsin (bovine pancreas ≥40 units/mg protein), 16 mg of trypsin (porcine pancreas 13,000- 20,000 BAEE units/mg protein) and 13 mg of protease (*Streptomyces griseus* ≥15 units/mg solid) mixed with 10 mL of deionized water was selected to simulate the digestive environment. The pH of multi-enzyme solution was adjusted to 8.0 using 0.1 M NaOH and HCl and maintained

in 37°C. Approximately 62.5 ± 0.5 mg of protein was mixed with deionized water then was stirred for 1 h at 37°C. The pH of the protein suspension was adjusted to pH 8.0 using 0.1 M NaOH and HCl. After that a 1 mL aliquot of the multi-enzyme solution be added with stirring to the protein suspension. The pH drop was monitored and recorded per 1 min over a 10 min period using a pH meter. *In vitro* protein digestibility was calculated from the Eq.3.9 below:

$$IVPD = 65.66 + 18.10 * \Delta pH_{10min} \quad \text{(Eq.3.9)}$$

ΔpH_{10min} is the pH drop from initial pH (8.0) to the final pH after 10 min.

3.2.10 Headspace solid phase microextraction

A headspace solid phase microextraction (HS-SPME) method was used for extracting the volatile compounds resulting from PPI and NBPI fermentation. A 2cm 50/30 divinylbenzene/carbo divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) Stable Flex SPME fiber (Sulpelco, Bellefonte, PA, USA) was used in order to adsorb a wide range of polar and non-polar compounds. The pulses protein isolate solutions (10%, w/w) were prepared under stirring at 500 rpm for 30 min at room temperature. About 30 g of the solution was transferred to an amber glass bottle (120 mL) that was sealed with a PTEF-faced silicone septum. Then, in order to release volatile compounds the protein solution was incubated in a water bath at 50°C for 30 min. After that, the SPME device was manually inserted into the headspace of the sample vial to adsorb volatile compounds by exposing 1 cm of the SPME fiber for 30 min at 50°C. Before each extraction, the SPME fiber was conditioned in the injection port of a gas chromatography at 250°C for 10 min.

3.2.11 Gas chromatography – mass spectrometer analysis

Gas chromatography–mass spectrometry (GC–MS) was used for analyzing the compounds of PPI and NBPI at different times of fermentation. An Agilent DB-5MS column (30 m × 0.25 mm, 0.25 μm film thickness was used according to the method proposed by Azarnia *et al*. (2011). A splitless GC injector equipped with a SPME taper (0.75 mm, 5190–4048 Ultra Inert Liner) was applied for desorption of the volatile compounds. The SPME fiber was immediately inserted into the splitless GC injector, and the compounds were thermally desorbed at 250°C for 2 min. The

initial flow rate and oven temperature were 1 mL/min of ultra-high purity helium and 40°C. After 5 min the temperature was increased at 5°C min/1 to 70°C, then 10°C min/1 increase rate up to 200°C, followed by 50°C min/1 increase rate to 250°C and was kept at that temperature for 15 min for the remainder of the analysis. Eventually, compounds were identified using the National Institute of Standards and Technology (NIST) and mass spectra Wiley libraries. To remove the distractive factors, the same condition was used for the SPME fiber blank. The relative peak area of the abundance (total area counts) represented the concentrations of volatile compounds.

3.2.12 Statistics

All measurements were conducted in duplicate, for each of the triplicate batches (n =3) and were reported as the mean ± standard deviation. IBM SPSS 21.0 statistics software was used to conduct the statistical tests. A one-way analysis of variance (ANOVA) and a Tukey's test was used to detect statistical differences in response to fermentation time within different measured variables.

4 RESULTS AND DISCUSSION

4.1 Study 1: Effect of fermentation time on the physicochemical, functional properties, and protein quality of pea protein isolate and navy bean protein isolate fermented by *Aspergillus oryzae.*

4.1.1 Determination of the pH and degree of hydrolysis during fermentation

The effect of fermentation time on the degree of hydrolysis (DH) and changes to pH were evaluated over 48 h for PPI and 72 h for NBPI. Fungal growth on PPI acted to increase the pH from 7.1 to 8.1 during 48-h, whereas for NBPI, pH decreased over the 72-h fermentation from 6.9 to 6.1 (Figure 4.1.1). In addition, DH increased for both protein isolates in the range of 0.0% to 9.3% and 0.0% to 4.4% for PPI and NBPI, respectively (Fig. 4.1.2).

The slight alkalization of the PPI through fermentation observed here has previously been reported in fermentation studies concerning different legumes with a high protein content and the absence of carbohydrates. It is hypothesized to be due to the release of basic peptides or ammonia. Ben-Harb *et al*. (2019) reported that the absence of carbohydrates in a pea protein enriched emulsion fermented by different microbial species contributed to alkaline or neutral fermentation.

Limón *et al*. (2015) reported during SSF of kidney bean by *B. subtilis*, pH significantly increased ($p \leq 0.05$) from 6.3 to 7.0, which could be attributed to the production of ammonia during the fermentation. Regarding the NBPI results, the decrease in pH could be due to fungi releasing possibly lactic acid and acetic acid leading to a low measured pH or the release of acidic peptides. Kumitch *et al*. (2020b) reported a similar gradual decrease in pH of the substrate over time for pea protein enriched flour fermented by *A. oryzae* and *A. niger* (SSF).

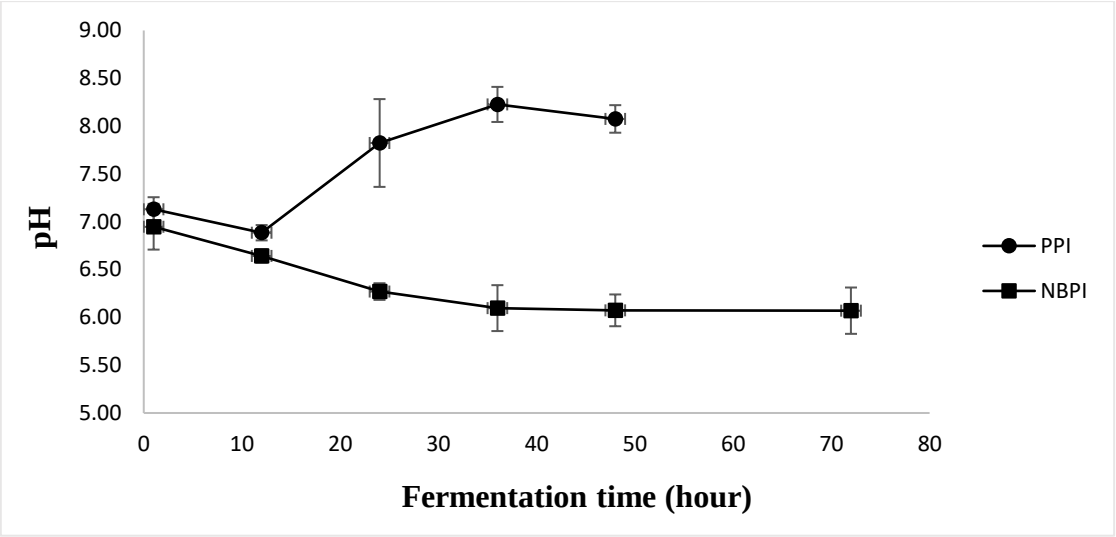

Figure 4.1.1 The effect of fermentation on pea protein isolate (PPI) and navy bean protein isolate (NBPI) inoculated with *A. oryzae* on pH over fermentation time. Data represent the mean values from duplicate samples from triplicate batches of PPI and NBPI samples ± one standard deviation (*n*=3).

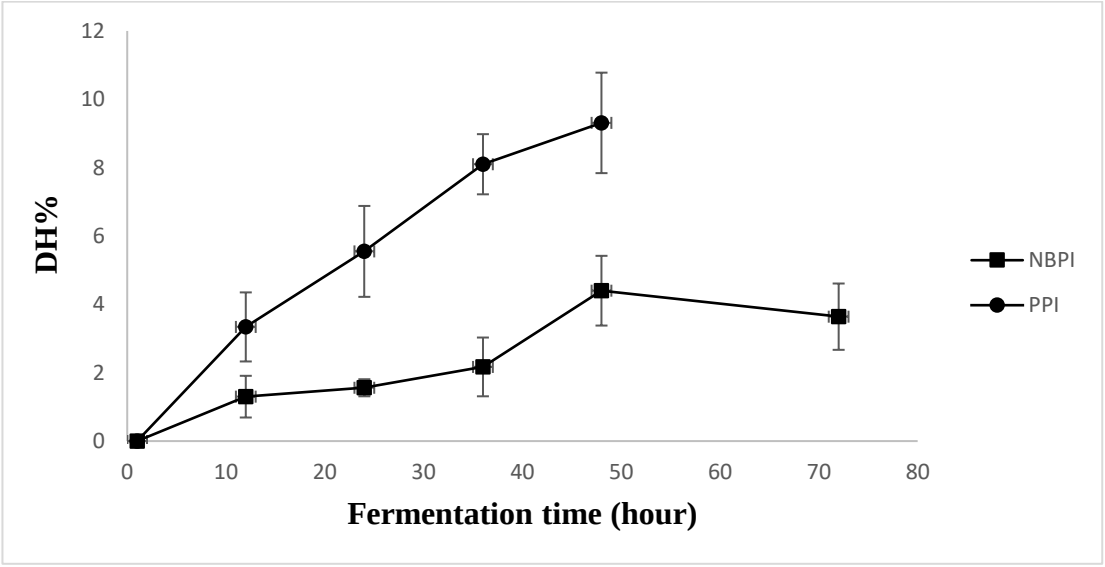

Figure 4.1.2 The effect of fermentation on pea protein isolate (PPI) and navy bean protein isolate (NBPI) inoculated with *A. oryzae* on degree of hydrolysis (%) over fermentation time. Data represent the mean values from duplicate samples from triplicate batches of PPI and NBPI samples ± one standard deviation (*n*=3).

Based on the DH results over the 48-h and 72-h period for PPI and NBPI, respectively, the hydrolysis of PPI was more extended compared to NBPI. This difference could be related to the fungus' action is dependent on the substrate; the fungus adjusts its enzymatic systems (hydrolase enzymes, oxide reductases, etc.) in response to the substrate's conditions, primarily the presence of carbon and nitrogen and the substrate's threshold availability for the fungus' growth. The substrate, hardness, and permeability all influence how fungal enzymes access the nutrients they require for growth (Espinosa-Páez *et al*., 2017). In this study, the texture, morphology, and composition of the PPI and NBPI substrates were different. The morphology of the NBPI substrate was compacted, and particles were closely packed after mixing with 50% moisture which could lead to reduced voidage or porosity of the substrate, causing particles to stick together and adversely impacting oxygen transfer to the fungi. Moreover, hyphae could have trouble penetrating through the compacted substrate. In contrast, the texture of the PPI substrate after mixing with 50% moisture was less compacted and had a more open structure. Consequently, the fungi growth was higher on PPI compared to NBPI. Chutmanop *et al*., (2008) used *A. oryzae* for SSF of rice bran; however, they indicated that rice bran used alone proved to have poor morphological characteristics for use as a substrate in large scale SSF. This was due to the small flat particles of rice bran which tended to pack together tightly to form a bed of low porosity that did not allow a good circulation of air. Additionally, navy bean consists of 54–79% of globulins composed of a major fraction of phaseolin (7S). Because phaseolin is known to be is resistant to enzymatic hydrolysis (Rui *et al*., 2015), it could be one of the main reasons of the low hydrolysis of NBPI. These results are in accordance with pH drop through fermentation of NBPI. Since phaseoline is resistant to hydrolysis, fungi could prefer carbohydrates as a source of energy which could contribute to acids production and pH decline.

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

The proteins from fermented PPI and NBPI were analyzed using SDS-PAGE and ImageJ software. The protein profile changes during fermentation using *A. oryzae* are shown in Fig. 4.1.3 and 4.1.4, and the analyzed percentages of major protein bands are presented in Table 4.1.1. The SDS-PAGE gel in Fig. 4.1.3a shows PPI to have a heterogeneous nature consisting of multiple major and minor bands representing a balance between the concentration of proteins with large molecular weight (>60 kDa) and proteins with small molecular weight (<30 kDa). As the

fermentation time increased the concentration of larger molecular weight peptides decreased. During fermentation, microorganisms produce enzymes that cleave the proteins and release smaller molecular peptides to give smaller-sized proteins. As a result, the content of mid to large-sized protein decreases. These changes to the primary structure can be observed and quantified in the chromatogram (Kumitch *et al*., 2020b).

