2020

Sonication assisted protein extraction from some legumes, and improvement of nutritional profile of ingredients through fermentation

Bibek Byanju
Iowa State University

Sonication assisted protein extraction from some legumes, and improvement of nutritional profile of ingredients through fermentation

by

Bibek Byanju

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Food Science and Technology

Program of Study Committee:
Buddhi Lamsal, Major Professor
Milagros P. Hojilla-Evangelista
Lester Wilson

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis globally accessible and will not permit alterations after a degree is conferred.

Iowa State University

Ames, Iowa

2020

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

NOMENCLATURE

ANFs	Anti-nutritional factors
BBPI	Black bean protein isolates
BAPA	Benzoyl-DL,-arginine-p-nitoanalide hydrochloric
CD	Circular dichroism
CPC	Chickpea protein isolates
GI	Glycemic index
GRAS	Generally recognized as safe
HPS	High-power sonication
IVPD	In vitro protein digestibility
MW	Molecular weight
MRS	De Man, Rogosa and Sharpe
PA	Phytic acid
PD	Power density
PPO	Polyphenol oxidase
PVPP	Polyvinyl-polypirrolidone
PPI	Pea protein isolates
RPI	Rice protein isolates
SPI	Soybean protein isolates
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SSF	Solid state fermentation
SmF	Submerged fermentation
SEM	Scanning electron microscopy

TIA	Trypsin inhibitor activity
TIU	Trypsin inhibitor unit
TPC	Total phenolic contents
UAE	Ultrasonic assisted extraction
WHC	Water holding capacity

ACKNOWLEDGMENTS

I would like to take this opportunity to express my gratefulness to those who helped me with various aspects of conducting research and writing of this thesis. First and foremost, Dr. Buddhi Lamsal for his guidance, patience, and support throughout this research and writing of this thesis. His insights and words of encouragement have often inspired me and renewed my hopes for completing my graduate education. I could not have asked for a better major professor.

I would also like to thank my committee members for their efforts and contributions to this work: Dr. Milagros P. Hojilla-Evangelista and Dr. Lester Wilson.

I would like to thank my friends, colleagues, the department faculty and staff for making my time at Iowa State University a wonderful experience.

At last, I would like to thank my family for their unconditional support and encouragement during this long adventurous journey. My accomplishment in research and academic endeavors would not have been possible without the love and support of my mother, father, and entire Byanju family.

ABSTRACT

The conventional protein extraction method uses alkali method by modulating pH in the range of 8.5- 9; however, the presence of cell wall, other polysaccharides, and location of protein inside the cell-matrix limits its extraction resulting in the recovery of approximately half of the available proteins. Several pretreatment or physical modification can be utilized to improve protein extraction. The present study aimed to apply physical modification such as high-power sonication and extrusion. Two studies were carried out: firstly, high-power sonication (HPS) was used as a pretreatment to improve the extraction of proteins and their effect on the physicochemical property was studied. Secondly, fermentation was used to improve the nutritional profile of physically modified (HPS and extrusion) legume-protein ingredients.

The impact of high-power sonication as a pretreatment on the extraction of proteins from soybean flakes, and flours from soybean, chickpea, and kidney bean, and changes in physicochemical properties were evaluated. The substrates were dispersed in distilled water (1:10 w/v) and sonicated at two power densities (PD) of 2.5 and 4.5 W/mL for 5 min continuously. Proteins were extracted at pH range 8–8.5. PD 2.5 and 4.5 W/mL significantly increased protein extraction yields from soy flakes to 29.03% and 25.87%, respectively, compared to 15.28% for unsonicated, but did not increase for flours. Free sulfhydryl content for both sonicated and unsonicated soy flakes and flour were similar but increased in proteins from chickpea and kidney bean when HPS of 4.5 W/mL was applied, indicating the unfolding of protein structure. The protein band patterns for sonicated and unsonicated legumes proteins were found to be similar, indicating no peptide profile alterations by HPS. However, circular dichroism analysis showed

changes in secondary structure composition in extracted kidney bean protein causing unfolding and destabilizing the native structure, but it remained unaffected for soy flakes and flour protein and chickpea protein.

Legumes are rich sources of protein, carbohydrates, dietary fibers, and minerals, but their utilization has been limited because of several anti-nutritional factors (ANFs) and lower protein digestibility. To reduce the ANFs, physical modification of the substrates along with subsequent fermentation by *L. plantarum* and *P. acidilactici* were evaluated in the second study.