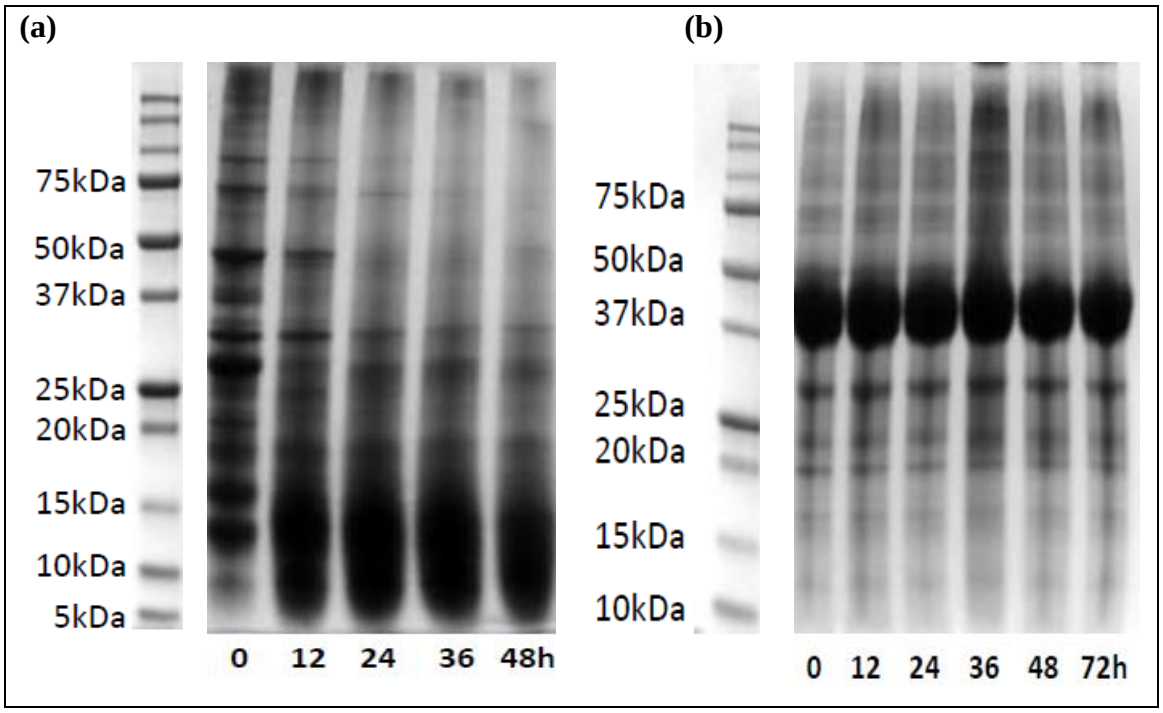

Figure 4.1.3 SDS-PAGE of solid-state fermented PPI (a) and NBPI (b) inoculated with *A. oryzae* during fermentation time.

Lipoxygenase (92.7 kDa), convicillin (72.4 -77.9 kDa), vicilin (44–50 kDa), and α-legume (34–39 kDa) usually can be found in PPI (Shi *et al*., 2021), and their band sizes are consistent with the bands indicated in non-fermented PPI sample in this research. Therefore, the protein degraded during the fermentation could be lipoxygenase, convicillin, vicilin and/or α-legume. The smaller protein fragments in the non-fermented PPI could be albumin (24–29 kDa), β-legumin (20–24 kDa), and other low molecular peptides (Reinkensmeier *et al.*, 2015 ; Barac *et al*., 2010).

The molecular mass of vicilin is approximately 150-170 kDa, with each monomer being ~44 50 kDa (Shi *et al*., 2021). It has been reported in previous studies that the product of the breakdown of vicilin could be the fraction with a molecular weight of 12-36 kDa (Kumitch *et al*., 2020b).

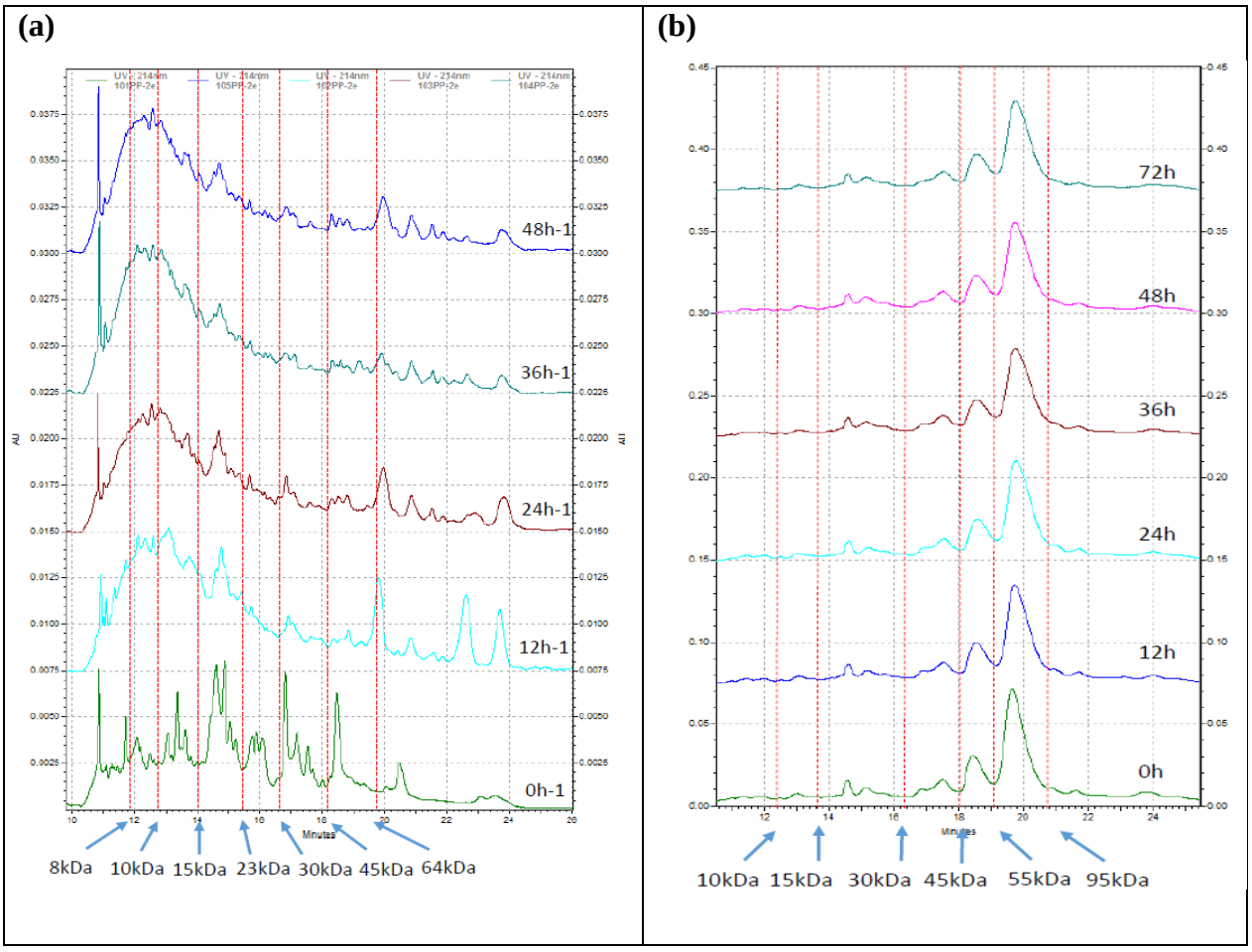

Figure 4.1.4 SDS-PAGE chromatogram of solid-state fermented PPI (a) and NBPI (b) inoculated with *A. oryzae* during fermentation time.

Table 4.1.1 SDS-PAGE ImageJ quantification of solid-state fermented PPI and NBPI inoculated with *A. oryzae* during fermentation time.

	Molecular weight (kDa) concentration (%)							
Protein source / Time (h)	**<8 k**	**8-10 k**	**10-15 k**	**15-23 k**	**23-30 k**	**30-45 k**	**45-64 k**	**>64 k**
a) Pea protein isolate								
0	9.3±1.1^{a}	8.5±0.9^{a}	14.6±0.3^{a}	22.8±0.3^{a}	9.4±0.6^{a}	15.7±2.1^{a}	10.5±0.8a	9.1±1.2^{a}
12	14.6±1.3^{b}	12.7±2.3^{b}	23.8±2.6^{bc}	18.4±1.8^{b}	6.3±1.4^{b}	7.7±2.0^{b}	5.2±1.1^{b}	11.3±4.1^{a}
24	14.0±2.9^{b}	15.7±2.9^{b}	25.7±2.4^{bc}	15.8±1.1^{c}	5.5±1.5^{b}	6.3±1.7^{b}	4.8±1.2b	12.0±3.1^{a}
36	14.00±1.9^{b}	18.2±3.1^{b}	26.2±0.4^{c}	16.0±1.6^{c}	5.7±0.9^{b}	6.7±0.8^{b}	4.6±0.8^{b}	8.8±2.3^{a}
48	13.4±1.2^{b}	17.7±3.1^{b}	22.7±2.6^{b}	16.4±2.2^{bc}	6.6±0.5^{b}	7.3±1.2^{b}	5.3±0.4^{b}	10.5±2.6^{a}
b) Navy bean protein isolate								
0	3.0±0.1^{a}	2.6±0.1^{a}	10.8±0.6^{a}	9.8±0.7 ^{a}	15.1±0.3^{a}	46.8±1.9^{a}	12.0±1.2^{a}	-
12	3.2±0.2^{a}	2.9±0.4^{a}	11.1±0.2 ^{a}	10.4±0.2 ^{a}	14.9±0.2 ^{a}	45.7±1.3^{ab}	11.8±0.7^{a}	-
24	3.2±0.1^{a}	2.7±0.1 ^{a}	11.0±0.9 ^{a}	10.4±0.5 ^{a}	15.0±0.2 ^{a}	44.3±3.6^{ab}	13.3±3.5^{a}	-
36	3.3±0.1^{a}	3.2±0.3 ^{a}	12.4±0.1 ^{a}	10.8±0.6 ^{a}	14.6±0.2 ^{a}	44.4±1.0^{ab}	11.2±0.2^{a}	-
48	3.8±0.6^{a}	3.3±0.7 ^{a}	13.4±1.4 ^{ab}	11.4±0.8 ^{a}	14.4±0.8 ^{a}	40.8±3.1^{b}	12.8±4.4^{a}	-
72	4.3±1.8^{a}	3.8±1.3 ^{a}	14.3±1.2^{b}	11.4±1.2 ^{a}	14.1±1.2 ^{a}	43.1±2.6^{ab}	8.8±0.8^{b}	-

Data represent the mean values from duplicate samples from triplicate batches of PPI and NBPI samples ± one standard deviation (n=3). Means followed with different letters within each column are significantly different ($p \leq 0.05$).

Another protein in unfermented PPI is convicilin, which declined with increasing fermentation time. Convicilin is different from vicilin in sulfur amino acid residues and contains no covalent bonds (Sikorski, 2000). The molecular mass of the hexameric legumin protein is approximately 300 to 400 kDa. Each subunit of legumin is 60 kDa, and it contains one acidic chain and one basic chain (Liang & Tang, 2013).

In some works of literature, it has been reported that endo-proteases prefer hydrolyzation of vicilin (44–50 kDa) over legumin (34–39 kDa) due to the compact structure of legumin. Indeed, the compact structure of legumin leads to difficulty for proteases activity (Gabriel *et al*., 2008; Barac *et al*., 2015). In this study, there was a significant decrease for protein bands related to both vicilin (44–50 kDa) and legumin (34–39 kDa) from 15.7 to 7.3 and from 10.5 to 5.3, respectively.

Regarding Fig 4.1.4a, the fermented PPI showed a decrease of larger molecular weight peptides with a concurrent increase in the concentration of lower molecular weight peptides compared to unfermented PPI. Specifically, there were reductions in the densities of the bands higher than ~15 kDa compared with earlier fermentation times (Fig 4.1.4a and Table 4.1.1). The SDS-PAGE results indicate that there was an alternation in protein distribution of the PPI sample, from bands of large-sized proteins (>60 kDa) to small sized or peptides (<30 kDa), which can be due to proteolytic activity. Microorganisms have a well-developed peptidase system that is able to hydrolyze larger proteins into smaller peptides and define the degree and specificity of hydrolysis of proteins into smaller peptides (Xiao *et al*., 2018). The modification of soybean, red bean, chickpea, and pea proteins during fermentation into smaller peptides, demonstrated using SDS-PAGE, has been reported in previous studies (Lim *et al.*, 2010; Xiao *et al*., 2015; Kumitch *et al*., 2020b). Specifically, Kumitch *et al*. (2020b) reported a similar trend of large to medium-sized proteins becoming reduced to smaller molecular weight via the proteolytic activity by *A. oryzae* and *A. niger* during SSF of pea protein-enriched flour. In addition, in Fig 4.1.4a, two major protein peaks appeared in the 12 h fermented sample, seen between 22 to 24 min. This could be related to secreted extracellular proteins or enzymes from *A. oryzae.* These bands are hydrolyzed as the fermentation time proceeds. This result can explain the increase in protein content of PPI during SSF.

The protein profile of NBPI did not indicate significant changes during fermentation except some minor but significant changes in concentration of polypeptides with molecular weight of 30-45, 10-15, and <8k. Fig. 3 b and Fig. 4.1.4b, concurrent the analyzed percentages of major protein

bands of NBPI did not show significant changes (Table 4.1.1). In general, there were no considerable alterations of protein profiles during the fermentation of NBPI by *A. oryzae*, which indicate low proteolytic activities. This was in accordance with our previous results of low DH% of NBPI. Navy beans consist of 54–79% of globulins, including a major fraction of phaseolin (7*S*) and a minor fraction of legumin (11*S*). The phaseolin of navy beans has three subunits, namely α, β, and γ type corresponding to the molecular mass of 45.75, 42.80, and 40.74 kDa, respectively. The proteolytic systems of *A. oryzae* did not cause potent degradation of phaseolins. The phaseolin is known to be resistant to enzymatic hydrolysis and thus is considered as one of the major causes of the low digestibility of bean proteins (Rui *et al*., 2015). In addition, proteins with lower molecular mass (~29 kDa) were also observed in NBPI, which could correspond to one of the major antinutritional proteins, phytohemagglutinin, according to previous mass spectrum results of navy bean proteins (Rui *et al*., 2015).

4.1.3 Physicochemical properties

(a) Proximate composition

The effect of fermentation on the proximate composition of PPI and NBPI is presented in Table 4.1.2. as a function of the fermentation time. The samples were made from fermenting triplicate batches at various times (0, 12, 24, 36, 48, and 72h), which were then oven-dried and ground into powder for proximate analysis. Overall, there were significant differences ($p \leq 0.05$) in protein and lipid contents among the protein isolate samples. For the PPI samples, the protein level increased slightly from 82.0% (0 h) to 83.8% (48 h), lipid content increased from 0.1 % (0 h) to 0.4% (48 h), and ash content did not change significantly ($p > 0.05$) over the fermentation duration by *A. oryzae*. Regarding NBPI, protein and ash levels did not have significant changes ($p > 0.05$); however, lipid amounts increased from 0.2 (0 h) to 1.8% (72 h). It could be related to fungal enzymes, that cleaved protein-lipid interactions, freeing up more lipid.

Changes in protein content can be due to fungi colonization and hyphae formation on the substrate surface during SSF. The higher amounts of protein can be related to the enzyme secretion from the fungi used in the fermentation or production of the cell mass of fungi and consequently the production of protein within the fungi population. The fungi utilize nutrients from the substrate

to grow and secrete enzymes. It is hypothesized that the increase in protein content is related to the fungi utilizing crude fiber content, as previously documented by Belewu *et al*. (2011) .

Table 4.1.2 Changes in the composition of pea protein isolate and navy bean protein isolate fermented with *A. oryzae* over fermentation time.

Fermentation time (h)	Protein content (%)		Lipid content (%)		Ash (%)	
	PPI	NBPI	PPI	NBPI	PPI	NBPI
0	82.0±1.0^{a}	81.5±0.4^{a}	0.1±0.0^{a}	0.2±0.1^{a}	3.8±0.2^{a}	6.4±0.0^{a}
12	83.3±0.9^{ab}	82.4±1.7^{a}	0.2±0.0^{ab}	0.2±0.0^{a}	3.8±0.0^{a}	6.5±0.4^{a}
24	82.7±1.3^{ab}	82.6±1.0^{a}	0.2±0.1^{ab}	0.2±0.0^{a}	3.9±0.6^{a}	6.5±0.4^{a}
36	83.9±1.2^{b}	82.8±1.0^{a}	0.3±0.0^{ab}	1.4±0.1^{b}	4.1±0.2^{a}	6.4±0.6^{a}
48	84.8±0.6^{c}	82.9±1.6^{a}	0.4±0.1^{b}	1.3±0.0^{b}	3.9±0.5^{a}	6.5±0.2^{a}
72	-	83.0±1.6^{a}	-	1.8±0.4^{b}	-	6.9±0.5^{a}

Data represent the mean values from duplicate samples from triplicate batches of PPI and NBPI samples ± one standard deviation (n=3). Means followed with different letters within each column are significantly different ($p \leq 0.05$).

(b) Surface charge (zeta potential)

The surface charge (zeta potential, ZP) of fermented PPI and NBPI are given in Table 4.1.3. The surface charge represents the repulsion or attraction between particles, leading to the stability of the fermented protein isolates in solution. When ZP values range between -30 and +30, the stability of the solution is low; however, at ZP lower than -30 and higher than +30, the stability of the solution is higher (Wu *et al*., 2015). In this study, the ZP ranged from -34.10 mV in the unfermented PPI to -38.77 mV in 48-h fermented PPI and -34.43 mV in the unfermented NBPI to -36.49 mV in 72-h fermented NBPI (Table 4.1.3). Overall, the ZP for both PPI and NBPI were negative due to pH of the solvents being adjusted to pH 7.0 as the proteins would be above the isoelectric point of pea and navy bean protein (pI~4.6). At pH values away from the pI, protein carries a high charge, and interactions between protein and water are more favorable which leads to improved protein solubility (Can Karaca *et al*., 2011). In addition, the high negative surface charge (mV) for PPI samples increased with fermentation time. However, there were no significant

changes to ZP of NBPI. Increase in surface charge leads to stronger repulsive forces between proteins. Therefore, strong repulsive forces, in the fermented PPI samples, between the proteins resulted in protein-water interactions to preferentially occur compared with protein-protein aggregation. As a result, solubility increased over fermentation time for PPI in this study as shown in table 4.1.3.

Table 4.1.3 Physicochemical properties of fermented pea protein isolate and navy bean protein isolate inoculated with *A. oryzae* over fermentation time.

Protein source / Time (h)	Zeta potential (mV)	Surface hydrophobicity (au)
a) Pea protein isolate		
0	-34.1 ± 2.2^{a}	19.3 ± 2.0^{a}
12	-36.0 ± 2.8^{ab}	25.0 ± 1.2^{b}
24	-36.3 ± 1.9^{ab}	29.3 ± 0.8^{c}
36	-39.0 ± 2.6^{b}	29.4 ± 2.1^{c}
48	-38.8 ± 1.6^{b}	33.4 ± 3.7^{c}
b) Navy bean protein isolate		
0	-34.4± 1.6^{a}	11.6 ± 1.9^{a}
12	-34.4± 1.4^{a}	13.3± 1.6^{a}
24	-35.0± 1.1^{a}	11.3 ± 1.5^{a}
36	-33.9± 1.1^{a}	11.7± 2.2^{a}
48	-35.7± 2.3^{a}	12.6± 3.5^{ab}
72	-36.5± 2.1^{a}	17.7± 2.6^{b}

Data represent the mean values from duplicate samples from triplicate batches of PPI and NBPI samples ± one standard (n=3). Abbreviations: PPI (pea protein isolate); NBPI (navy bean protein isolate); mV (millivolts), and a.u. (arbitrary units). Means followed with different letters within each column are significantly different ($p \leq 0.05$).

Increase in surface charge can be due to the fungi releasing protease enzymes that hydrolyzed the protein, exposing a higher proportion of negatively charged groups at the PPI and

NBPI surfaces. In addition, a high negative ZP could be due to a low ratio of basic to acidic amino acid clusters (Tirgar *et al*., 2017). An increase in ZP at pH 7.0 with increasing fermentation time was reportd by Kumitch *et al*. (2020b), where pea protein-enriched flour was fermented via *A. niger* and *A. oryzae* (Kumitch *et al*., 2020b).

(c) Surface hydrophobicity

Hydrophobic interactions play an important role in stabilizing intra- and intermolecular interactions involving proteins and aggregates. Hydrophobicity is the affinity of non-polar solutes to adhere to one another in aqueous environments. Therefore, hydrophobic sites on the protein can determine the conformation and number of protein-protein interactions that occur. Protein functionality such as solubility, oil holding, emulsification, and foaming can be influenced by the protein's surface hydrophobicity (Cardamone & Puri, 1992). The surface hydrophobicity depends on the degree of the unfolding of protein in solution, the conformation of surface hydrophobic "patches" of the protein, and the presence of aromatic amino acids, including tryptophan, tyrosine, and phenylalanine (Wang *et al*., 2014). Surface hydrophobicity of fermented PPI and NBPI samples at pH 7.0 are presented in Table 4.1.3. The surface hydrophobicity increased from 19.30 to 33.24 a.u. (arbitrary unit) and from 11.62 to 17.70 a.u., for PPI and NBPI, respectively (Table 4.1.3). Overall, the fermented PPI and NBPI surface is more hydrophobic than the controls due to the proteolytic activity of the fungi. Specifically, fungal enzymes produced during SSF hydrolyzed the PPI and NBPI proteins, leading to a partial unraveling of the protein and release of peptides which exposed buried reactive charged and hydrophobic sites.

Besides, different factors related to protein structure, environmental conditions (temperature, pH, ionic strength), reactions with other components (different proteins, saccharides, lipids), fungal species, as well as the age of fungi could affect the surface hydrophobicity (Konieczny & Uchman, 2002). For instance, hyphae, which are produced by fungi during fermentation, could influence hydrophobicity. During fungal adaptation and attachment, the hyphae hydrophobicity can change from hydrophilic to hydrophobic(Vujanovic & Kim, 2018). Another factor that could affect hydrophobicity is the ratio of the legumin to vicilin. The legumin is more hydrophobic because it contains a greater amount of tryptophan than vicilin. In contrast,

vicilin is more hydrophilic due to its N-terminal extension made up of polar amino acid residues (serine, glutamic acid, and aspartic acid) (Liang & Tang, 2013).

There are limited pieces of literature available on the surface hydrophobicity of fermented navy bean isolate. Guldiken *et al*. (2021) reported the hydrophobicity of unfermented flours from six navy bean varieties cultivated in two different locations ranged from 6.0 a.u. to 19.5 a.u. Zhang & Romero, (2020) reported a surface hydrophobicity value of 73.0 a.u. for an unfermented navy bean protein concentrate. Regarding PPI, Can Karaca *et al*. (2011) reported pea protein isolate surface hydrophobicity of 84.8 a.u. and 77.8 a.u., prepared by isoelectric precipitation and salt extraction, respectively (unfermented). Tirgar *et al*. (2017) found the surface hydrophobicity of pea protein concentrates to be 68.5 a.u., which was higher than determined values for fermented PPI samples at all fermentation times in this study. Çabuk *et al*. (2018) reported that surface hydrophobicity significantly increased from approx. 9 a.u. to ~21 a.u. at pH 4.0 as a function of fermentation time (12 h) for pea protein-enriched flour (PPEF) fermented by *Lactobacillus plantarum*, whereas at pH=7.0, there was a slight decrease in surface hydrophobicity of fermented PPEF. Kumitch *et al*. (2020b) reported that surface hydrophobicity decreased in PPEF from 14.1 to 8.4 a.u. and from 21.6 to 13.9 a.u. when fermented with *A. oryzae* and *A. niger*, respectively. The differences among surface hydrophobicity values of different studies on pea and navy bean protein are due to different processing methods used in these studies.