ANFs like phytic acid, tannins, and enzyme inhibitors impact the availability of nutritional compounds and can be reduced or modified with physical/ biochemical processes, for example, extrusion, sonication, and fermentation. In this study, the effect of a combination of physical treatments (sonication/extrusion) and fermentation on some legume ANFs was evaluated. Flours of soybean, lentil, and green peas were sonicated for 2 and 4 min (power density ~ 2.5 W/mL) at a 1:8 (w/v) ratio (substrate: water) and fermented. Physically modified flours were fermented with probiotic bacteria namely *Lactobacillus plantarum* and *Pediococcus acidilactici* in shake flasks for 72 h at 37°C, and 200 rpm. All the substrates, modified and unmodified, effectively supported microbial growth which reached a peak of around 10^{13} CFU/mL at 24 h. Trypsin inhibitor activity (TIA) was reduced significantly for all the substrates except for unsonicated soybean, and lentil fermented with both microbes. TIA decreased when physical processing was done. Phytic acid content decreased notably for physically modified soybean and lentil but not for green pea. Total phenolic contents were significantly ($p<0.05$) reduced for all physically modified and fermented substrates compared to non-fermented controls. Even though there was a decrease in ANFs, there was no significant change in in vitro protein digestibility.

CHAPTER 1: GENERAL INTRODUCTION

Plant-based ingredients are widely used as its healthier and cheaper sources of nutrients like proteins, carbohydrates, fibers, and minerals. Pulses/ legumes such as dry beans, peas, soybean, and lentils are major sources of these plant-based ingredients. They are of growing interest in the food and feed industry as they have high protein contents and other nutritional components than those of animal origin (Adebo et al., 2017). Extraction, isolation, purification, and improvement of nutritional quality of proteins are the major areas of research on utilization as ingredients. Conventionally, extraction of protein is done by pH modulation at the range of 8.5-9.0, which results in the recovery of about half of the available proteins (Karki et al., 2010). The presence of cell wall polysaccharide and the location of desired proteins inside the cell-matrix are the limiting factors in extraction. Also, several other extraction methods like acid extraction, air classification, and salt extraction provide its advantages and disadvantages. To improve the extraction, pretreatments like microwave heating, enzymatic modification, and chemical modification have also been employed (Jung et al., 2006; Sari et al., 2013). These pretreatment results in an alteration in structure of the extracted protein, leading to changes in functional properties that play a vital role in food formulation and processing.

The consumption of legumes or pulses provides energy, dietary fibers, proteins, and minerals that benefit human health including moderating diabetes, reduced risk of cardiovascular disease, cancer, hypertension, and, reduction of low-density lipoprotein (LDL) cholesterol (Tharanathan & Mahadevamma, 2003). Such studies have contributed to raising awareness of the importance of including pulses and pulses derived foods in the diets. Even with the presence of several nutritional factors, the use of pulse in the food industry has been limited due to the presence

of off-flavors and several anti-nutritional factors (ANFs), for example, tannins, trypsin inhibitors, phytic acid, and flatulence causing compounds (stachyose, raffinose, and verbascose). An increase in the use of whole pulse, flour, protein, starch, and bioactive compounds for many food applications has driven the research on pulse-based processing technologies.

Apart from conventional methods, physical modification or pretreatments like high power sonication (HPS) and extrusion can be used as a potential alternative method for the extraction of proteins as well as modification. The combination of physical pretreatment and fermentation with probiotic bacteria will change the molecular structure and functions, reduce or eliminate anti-nutritional factors, and, hence improve the quality of protein ingredient (Kiers et al., 2000; Ojokoh et al., 2011). Physical processing using thermal treatment can improve reduce ANFs, while HPS disrupts plant matrices and facilitates the extraction of protein, carbohydrates, and, other bioactive compounds using cavitation phenomenon (Vilkhu et al., 2008). HPS can release carbohydrates and sugars which can be utilized in fermentation to produce hydrolytic enzymes and thus, modify substrates. Fermentation has been utilized as a low-cost processing method to enrich the substrates with essential amino acids, bioactive compounds and reducing anti-nutritional factors (Liu et al., 2011; Khattab et al.,2009). Even though there are several other modifications, fermentation is significant as it can provide improved sensory quality, reduce pathogenic microorganisms, and enhance functionality as well as nutritional properties (Adebo et al., 2017).

1.1 Research questions and hypotheses

The high-power sonication induced cavitation disrupts cell matrix which led to our first research question on how important sonication parameters (power density and time) affect the extraction yield of some legume proteins. Secondly, how HPS impacts the molecular structure of an extracted protein and physicochemical properties of protein ingredients.

Physical modification (sonication and extrusion) affects plant substrate, and fermentation produces several hydrolytic enzymes, thus, the third research question, whether fermentation of modified legume-protein ingredient increases the nutritional profile.

Our hypothesis is that high-power sonication affects the integrity of plant cell matrix resulting in improved protein extraction and alters the molecular structure of resulting proteins. Another hypothesis is physical modification of the cell-matrix supports microbial fermentation, which results in a decrease in major anti-nutritional factors.