4.1.4 Functionality

(a) Nitrogen solubility

The nitrogen solubility of fermented PPI and NBPI were measured at pH 7.0 and summarized in Table 4.1.4. Protein solubility represents the amount of water-soluble nitrogen in a protein (Adler-Nissen, 1976). This parameter is important since it can affect other functionalities such as foaming and emulsification (Kinsella & Melachouris, 2009). Highly soluble proteins can be used in a wide range of applications in the food industry. The protein solubility depends on hydrophilic interactions, which lead to protein-solvent interactions rather than protein-protein interactions. In addition, solubility depends on pH; protein-protein interactions are favored at pH values close to the pI leading to aggregation and lower solubility; however, protein-solvent interactions are favored at pH values above and below the pI since the electrostatic repulsive forces increase at these pH values (Wu *et al*., 1998). The protein solubility increased significantly

($p \leq 0.05$) over fermentation time (0-48 h) 8.74 to 34.19% for PPI; however, NBPI protein solubility did not change significantly over fermentation time (0-72 h) and remained about 62% ($p > 0.05$) (Table 4.1.4). These results are consistent with the SDS-PAGE results (Fig. 4.1.3a).

The fermentation treatment caused a decrease in protein size of PPI, which explains the observed increase in water solubility of the PPI protein. During fermentation, the fungi release the proteolytic enzymes, which lead to the cleaving of proteins to smaller peptides and exposure of hydrophobic and hydrophilic sites on the protein. The exposed hydrophilic or hydrophobic properties on the protein impact the protein solubility.

Table 4.1.4 Protein solubility of pea protein isolate and navy bean protein isolate samples inoculated with *A. oryzae* over fermentation time.

Fermentation time (h)	**Solubility (%)**	
	Pea protein isolate	**Navy bean protein isolate**
0	8.7 ± 1.5^{a}	61.7 ± 2.9^{a}
12	27.7 ± 1.3^{b}	61.1 ± 2.0^{a}
24	29.9 ± 2.2^{b}	60.7 ± 1.3^{a}
36	33.9 ± 1.4^{c}	63.7 ± 1.6^{a}
48	34.2 ± 0.5^{c}	64.0 ± 1.7^{a}
72	-	62.6 ± 1.8^{a}

Data represent the mean values from duplicate samples from triplicate batches of PPI and NBPI samples ± one standard deviation (n=3). Different letters within each column are significantly different ($p \leq 0.05$).

Higher solubility can result from molecular size reduction and exposure of hydrophilic moieties due to fungal protease cleavage (Prinyawiwatkul *et al*., 1997). Akubor & Chukwu (1999) reported nitrogen solubility in water increased in fermented oil bean seed flour compared to non fermented flour due to proteolytic activity yielding peptides and free amino acids. The increase in solubility is in accordance with an increase in DH% of PPI after 48h to 9.31%, as shown in Fig. 4.1.2. However, limited changes in DH% of NBPI (~3.64% after 72h) resulted in no significant change in solubility.

Limited studies have been focused on DH% of PPI and NBPI through fermentation and its effect on solubility. Barac *et al*. (2010) reported that the limited hydrolysis of protein could lead to the production of smaller and less compacted peptides compared to the original, which contributes to legume protein modification. Tsumura *et al*., (2005) indicated that functional properties were improved after limited DH in the range of 2%-8% of protein. In other studies, an even lower DH, between 2%-4%, by the endo-protease treatment of soy protein causes an improvement in functional properties (Jung, 2005;Yu *et al*., 2007). Therefore, partial hydrolysis of protein in this study could improve solubility. Thus, the solubility of the final product can be improved by partial hydrolysis of protein.

The surface charge and surface hydrophobicity are other factors that could affect solubility (Arteaga *et al*., 2021). The surface charge of the fermented PPI increased through fermentation time, ranging from -34.10 to -38.77. The high net charge favors protein-solvent interactions over protein-protein interactions, reducing the aggregation and precipitation of protein. A similar phenomenon could occur in PPI fermented medium. However, surface hydrophobicity increased with fermentation time (Table 4.1.3) hypothetically due to exposing hydrophobic patches. The increase in surface hydrophobicity could increase protein-protein interactions and decrease the protein solubility; however, in this study, solubility increased. The increase of protein solubility by fermentation might be related to factors such as the increase in DH%, leading to smaller and more soluble peptides, and an increase in the surface charge of the samples leading to water-protein interactions.

(b) Emulsifying properties

Emulsion stability (ES) represents the ability of the emulsion to stay unchanged over time in response to gravitational forces. The ES depends on the properties of the protein interface (Shevkani *et al*., 2015), protein flexibility, and surface hydrophobicity (Ahmed *et al*., 2011). ES was determined for fermented PPI and NBPI, as shown in Table 4.1.5. ES values of fermented PPI was 81.1% initially, and after 48 h, fermentation declined to 65.2%, whereas ES values of fermented NBPI did not significantly change during the fermentation process and remained constantly around 81.88% (Table 4.1.5).

In a similar study, Kumitch *et al*. (2020b) reported no change in emulsifying properties of PPEF after fermentation using *A. oryzae* and *A. niger*. Shi *et al*. (2021) reported no significant

difference in emulsifying properties for PPI fermented by lactic acid bacteria sample and the control. It was hypothesized that the lack of significant changes in ES of NBPI in the present study is related to the limited DH% (~5%). In contrast, the ES of fermented PPI was shown to decrease significantly over the first 12 h ($p \leq 0.05$) (Table 4.1.5. The ES could be improved by adsorbing more flexible peptide molecules at the interface of oil and water (Xiao *et al*., 2018). Thus, a lower amount of the hydrophilic and flexible proteins that formed during the SSF may have induced the decrease in ES of the PPI.

Table 4.1.5 ES of fermented pea protein isolate and navy bean protein isolate inoculated by *A. oryzae* over fermentation time.

Fermentation time (h)	**ES (after 30 min)**	**ES (after 30 min)**
	Pea protein isolate	**Navy bean protein isolate**
0	81.1±1.2[a]	83.0±1.0[a]
12	59.0±2.8[b]	81.5±1.7[a]
24	60.0±1.5[b]	81.5±1.3[a]
36	60.3±2.0[b]	80.4±2.6[a]
48	61.2±2.6[b]	83.5±2.4[a]
72	-	83.0±3.2[a]

Data represent the mean values from duplicate samples from triplicate batches of PPI and NBPI samples ± one standard deviation (n=3). Means followed with different letters within each column are significantly different ($p \leq 0.05$). Abbreviation: ES (emulsifying stability).

(c) Foaming properties

Foaming capacity (FC) and stability (FS) are important functional properties of food ingredients that determine their application in food systems where aeration and overrun are important factors (e.g., baked foods, whipped toppings, and ice-cream mixes) (Shevkani *et al*., 2015). The FC represents the amount of air entrapped within the protein matrix, which is indicated by the maximum volume increase because of dispersed proteins (Sathe, 1982). The FS represents the ability of the foam to keep the same volume over a designated period of time (Wouters *et al*., 2016). The FC and FS of fermented PPI and NBPI are indicated in Table 4.1.6. The FC of the fermented samples significantly ($p \leq 0.05$) increased compared to non-fermented PPI. The FC

values for PPI increased from 155% to 175 % after fermentation for 48 h. However, there were no significant changes in FC of NBPI samples and it remained approximately 151%.

Solubility has a great effect on foaming properties. During fermentation, the solubility of the samples increased by fermentation time, which could lead to the enhancement in PPI's foaming capacity. The soluble globular proteins could diffuse to the air-water interface, contributing to surface tension reduction. If the protein's solubility increases during fermentation, then its ability to diffuse at the air-water interface increases. The unfolded proteins reorient their polar ends at this interface, forming a stable film around the air bubbles by the protein-protein interactions, occurring through electrostatic, hydrophobic interactions, and hydrogen bonds (Zayas, 1997). In addition, the foaming capacity of proteins depends on different physicochemical characteristics including, surface tension and hydrophobicity, electrostatic repulsion, and molecular weight (Zayas, 1997).

Table 4.1.6 Foaming capacity and foaming stability of fermented pea protein isolate and navy bean protein isolate inoculated by *A. oryzae* over fermentation time.

	Foaming capacity (%)		**Foaming stability (%)**	
Protein source/Time (h)	**PPI**	**NBPI**	**PPI**	**NBPI**
0	155±8^{a}	156±6^{a}	33±1^{a}	46±2^{a}
12	147±8^{a}	148.3±6^{a}	34±4^{a}	40±4^{a}
24	162±8^{ab}	148.9±8^{a}	71±7^{b}	42±2^{a}
36	171±9^{b}	153.3±7^{a}	69±9^{b}	42±4^{a}
48	176±8^{b}	151.1±4^{a}	69±8^{b}	45±6^{a}
72	-	148.9±4^{a}	-	43±3^{a}

Data represent the mean values from duplicate samples from triplicate batches of PPI and NBPI samples ± one standard deviation (n=3). Means followed with different letters within each column are significantly different ($p \leq 0.05$).

The FS of NBPI showed no significant difference during fermentation time and remained constantly around 43.05%, but fermentation significantly increased FS of PPI from 33.10% to 69.52% (Table 4.1.6). The higher FS of fermented PPI could be related to the high adsorption and surface activities of soluble proteins in the formed liquid phases (Kaur & Singh, 2005). Elkhalifa & Bernhardt (2005) reported that conformational changes created during the bioprocessing of the

proteins could affect the FS of cereal flours. This could partially explain the higher FS of the PPI. Thus, the enhanced FS might be related to the structural changes of protein during SSF, which leads to an increase in the intermolecular interactions and, as a result, increased the formed viscoelastic film (Kaur & Singh, 2005). The unfolded proteins produced during fermentation should reorient around the foam and stabilize the bubbles via electrostatic repulsion, hydrophobic interactions, and hydrogen bonds (Zayas, 1997). An increase in FS could be related to stronger protein-protein interactions in fermented PPI compared to non-fermented PPI.

Previous studies have reported the influence of SSF on FC and FS of legume and cereal isolates and flours (Elkhalifa *et al*., 2005; Adebowale & Maliki, 2011;Chandra-Hioe *et al*., 2016; Chawla *et al*., 2017; Xiao *et al*., 2018). In accordance with fermented PPI, Chawla et al. (2017) reported an increase in FC and FS of fermented black-eyed pea flour, which could result from the significant increase in the electrostatic charges of samples Chawla *et al.* (2017). Meinlschmidt *et al.* (2016) reported that the foaming activity, density, and stability of non-fermented soy protein isolate were nearly doubled after fermentation due to the partial hydrolysis of soy proteins, in which more flexible low-molecular weight peptides were formed. Therefore, peptides could transfer to the air-water interface more rapidly, and the protein-protein interactions improved the strength of the viscoelastic cohesive film, dropping the surface tension (Tsumura *et al*., 2005). Ferreira *et al*. (2018)reported an increase in foaming and reduction in foam degradation of moldy and fermented black beans, as compared to untreated black beans, which could be attributed to the globulins, the predominant fraction of bean proteins. The globulins in their original form have a rigid and compact structure; but, after storage and fermentation, modification of the conformation and relaxation of the structure of globulins may be responsible for the increase in the FC and FS (Martin *et al.,* 2002). In other studies, no effect or even a decrease in foaming capacity after fermentation of PPEF was reported, which could be attributed to the more-compact structure and low solubility of the fermented flour (Çabuk *et al*., 2018; Kumitch *et al*., 2020b). In a study by Arteaga *et al*. (2021), the unfermented and fermented PPI could not form foams. The lack of foam formation by the unfermented PPI might be attributed to the alkaline extraction method (Arteaga *et al*., 2021). This is in contrast with the current study since the PPI could produce foam; this may be related to the pea cultivar, environmental factors, and plant genetics influencing foaming. Xiao *et al*. (2018) reported no significant difference in FC of fermented red bean flour (RBF) compared to unfermented RBF. However, FS of fermented RBF increased in fermented samples. This was due

to structural changes of proteins during SSF, leading to higher solubility in the liquid phase, enhancing their adsorption and surface activities. This is in accordance with the current study in which PPI solubility increased during fermentation which attributes to an increase in foaming capacity. A decrease in FC and FS with an increase in fermentation time occurred in fermented pigeon pea seed flour, which contrasts with PPI (Adebowale & Maliki, 2011). In a study, three fermented legume varieties including, faba bean, desi, and kabuli chickpeas, were reported to possess different foaming properties depending upon the type of legume (Chandra-Hioe *et al*., 2016). It might indicate that foaming properties are dependent on the legume used and the cultivation of the legume.

(d) Oil holding and water hydration capacities

Water hydration (WHC) and oil-holding (OHC) capacities are two important functional properties in food applications. WHC is the amount of water absorbed into one gram of protein, whereas OHC is the amount of oil absorbed into one gram of protein (Boye *et al*., 2010). The OHC and WHC can affect storage, product shelf-life, and food structure. The OHC and WHC of fermented PPI and NBPI inoculated with fungal strain *A. oryzae* are shown in Figures 4.1.5 and 4.1.6. The WHC of fermented PPI decreased over the fermentation time from 3.57 g/g (0 h) to 2.21 g/g (48 h). However, the WHC of fermented NBPI increased throughout fermentation from 1.55 g/g (0 h) to 2.02 g/g (72 h).

In some studies, increases in WHC after fermentation have been reported, such as in fermented chickpea (Reyes-Moreno *et al*., 2004); red bean (Xiao *et al*., 2018); black-eyed pea (Chawla *et al*., 2017); oil bean (Akubor & Chukwu, 1999), and *Moringa oleifera* (Oloyede *et al*., 2016).

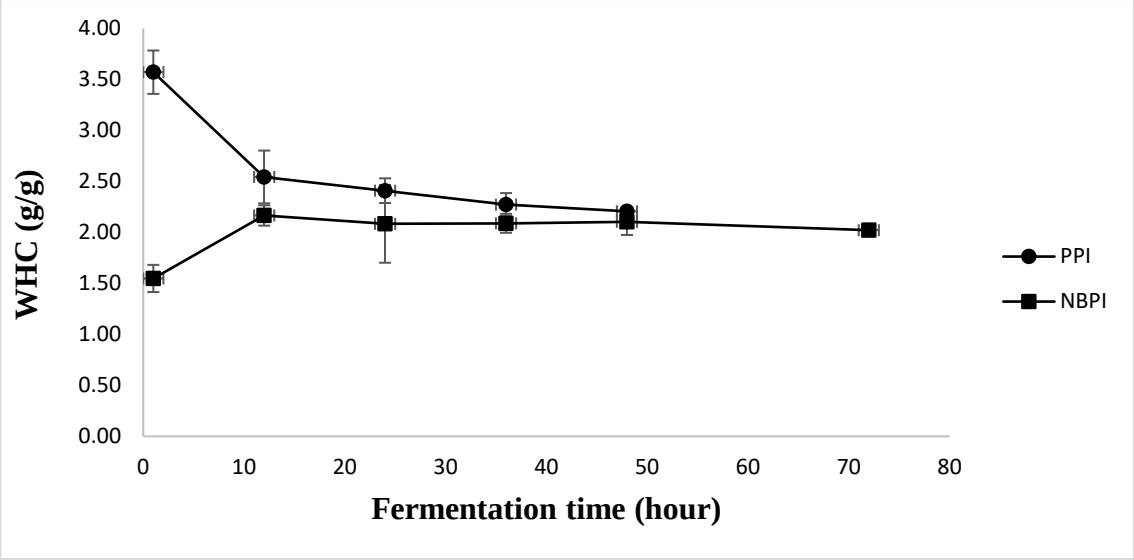

Figure 4.1.5 Water hydration capacity (WHC) of fermented pea protein isolate (PPI) and navy bean protein isolate (NBPI) inoculated with *A. oryzae* during fermentation time. Data represent the mean values from duplicate samples from triplicate batches of PPI and NBPI samples ± one standard deviation (*n*=3).

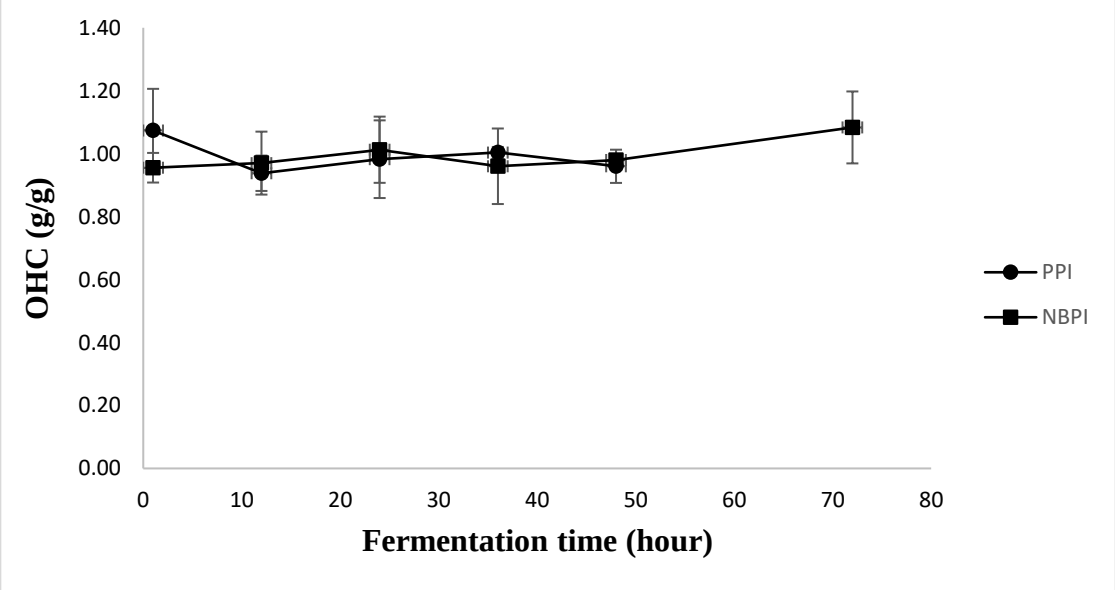

Figure 4.1.6 Oil holding capacity (OHC) of fermented pea protein isolate (PPI) and navy bean protein isolate (NBPI) inoculated with *A. oryzae* during fermentation time. Data represent the mean values from duplicate samples from triplicate batches of PPI and NBPI samples ± one standard deviation (*n*=3).

The WHC improvement of fermented flours is due to the proteolytic activity, which changes the protein conformation. The peptide bonds hydrolyzed during fermentation due to protease activity lead to the exposure of more polar amino acid side chains with strong water hydration ability. Oloyede *et al*. (2016) indicated that WHC of *Moringa oleifera* seed flour had been increased from 0.86 g/mL to 1.81 g/mL during 72 h fermentation time (Oloyede *et al*., 2016). However, the WHC of pigeon pea decreased from 1.42 g/g to 1.13 g/g after 5 days of fermentation (Adebowale & Maliki, 2011). The WHC reduction could result from the alteration of protein conformation during fermentation, exposing fewer hydrophilic groups than hydrophobic groups. Meinlschmidt *et al*. (2016) also reported that fermentation of soy protein isolate increased WHC and decreased protein solubility at pH 7.0. WHC of fermented SPI samples increased up to 3.5 (g/g) after 48 h, while non-fermented SPI had a WHC of approx. 2.6 (g/g). An increase in WHC can be related to a decrease in solubility. The higher amount of insoluble proteins could bind more water molecules (Meinlschmidt *et al*., 2016).

The OHC of both protein isolates was approx. 0.99 g/g for fermented and non-fermented samples and did not change significantly (p>0.05). This result is consistent with that reported by Udensi & Okoronkwo (2006) for mucuna bean protein isolates. The OHC of mucuna bean protein isolates remained constant during fermentation. Some studies were conducted on the effect of fermentation on PPI and PPEF; however, few studies were conducted on the OHC of fermented navy bean. In contrast to our results, Shi *et al*. (2021) indicated that from 0 to 10 h of fermentation, the OHC increased from 1.96 to 6.13 g/g. This is because the fermentation changed the protein folding and increased the exposure of the hydrophobic region. Starting from 15 h of fermentation, OHC then decreased to 3.64 g/g. In another study by Kumitch *et al*. (2020b) the OHC of the PPEF increased from 1.25 to 1.39 g/g and 1.18 to 2.27 g/g via *A. oryzae* and *A. niger* inoculation, respectively. Xiao *et al*. (2018) reported fermentation significantly increased the OHC of red bean flour by 29.9%.

For both PPI and NBPI, Table 4.1.7 indicate the correlations between surface properties (surface charge and surface hydrophobicity) and functional properties (OHC, WHC, solubility, foaming capacity, foaming stability, and emulsion stability). For PPI the correlations between surface properties (surface charge and surface hydrophobicity) and functional properties (OHC, WHC, solubility, foaming capacity, foaming stability, and emulsion stability). Surface hydrophobicity has a considerable impact on WHC, foaming capacity, and foaming stability in

PPI. However, there is a weak relationship between surface charge and functional properties. Interaction of surface charge and surface hydrophobicity also moderately change foaming properties and WHC.

Surface charge has a considerable impact on WHC, OHC, foaming capacity, and emulsion stability in NBPI. However, there is a weak relationship between surface hydrophobicity and functional properties. Interaction of surface charge and hydrophobicity also moderately change foaming capacity.

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Table 4.1.7 Correlation between surface properties and functional properties of (a) fermented pea protein isolate and (b) fermented navy bean protein isolate.

(a)	Functional properties					
	OHC	WHC	Solubility	Foaming capacity	Foaming stability	Emulsion stability
Surface charge	0.07	-0.25	0.30	0.09	0.12	-0.39
Surface hydrophobicity	-0.34	-0.82	0.87	0.65	0.67	-0.47
Surface charge * Surface hydrophobicity	0.33	0.66	-0.69	-0.54	-0.57	0.27

(b)	Functional properties					
	OHC	WHC	Solubility	Foaming capacity	Foaming stability	Emulsion stability
Surface charge	0.61	0.81	0.24	0.83	0.09	0.62
Surface hydrophobicity	0.20	0.16	0.17	0.47	0.35	0.31
Surface charge * Surface hydrophobicity	-0.29	0.17	0.11	0.51	0.18	0.28

4.1.5 Protein quality

Protein quality is a major factor for health and adequate nutrition. However, the main issue surrounding pulse protein, such as that from pea and navy bean, is their low digestibility. The protein digestibility can be affected by different factors such as globular structure, the conformation of the protein, and bioactive compounds (Tinus *et al*., 2012). The full amino acid composition of fermented PPI and NBPI, reported in grams per 100 g of sample, is indicated in Table 4.1.8. There were no significant ($p>0.05$) or very slight significant changes ($p\leq0.05$) to amino acid composition of PPI and NBPI.

The amino acid scores of fermented PPI and NBPI is indicated in Table 4.1.9. The amino acid scores did not significantly change for PPI and NBPI during fermentation time. Before and after fermentation, the limiting amino acids of both PPI and NBPI were methionine and cysteine (Table 4.1.10). The limiting amino acid values ranged from 0.68 to 0.71 in PPI and 0.62 to 0.63 in NBPI. The limiting amino acid scores for fermented PPI and NBPI are in agreement with previous observations for legumes (Nosworthy *et al*., 2018).

Nosworthy *et al*. (2018) reported methionine and cysteine to be the limiting amino acids in red and green lentils while using different processing methods, including extrusion, cooking, and baking. The limiting amino acids ranged from 0.57 for baked green lentil, to 0.68 for extruded red lentil. In addition, tryptophan was also indicated to be limiting in the processed lentil samples ranging from 0.69 to 0.78 for extruded green lentils and red lentil, respectively (Nosworthy *et al*., 2018).