1.2 Dissertation organization

This dissertation is organized into five different chapters. The first chapter is a general introduction which includes objectives and hypothesis for further research. The second chapter is the literature review. Chapters 3, and 4 are presented as manuscripts for publication in various international journals. The third chapter has been published in the *International Journal of Biological Macromolecules* in February 2020 and is focused on the effects of high-power sonication and its effect on extraction and some physicochemical properties of plant-based proteins. The fourth chapter will be submitted in a peer-review journal. General conclusions for this dissertation are presented in chapter five.

1.3 References

Adebo, O. A., Njobeh, P. B., Adebiyi, J. A., Gbashi, S., Phoku, J. Z., & Kayitesi, E. (2017). Fermented pulse-based food products in developing nations as functional foods and ingredients. Johannesburg, Gauteng, South Africa. https://doi.org/10.5772/intechopen.69170

Jung, S., Lamsal, B., Stepien, V., Johnson, L., & Murphy, P. A. (2006). Functionality of soy protein produced by enzyme-assisted extraction. *Journal of the American Oil Chemists' Society*, *83*(1), 71–78

Karki, B., Lamsal, B. P., Jung, S., van Leeuwen, J. (Hans), Pometto, A. L., Grewell, D., & Khanal, S. K. (2010). Enhancing protein and sugar release from defatted soy flakes using ultrasound technology. *Journal of Food Engineering*, *96*(2), 270–278. https://doi.org/10.1016/J.Jfoodeng.2009.07.023

Khattab, R. Y., & Arntfield, S. D. (2009). Nutritional quality of legume seeds as affected by some physical treatments 2.Antinutritional factors. *LWT - Food Science and Technology*, *42*(6), 1113–1118. https://doi.org/10.1016/j.lwt.2009.02.004

Kiers, J. L., Van Laeken, A. E. A., Rombouts, F. M., & Nout, M. J. R. (2000). In vitro digestibility of Bacillus fermented soya bean. *International Journal of Food Microbiology*, *60*, 163–169.

Liu, S., Han, Y., & Zhou, Z. (2011). Lactic acid bacteria in traditional fermented Chinese foods. *Food Research International*, *44*(3), 643–651. https://doi.org/10.1016/j.foodres.2010.12.034

Ojokoh, A. O., & Yimin, W. (2011). Effect of fermentation on chemical composition and nutritional quality of extruded and fermented soya products. *International Journal of Food Engineering*, *7*(4), 1–16. https://doi.org/10.2202/1556-3758.1857

Sari, Y. W., Bruins, M. E., & Sanders, J. P. M. (2013). Enzyme assisted protein extraction from rapeseed, soybean, and microalgae meals. *Industrial Crops and Products*, *43*, 78–83. https://doi.org/10.1016/j.indcrop.2012.07.014

Tharanathan, R. N., & Mahadevamma, S. (2003). Grain legumes - A boon to human nutrition. *Trends in Food Science and Technology*, *14*(12), 507–518. https://doi.org/10.1016/j.tifs.2003.07.002

Vilkhu, K., Mawson, R., Simons, L., & Bates, D. (2008). Applications and opportunities for ultrasound assisted extraction in the food industry-A review. *Innovative Food Science and Emerging Technologies*, *9*, 161–169. https://doi.org/10.1016/j.ifset.2007.04.014

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

According to the Food and Agricultural Organization of United Nations pulses are defined as “Leguminosae crops harvested exclusively for their grains including dry beans, peas, and lentils”. This definition excludes legumes that are specially used for its oil extraction like soybean, peanuts or those that are harvested green for food like green peas and green beans. About 73 million metric tons (MMT) of pulses are produced globally with 52 MMT accounting for dry beans, chickpea, lentils, and dry pea (Patterson et al., 2017). Pulses are important crops that have a balanced nutritional composition, inexpensive and are easily available sources of carbohydrates, proteins, dietary fibers, vitamins, and minerals (Adebo et al., 2017). These cheaper pulse proteins can be substituted for animal proteins and other essential nutritional components.

Pulses are a rich source of carbohydrates but provide low energy density of around 1.3 kcal/g and are slowly digested, thereby keeping them on the lower glycemic index (GI) category. They also contain dietary fibers that are mostly insoluble. Pulse proteins are also high in lysine, aspartic acid, leucine, glutamic acid, and arginine but have a shortage of methionine, cysteine, and tryptophan (Boye et al., 2010). In addition, pulses are an excellent source of micronutrients. The consumption of pulses provides energy, dietary fibers, proteins, and minerals which benefits human health including management of diabetes, reduced risk of cardiovascular disease, cancer, hypertension, reduction of LDL cholesterol (Tharanathan & Mahadevamma, 2003). Such studies have contributed to raising awareness of the usefulness of including pulses and pulses derived foods in the diets.