In vitro protein digestibility amino acid score (IV-PDCAAS) was also determined and reported in Table 4.1.11. The in vitro protein digestibility (IVPD) represents the pH drop due to protein digestion in which amino acids and peptides are produced (Tinus *et al*., 2012). The IVPD of fermented PPI decreased over fermentation time from 88.19 to 75.25%; however, there was no significant changes for IVPD of NBPI (~75%) ($p>0.05$). Olukomaiya *et al*. (2020) reported a reduction in IVPD through fermentation of lupin flour, where IVPD decreased as fermentation time increased. In addition, proso millet flour was also observed to have reduced IVPD after fermentation (Gulati *et al*., 2018).

Table 4.1.8 Amino acid composition (g per 100 g of protein isolate, on an as is basis) for fermented (a) pea protein isolate and (b) navy bean protein isolate inoculated with *A. oryzae*.

(a) Pea protein isolate	**Fermentation time (h)**				
	0	**12**	**24**	**36**	**48**
Amino acid					
Aspartic acid/asparagine	8.7±0.2 [a]	9.1±0.5 [a]	8.4±0.4 [a]	8.6±0.2 [a]	8.5±0.1 [a]
Glutamic acid/glutamine	12.8±0.2 [a]	13.5±0.7 [a]	13.1±0.5 [a]	13.8±0.4 [a]	13.6±0.3 [a]
Serine	3.9±0.1 [a]	4.1±0.2 [a]	3.9±0.1 [a]	3.8±0.1 [a]	3.8±0.0 [a]
Glycine	3.0±0.1 [a]	3.1±0.1 [a]	3.1±0.1 [a]	3.2±0.1 [a]	3.1±0.0 [a]
Histidine‡	1.8±0.1 [a]	2.0±0.2[a]	2.0±0.1 [a]	2.0±0.1 [a]	1.9±0.1[ab]
Arginine	6.2±0.1 [a]	6.5±0.3 [a]	6.0±0.2 [ab]	5.8±0.1[b]	5.7±0.1 [b]
Threonine‡	2.8±0.0 [a]	2.9±0.1 [ab]	2.9±0.0 [b]	2.9±0.1[b]	2.9±0.0[b]
Alanine	3.2±0.0[a]	3.3±0.1[a]	3.2±0.1 [a]	3.3±0.1 [a]	3.3±0.0 [a]
Proline	3.3±0.0[a]	3.4±0.1 [a]	3.4±0.1 [a]	3.4±0.0 [a]	3.3±0.0 [a]
Tyrosine	2.5±0.1[a]	2.7±0.2 [a]	2.5±0.2 [a]	2.5±0.1 [a]	2.6±0.0 [a]
Valine‡	3.9±0.0 [a]	4.1±0.2 [a]	3.9±0.1 [a]	3.9±0.0 [a]	3.9±0.1 [a]
Methionine*‡	0.9±0.0 [a]	0.9±0.0 [a]	0.9±0.0 [a]	0.9±0.0 [a]	0.9±0.0 [a]
Cysteine*	0.5±0.0 [a]	0.5±0.0 [a]	0.5±0.0 [a]	0.5±0.0 [a]	0.5±0.1 [a]
Isoleucine‡	3.7±0.1 [a]	3.8±0.1 [a]	3.6±0.1 [a]	3.7±0.1 [a]	3.6±0.0 [a]
Leucine‡	6.3±0.1 [a]	6.5±0.3 [a]	5.9±0.1 [a]	5.8±0.0 [ab]	5.7±0.0 [b]
Phenylalanine‡	3.8±0.1 [a]	3.8±0.1 [a]	3.5±0.1 [ab]	3.4±0.0[b]	3.4±0.0[b]
Lysine‡	5.8±0.1 [a]	6.0±0.2 [a]	5.7±0.2 [a]	5.8±0.1 [a]	5.5±0.1 [a]
Tryptophan‡	0.8±0.0 [a]	0.8±0.0 [a]	0.9±0.0 [b]	0.9±0.0 [b]	0.9±0.0 [b]

Data represent the mean values from duplicate samples from triplicate batches of PPI and NBPI samples ± one standard deviation (n=3). Means followed with different letters within each row are significantly different ($p \leq 0.05$).

(b) Navy bean protein isolate	Fermentation time (h)					
	0	**12**	**24**	**36**	**48**	**72**
Amino Acid						
Aspartic Acid/asparagine	7.9±1.1[a]	8.4±0.3[a]	8.4±0.3[a]	8.4±0.2[a]	8.2±0.2[a]	8.2±0.2[a]
Glutamic Acid/glutamine	10.5±0.6[a]	10.9±0.5[a]	10.7±0.3[a]	10.7±0.4[a]	10.6±0.2[a]	10.6±0.3[a]
Serine	4.1±0.7[a]	4.4±0.1[a]	4.4±0.1[a]	4.4±0.1[a]	4.3±0.2[a]	4.3±0.1[a]
Glycine	2.6±0.2[a]	2.7±0.1[a]	2.7±0.1[a]	2.7±0.1[a]	2.7±0.1[a]	2.7±0.1[a]
Histidine‡	2.0±0.1[a]	2.0±0.1[a]	1.9±0.1[a]	2.0±0.1[a]	2.0±0.1[a]	2.0±0.2[a]
Arginine	4.0±0.1[a]	4.0±0.2[a]	3.9±0.2[a]	3.8±0.1[a]	3.8±0.1[a]	3.8±0.1[a]
Threonine‡	2.7±0.3[a]	2.8±0.1[a]	2.8±0.1[a]	2.8±0.1[a]	2.8±0.1[a]	2.8±0.1[a]
Alanine	2.7±0.0[a]	2.8±0.1[a]	2.8±0.1[a]	2.8±0[a].0	2.8±0.0[a]	2.8±0.0[a]
Proline	2.7±0.1[a]	2.8±0.1[a]	2.7±0.1[a]	2.8±0.1[a]	2.7±0.1[a]	2.7±0.1[a]
Tyrosine	2.5±0.3[a]	2.5±0.1[a]	2.5±0.3[a]	2.5±0.2[a]	2.5±0.2[a]	2.4±0.1[a]
Valine‡	3.5±0.5[a]	3.8±0.1[a]	3.7±0.1[a]	3.7±0.1[a]	3.7±0.0[a]	3.7±0.1[a]
Methionine*‡	1.0±0.0[a]	1.0±0.0[a]	1.0±0.0[a]	1.0±0.0	1.0±0.0[a]	1.0±0.0[a]
Cysteine*	0.3±0.0[a]	0.3±0.0[a]	0.3±0.0[a]	0.3±0.0	0.3±0.0[a]	0.3±0.0[a]
Isoleucine‡	3.3±0.4[a]	3.4±0.1[a]	3.4±0.1[a]	3.4±0.1[a]	3.4±0.0[a]	3.4±0.1[a]
Leucine‡	5.8±0.9[a]	6.2±0.2[a]	6.2±0.2[a]	6.2±0.1[a]	6.0±0.1[a]	6.0±0.1[a]
Phenylalanine‡	3.9±0.6[a]	4.1±0.2[a]	4.1±0.2[a]	4.1±0.1[a]	4.0±0.1[a]	4.0±0.1[a]
Lysine‡	4.8±0.8[a]	5.1±0.2[a]	5.1±0.2[a]	5.2±0.2[a]	5.0±0.1[a]	5.0±0.1[a]
Tryptophan‡	1.2±0.5[a]	1.2±0.5[a]	1.2±0.5[a]	1.6±0.5[a]	1.6±0.5[a]	1.2±0.5[a]

Measurements were performed twice on one sample from each of triplicate batches of protein isolates. (*) Indicates sulfur amino acid. (‡) Indicates essential amino acids. Means followed with different letters within each row are significantly different ($p \leq 0.05$).

Table 4.1.9 Amino acid scores for fermented pea protein isolate and navy bean protein isolate, inoculated with *A. oryzae*.

	Amino acids								
Time (h)	**THR**	**VAL**	**MET + CYS**	**ILE**	**LEU**	**PHE + TYR**	**HIS**	**LYS**	**TRP**
a) Pea protein isolate									
0	1.0	1.4	*0.7	1.6	1.2	1.2	1.2	1.2	0.9
12	1.0	1.4	*0.7	1.6	1.2	1.2	1.2	1.2	0.9
24	1.0	1.3	*0.7	1.6	1.1	1.1	1.2	1.2	1.0
36	1.0	1.3	*0.7	1.6	1.0	1.7	1.3	1.2	1.0
48	1.0	1.3	*0.7	1.5	1.0	1.7	1.2	1.1	1.0
b) Navy bean protein isolate									
0	1.0	1.3	*0.6	1.5	1.1	1.3	1.3	1.0	1.3
12	1.0	1.3	*0.6	1.5	1.1	1.3	1.2	1.1	1.3
24	1.0	1.3	*0.6	1.5	1.1	1.3	1.2	1.0	1.3
36	1.0	1.3	*0.6	1.5	1.1	1.3	1.3	1.1	1.7
48	1.0	1.3	*0.6	1.4	1.1	1.3	1.3	1.0	1.7
72	1.0	1.3	*0.6	1.4	1.1	1.3	1.3	1.0	1.3

Measurements were performed twice on one sample from the each of triplicate batches of protein isolates (*n*=3). (*) Indicates the first limiting amino acid. Abbreviations: THR (threonine); CYS (cysteine); VAL (valine); MET (methionine); ILE (isoleucine); LEU (leucine); TYR (tyrosine); PHE (phenylalanine); HIS (histidine); LYS (lysine); and TRP (tryptophan).

Table 4.1.10 Essential amino acid concentration (mg/g protein) for fermented pea protein isolate and navy bean protein isolate inoculated with *A. oryzae*.

Time (h)	Amino acids								
	THR	**VAL**	**MET + CYS**	**ILE**	**LEU**	**PHE + TYR**	**HIS**	**LYS**	**TRP**
a) Pea protein isolate									
0	34	48	17	45	77	77	23	71	10
12	35	49	17	45	78	79	24	72	10
24	35	47	18	44	72	73	24	69	11
36	35	47	17	44	69	107	24	69	11
48	35	47	17	43	69	107	23	66	11
b) Navy bean protein isolate									
0	34	45	16	41	74	84	24	61	15
12	34	45	16	41	74	83	24	61	14
24	34	44	16	41	74	83	23	61	14
36	34	45	16	41	74	82	24	62	19
48	33	44	16	40	72	81	24	59	19
72	33	44	16	41	72	81	24	60	15
FAO reference	**34**	**35**	**25**	**28**	**66**	**63**	**19**	**58**	**11**

Measurements were performed twice on one sample from the each of triplicate batches of protein isolates (*n*=3). Abbreviations: THR (threonine); CYS (cysteine); VAL (valine); MET (methionine); ILE (isoleucine); LEU (leucine); TYR (tyrosine); PHE (phenylalanine); HIS (histidine); LYS (lysine); and TRP (tryptophan).

The unexpected reduction in IVPD of the fermented protein isolate may be due to the presence of anti-nutrients such as total phenolic compounds. During fermentation proteolytic activity could cleave polyphenols from protein complexes and release polyphenols. Polyphenols

have antinutritional activity and could decrease IVPD. Kumitch *et al*. (2020b) reported total phenolic compounds in fermented PPEF increased throughout the fermentation time course, by *A. oryzae* and *A. niger*. Furthermore, the protein digestibility reduction can be due to an inactive protein complex between trypsin inhibitors and endopeptidase trypsin. Additionally, the protocol used to determine IVPD for the fermented PPI and NBPI has been criticized because the equation IVPD (%) = 65.66 + 18.10 ∗ ΔpH_{10min} (Eq. 1) shows the minimum of 65.66 for the IVPD even if there is no change in pH during 10 min of the experiment (Tinus *et al*., 2012). Based on this equation, values more than 100% could be calculated for IVPD (Tinus *et al*., 2012).

Table 4.1.11 Amino acid scores and protein data of fermented pea protein isolate and navy bean protein isolate inoculated with *A. oryzae.*

Time (h)	Limiting AA	Limiting AA score	IVPD (%)	IV-PDCAAS (%)
a) Pea protein isolate				
0	MET+CYS	0.7	88.2±1.4[a]	60.0±0.9[a]
12	MET+CYS	0.7	80.8±1.8[b]	55.0±1.2[b]
24	MET+CYS	0.7	76.5±1.4[c]	54.4±0.9[b]
36	MET+CYS	0.7	75.7±1.1[c]	52.4±0.8[c]
48	MET+CYS	0.7	75.2±1.2[c]	52.1±0.8[c]
b) Navy bean protein isolate				
0	MET+CYS	0.6	75.8±0.7[a]	47.4±0.4[a]
12	MET+CYS	0.6	75.6±0.6[a]	47.3±0.4[a]
24	MET+CYS	0.6	75.1±0.7[a]	47.0±0.4[a]
36	MET+CYS	0.6	75.7±1.0[a]	46.9±0.6[a]
48	MET+CYS	0.6	74.4±0.5[a]	46.9±0.3[a]
72	MET+CYS	0.6	73.4±1.5[a]	46.3±0.9[a]

Measurements were performed twice on one sample from each of triplicate batches of protein isolates. Data represent the mean ± one standard deviation. Abbreviations: AA (amino acid); MET (methionine); CYS (cysteine); IVPD (In vitro protein digestibility); IV-PDCAAS (In vitro protein digestibility corrected amino acid score).

The Food and Agriculture Organization of the United Nations (FAO) uses PDCAAS for assessing global food protein quality because it includes both the most limiting essential amino acid and digestibility data. The IV-PDCAAS had a statistically significant decrease for PPI over the fermentation time from 66.68% to 52% ($p \leq 0.05$). However, IV-PDCAAS of NBPI remained around 47%, during fermentation. The decrease in IV-PDCAAS scores is due to the reduction in IVPD as the limiting amino acid scores were fairly constant for both protein isolates during fermentation.

Nosworthy *et al*. (2018) reported IV-PDCAAS values ranging from 49.81% to 70.19% for cooked faba bean and pinto bean, respectively. However, IV-PDCAAS values for yellow pea ranged from 62.27% to 67.44% using extrusion and cooking process, respectively (Nosworthy *et al*., 2018). The fermented PPI values were within the range of these two studies, ranging from the highest value of 60% at 0 h to the lowest value of 52% for 48 h fermentation. In contrast, the IV-PDCASS value for NBPI is approx. 47% which is a little lower than these two studies.

Nosworthy *et al*. (2020) indicated that the processing method strongly determined the optimum protein quality. In another study by Kumitch *et al*., (2020a) IVPD increased over the fermentation time for PPEF inoculated by *A. oryzae* and *A. niger*. However, IV-PDCASS decreased about 5%-15% after 6 h of fermentation. Fermentation was not an effective processing method to increase digestibility, as it negatively affected the overall IV-PDCAAS score; however, the limiting amino acids did not change during fermentation (via fungi utilization).

4.1.6 Conclusion

In study 1 the physicochemical, functional properties, and protein quality of fermented PPI and NBPI inoculated with *A. oryzae* were evaluated duration fermentation time. As a result, fermentation of PPI altered the protein structure via proteolytic enzymes secreted from the fungi, thus smaller sized peptides were produced leading to the higher solubility. Higher solubility during fermentation attributed to higher foaming stability and capacity. Additionally, fungal fermentation exposed hydrophobic moieties and increased surface charge for PPI. Emulsifying properties, WHC, and IVPD of PPI were negatively affected by SSF. However, OHC remained unchanged. In overall, fermentation of NBPI affected its structure, functional properties, and protein quality less than PPI. For NBPI, WHC and surface hydrophobicity improved with SSF throughout the fermentation and other functional properties and IVPD remained unchanged.

Findings suggested that SSF was effective at modification of PPI's physicochemical and functional properties. Specifically, solubility and foaming properties for PPI were significantly improved through fermentation and ultimately, fermented PPI could potentially be used as an ingredient in food products where improved solubility and foaming properties are of needed.

4.1.7 Connection to study 2

Study one was conducted to evaluate the proximate content, functionality, amino acid content, and protein quality of fermented PPI and NBPI. The PPI and NBPI were modified by SSF, and their functional properties could be changed by fermentation. Evaluating the functional properties could help to understand whether these changes were positive or negative and whether fermented protein isolates have the potential to be used as an ingredient in specific foods. Fermented PPI and NBPI could be incorporated in foods such as emulsions and foams; in this case, protein functional properties greatly influence the structure and stability of food (Foegeding & Davis, 2011). In addition, changes to amino acid content and protein digestibility were evaluated due to the significance of nutritional properties of fermented PPI and NBPI when used as a food ingredient. However, study one alone does not fully indicate the potential of fermented PPI and NBPI as food ingredients. The fermentation could potentially modify the flavour and volatile compounds of PPI and NBPI. A study was conducted to highlight the significance and impact of flavor on food ingredients.

4.2 Study 2: Effect of fermentation time on the aroma compounds of pea protein isolate and navy bean protein isolate fermented by *Aspergillus oryzae*

4.2.1 Aroma compounds analysis

During fermentation, fungi enzymes break down substrate nutrients, including proteins, carbohydrates, lipids, organic and amino acids, which act as precursors of non-volatile and volatile compounds that generate aromas and flavours. In substrates with a high protein content, enzymatic degradation can be particularly critical (Ben-Harb *et al.*, 2019). The activity of proteases, such as amino carboxypeptidases and decarboxylases, can produce different peptides, free amino acids,

and α-keto-acids and consequently modify the substrate's organoleptic properties (e.g., aroma and texture) (Curioni & Bosset, 2002; Visessanguan *et al*., 2005).

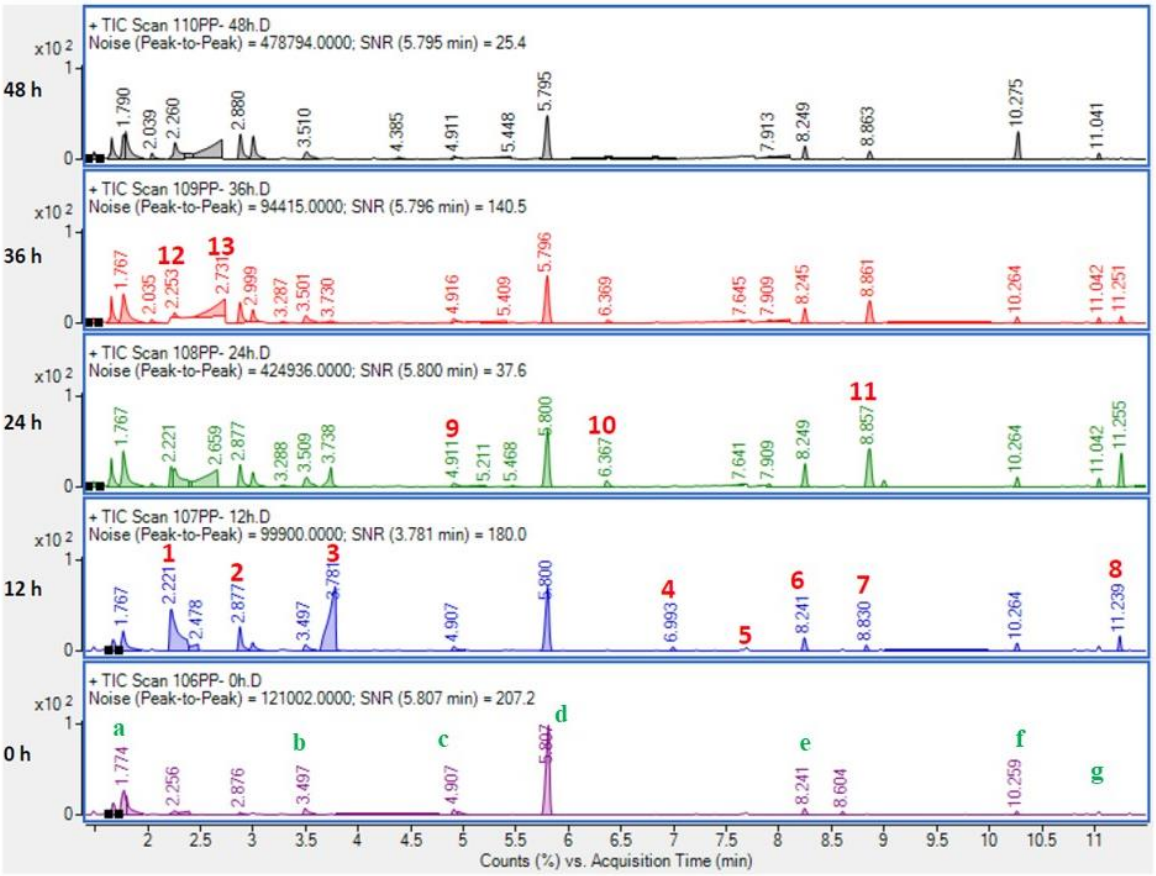

Figure 4.2.1. Chromatogram of pea protein isolate through 48 h of fermentation by *Aspergillus oryzae* showing volatile compound scan over a 12 min response period. 1: acetic acid ethenyl ester; 2: 3-methyl butanal; 3: acetoin; 4: acetic acid diethyl; 5: 1-chloro hexane; 6: 2-Heptanone; 7; 2,5-dimethyl-pyrazine; 8: trimethyl-pyrazine; 9: 1,3,5-cycloheptatriene; 10: methyl-pyrazine; 11: 2,6-dimethyl- pyrazine; 12: 2-butanone; 13: acetic acid. a: Trimethyl-Oxirane; b: Pentanal; c: Toluene; d: Hexanal; e: Propanoic acid; f: Benzaldehyde; g: 2-pentyl-Furan

In this study, GC-MS was used to identify the volatile compounds produced during the fermentation of both PPI and NBPI using *A. oryzae*. Based on the results, hexanal was the principal volatile compound found in unfermented isolates (Fig. 4.2.1 and Fig. 4.2.2). Hexanal contributes to the greeny and grassy flavor of pulses. Shi *et al*. (2021) reported hexanal as the most abundant

aromatic compound in unfermented PPI. Ferawati *et al*. (2020) also reported hexanal to be the main compound found in flours from boiled yellow and gray peas and was detected at significantly higher levels in boiled peas than in the raw pea flours (Ma *et al*., 2016). The presence of hexanal has been previously reported in beans and peas seeds (Ruth *et al*., 1995; Azarnia *et al*., 2011). In the raw seeds, linoleic acid is oxidized to hydroperoxides in the presence of oxygen; hexanal may be formed by the cleavage of 13-hydroperoxylinoleic acid by lyases. This process often occurs in pulse seeds that are disrupted during processing (Murat *et al*., 2013).

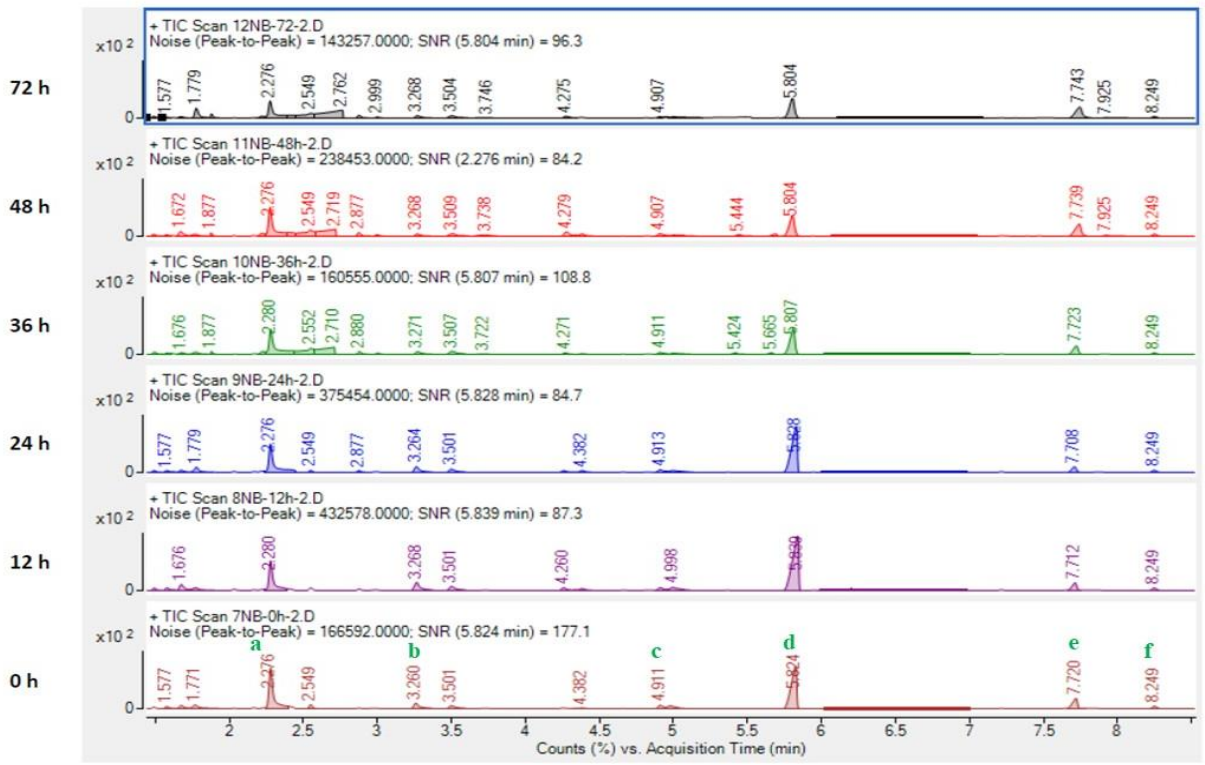

Figure 4.2.2. Chromatogram of navy bean protein isolates through 72 h of fermentation by *A. oryzae* showing volatile compound scan over a 12 min response period. a: n-Hexane; b: 1-Penten-3-ol; c: Toluene; d: Hexanal; e: 1-Hexanol; f: Bicyclo [4.2.0] octa-1,3,5-triene

The analysis of volatile compounds indicated that the levels of initial compounds did not have major changes after fermentation in both the PPI and NBPI (Fig 4.2.1 and Fig 4.2.2); however, the diversity and abundance of aroma compounds increased for fermented PPI. Many new volatile compounds with favourable sensory attributes were added to the fermented PPI substrate that were

produced by *A. oryzae.* These compounds could mask the off-flavour or add new favourable sensory properties to the fermented PPI (Table 4.2.2 and Fig. 4.2.1). Whereas there were no significant changes for the volatile compound profile of NBPI after fermentation. The *A. oryzae* has been reported to produce aromatic and volatile compounds with favourable sensory attributes (Permana *et al*., 2020; Zhao *et al*., 2020). A total of 13 new odourants were detected in fermented PPI (Table 4.2.2).

Table 4.2.1. Volatile compounds of non-fermented navy bean protein isolate and pea protein isolate.

Peak identification number	Compound	Retention time (min)
a) Navy bean protein isolate		
1	n-Hexane	2.276
2	1-Penten-3-ol	3.260
3	Toluene	4.911
4	Hexanal	5.824
5	1-Hexanol	7.720
6	Bicyclo [4.2.0] octa-1,3,5-triene	8.249
b) Pea protein isolate		
1	Trimethyl-Oxirane	1.774
2	Pentanal	1.497
3	Toluene	4.907
4	Hexanal	5.807
5	Propanoic acid	8.241
6	Benzaldehyde	10.259
7	2-pentyl-Furan	11.042

In fermented PPI, acetic acid ethenyl ester, 3-methyl butanal, acetoin, acetic acid diethyl, 1-chloro hexane, 2-Heptanone, 2,5-dimethyl-pyrazine, and trimethyl-pyrazine were first identified after 12 h fermentation. The compounds 1,3,5-cycloheptatriene, methyl-pyrazine, and 2,6-dimethyl-pyrazine were first identified after 24 h, and 2-butanone and acetic acid were first

identified after 48 h fermentation in PPI samples. The 3-methyl butanal, which is derived from the metabolism of the branched amino acids, leucine, and isoleucine, contributes malty, nutty, and caramel notes to the aroma profile (Curioni & Bosset, 2002; Smit *et al*., 2005).

Table 4.2.2. Volatile compounds of fermented pea protein isolate inoculated with *A. oryzae.*

No.	Compound	Retention time (min)	Odour
1	Acetic acid ethenyl ester	2.221	Sweet, pleasant, fruity
2	3-methyl butanal	2.877	Malty
3	Acetoin	3.781	Pleasant buttery
4	Diethyl acetic acid	6.993	Fruity
5	1-chloro hexane	7.692	-
6	2-Heptanone	8.241	Banana-like
7	2,5-dimethyl-pyrazine,	8.830	Cocoa, chocolate, nutty
8	Trimethyl-Pyrazine	11.239	Nutty
9	1,3,5-Cycloheptatriene	4.911	-
10	Methyl-Pyrazine,	6.367	Nutty, cocoa
11	2,6-dimethyl pyrazine,	8.857	Chocolate, nutty
12	2-Butanone	2.253	Ethereal fruity camphor
13	Acetic acid	2.731	Acidic, sour

As shown in Tables 4.2.1, none of the nitrogenous flavor and pyrazine compounds were identified in the non-fermented samples; instead, they were generally formed after fermentation. A diverse group of pyrazines was found in fermented PPI including, methyl-pyrazine, 2,5-dimethyl-pyrazine, 2,6-dimethyl-pyrazine, trimethyl-pyrazine (Tables 4.2.2). These compounds generally have a chocolate, roasted nut flavor, and a sharp taste (Ma *et al*., 2016). Ketones are carbonyl compounds that are formed by lipoxygenase activity from the breakdown of unsaturated fatty acid hydroperoxides. As shown in Tables 4.2.2, 2-butanone, which has an ethereal fruity camphor odor (Kim *et al*., 2021), was found in the fermented PPI samples. Fermentation produced other ketones

in the PPI, including 2-heptanone which has a fruity, spicy, cinnamon, banana, slightly spicy odor (Azarnia *et al*., 2011). These compounds have distinctive characteristics that can affect the overall flavor of PPI. 1,3,5-cycloheptatriene was detected in the PPI samples after 24 h fermentation (Tables 4.2.1). Aromatic compounds (cyclic compounds containing a certain number of double bonds) are present in a wide variety of foods in small quantities(Ma *et al*., 2016).

4.2.2 Conclusion

Overall, hexanal was the principal volatile compound found in unfermented isolates which contributes to the greeny and grassy flavor of pulses. The volatile compounds profile analysis showed that the initial volatile compounds did not significantly change during fermentation for protein isolates. Whereas about 13 new desirable volatile compounds were produced in fermented PPI including acids, alcohols, aldehydes, and ketones. These compounds could mask the off flavour and add new desirable sensory properties to the fermented PPI.

5 OVERALL CONCLUSION

Pulses, including pea and navy bean, are farmed as a staple crop all throughout the world. Being rich in essential amino acids and total phenolic compounds pulses have the potential to be included into the human diet as a healthy element. In addition, pulses' proteins have good functional properties to be used as food ingredient. However, consumption of pulses is limited due to the presence of anti-nutritional factors such as trypsin and chymotrypsin inhibitors and strong beany flavour. The SSF is a biotechnological process which could improve functional properties and protein quality and alter their sensory properties such as flavour compounds

In order to have a successful new food ingredient it is important to determine its functional properties for food application. The functionality properties including solubility, emulsification, foaming and water/oil holding capacities might alter trough fermentation which could be either beneficial or impractical for a food ingredient. In addition, protein quality is a pivotal factor which could change either in a positive or negative way by fermentation process. Moreover, a product success is based on consumer acceptability which depends on product's flavor. Fermentation could change the strong beany flavour of PPI and NBPI. Therefore, this study evaluated the effect of SSF on the functionality, protein quality, and volatile compounds of PPI and NBPI.

In the first study, the physicochemical, functional properties, and protein quality of fermented PPI and NBPI inoculated with *A. oryzae* were measured over a 48 h and 72 h fermentation period, respectively. Overall, fermented PPI had higher DH% through 48 h fermentation compared to NBPI. The protein structure of PPI had more changes compared to NBPI via proteolytic enzymes secreted from the fungi. SDS-PAGE indicated the extent of proteolytic hydrolysis because of changes in the legumin (11*S*) and vicilin (7*S*) ratio and the size of peptides. The smaller peptides size leads to higher solubility which attributes to an increase in foaming properties. The fungi release the proteolytic enzymes contributes to exposure of hydrophobic sites on the proteins of PPI. Surface hydrophobicity, zeta potential and nitrogen solubility, and foaming properties of PPI showed increase over fermentation time. As a result,

fermented PPI could be used in products which need higher protein solubility or foaming properties. The WHC of PPI decreased with fermentation time; whereas the WHC of NBPI was improved and OHC remained unchanged for both the protein isolates. The protein quality of fermented PPI and NBPI were analysed through analysis of IVPD and IV-PDCAAS. Overall, the limiting amino acids, methionine and cysteine, remained constant during fermentation for both protein isolates. Whereas the protein quality (IVPD) and IV-PDCAAS decreased through fermentation time for PPI.

In the second study, the changes to the volatile compounds of PPI and NBPI during fermentation were analyzed. The GC mass showed that initial flavor compounds in PPI and NBPI did not change significantly. However, SSF enhanced new desirable flavour compounds in PPI which could contribute to masking undesirable compounds. It is notable that the success of one product ultimately depends on consumer acceptability and consumer acceptability depends on sensorial properties. Thus, fermentation could be a beneficial process and a viable approach to improve PPI flavor and consumer acceptability.

6 FUTURE STUDIES

Based on the results, SSF had affected protein functionality, quality, and flavour compounds. This study could explain the effect of SSF modification on PPI and NBPI. However, providing insights into what happens during fermentation such ask could be beneficial to better understanding fermentation by *A. oryzae*.

In this thesis research *A. oryzae* fermentation was investigated; however, different microorganisms could create a variety of products, such as enzymes and bioactive substances. Additionally, another genus could produce more enzymes to hydrolyze the protein more effectively or cleave at other sites to improve the food product. Therefore, future research could evaluate other fungal genera or other *Aspergillus* species.

In this thesis research, the number of different enzymes involved in the fungal fermentation process was not evaluated. The protease activity investigation could help to better understanding of the protein hydrolysis throughout fermentation. In order to improve efficiency of the SSF, the amount of protease needed for a specific DH% and the required time to produce this amount of protease should be investigated. Furthermore, investigating the produced enzymes and their amounts during SSF of NBPI could improve the knowledge about the factors that prevent protein hydrolysis of NBPI substrate and could contribute to greater SSF efficiency in future.

Additionally, more investigation about nutritional value, such as information about phytic acid and saponins, and poly phenolic compounds could assist to better understanding fermented PPI and NBPI as an ingredient. Assessment of these properties could also further explain the alteration in protein digestibility that was observed in this study.

Furthermore, analyzing the activity of angiotensin-converting enzyme inhibitors (ACE inhibitors) might assist better understanding the nutritional value of PPI as a food additive. ACE inhibitors are bioactive peptides that have the potential to lower blood pressure and enhance cardiovascular health.

Because ACE inhibitory peptides can be generated using SSF with a legume substrate, this might be advantageous for hypertension management (Xiao *et al*., 2018). The hydrolysis of legume proteins results in the formation of ACE inhibitory peptides, and because protein hydrolysis happens throughout the fermentation process, bioactive peptides with ACE inhibitory activity may be freed and help in the decrease of hypertension. ACE inhibitory activity has the potential to be a value-added element that improves PPI use.

Since the ultimate product's goal is to be used as a food additive, a sensory evaluation of the colour, flavour, and texture of fermented PPI should be undertaken in the future. In this study, the SSF could produce desirable flavour compounds in PPI. However, a sensory evaluation could be helpful to better understand how these new compounds affect the initial flavour compounds of peas while masking the bitterness and beany flavour; consequently, having a sensory panel analyse this product might assist determine if using fermented PPI is a viable option. It is worth noting that, while a food product with high functionality, nutritional value, and digestibility is possible, consumer acceptance is ultimately the major factor of whether a product will succeed. It is notable that the success of one product ultimately depends on consumer acceptability.

Furthermore, there can be some limitations for scaling up the SSF. Therefore, understanding these limitations of scaling-up process would assist to produce a consistent fermented PPI and NBPI product. Additionally, the results of protein functionality in different studies extremely depends on the used methods. Therefore, standardization of the methods used to measure functionality, including water holding capacity, oil holding capacity, emulsification and foaming would be very helpful to compare results and benchmark further research. In addition, the analysis could be performed, and comparison of results would be easily obtained, if there was a standardized method formulated based on the type of protein (flour, concentrate or isolate).

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