Despite the health benefits, the consumption of pulses is still not prevalent globally due to the presence of several ANFs, for example, tannins, trypsin inhibitors, phytic acid, off-flavors, and flatulence causing compounds (stachyose, raffinose, and verbascose). Phytic acid binds to essential

dietary minerals, protein, and starch which reduces bioavailability in humans. Tannins and trypsin inhibitors strongly inhibit the digestive enzymes reducing the digestion and absorption of dietary proteins and carbohydrates (Khattab & Arntfield, 2009). Several physical and biochemical processing of pulses may favorably modify some of the physicochemical characters of ingredients resulting in a higher nutritional profile.

Plant-based protein ingredients have been increasingly utilized in various food applications due to their high protein, carbohydrates, fibers, and mineral contents. These products have been made more palatable with advanced technologies that have altered the plant products into desirable foods. Due to the nutritional health benefits of plant-based ingredients in the formulation of products, it has been one of the booming markets recently in many developed countries. Pulses have been utilized as a meat analog for animal-free diet, used in meat products to increase protein content, baked products, pasta, noodles, sports beverages, and high protein bars. A summary of the applications of pulses as emerging food/ingredient is provided below.

2.2 Applications of pulse-based protein ingredients

2.2.1 Meat products and analog

Ingredients derived from plant sources have been used as fillers, extenders, binders, and emulsifiers. The principle protein functionalities utilized are gelation, emulsification, water holding capacity (WHC), and oil absorption capacity (OAC). Pulse protein concentrates can be used as an ingredient in processed meats to improve the protein contents as well as textural properties. Ghribi et al. (2018) used three different concentrations of chickpea protein concentrates (CPC), at 1.5, 2.5, and 5% w/w, as an ingredient to produce “Merguez” sausage to enhance the sensory profile. The addition of CPC to sausages increased the protein contents, textural properties, and global acceptability, while also decreasing the cooking loss without changing the taste.

Similarly, Thushan Sanjeewa et al. (2010) incorporated the chickpea flour to produce low-fat pork bologna. Chickpea flour (2.5 and 4%) were used as non-meat ingredients to enhance texture and WHC while retaining taste and functionality and also helping to reduce high-fat contents. Chickpea has the potential to be used as an extender in meat products. Impossible^{TM} burger and pork were created using plant-based proteins as an ingredient to make the meat-free products (Impossible Foods., 2020). Texturized vegetable products have also been used as meat analogs which provide high protein and functional food ingredient (Joshi & Kumar, 2015).

2.2.2 Imitation milk

Imitation milk, plant-based milk or non-dairy milk is another booming application for plant-based proteins as an alternative to dairy produced milk. As people nowadays are at high risk of lactose intolerance and preference for vegetarian and vegan diets, an alternative to cow milk is flourishing, but several issues like nutritional balance, preservation, and sensory acceptability must be resolved for ideal imitation milk. One of the most popular plant-based beverage products is soymilk, which is a good source of essential mono and poly-saturated fatty acids, isoflavones, proteins, and carbohydrates (Sethi et al., 2016). Boye et al. (2010) reported that using lentil protein isolates produced intermediate quality milk, while pea protein produced poorer quality milk compared to soymilk.

2.2.3 Baked goods

Bakery products are another potential application of the pulses. With their low amounts of sulfur-containing amino acids like cysteine and methionine, pulses can be mixed with wheat flours to compensate for those essential amino acids (Boye et al., 2010). The functional property, like WHC and foaming, plays an essential role in baked goods such as bread, cake, and confectionery. Millar et al. (2017) mixed broad bean, green pea, and yellow pea flours and mixed it with 40%

wheat flour to produce a cracker. In sensory evaluations, panelists preferred the yellow pea and broad bean crackers. Similarly, bread was produced using fermented split yellow pea flour as a partial replacement to wheat flour (Bourré et al., 2019). Three ferment inclusion (25, 35, and 45%) were used which improved the flavor of bread by masking distinctive pea flavor. Simons & Hall (2018) germinated, cooked, and steam blanched pinto bean flours to make gluten-free cookies and their consumer acceptability were analyzed. The overall acceptability score was 6.0 out of 9.0 scale which was considered good.

2.2.4 Beverages

Pulse proteins have also been utilized in beverages. A product like Ripple and Dream utilized yellow pea protein along with other ingredients; customers were reportedly pleased with the much creamier texture and mouthfeel (Nutrilicious, 2020). InovoPro Company has produced a chickpea protein concentrate called CP-pro 70 ® (protein content-70%) which is used in non-dairy milk beverages having rich mouthfeel due to the protein concentrate's higher water and fat-binding capacity. It also provides a clean label, GMO-free, and dairy-free products (InnovoPro, 2017).

2.2.5 Nutrition bar

High protein bars are popular for portability as well as health implications. As people are concerned more and more about wellness, consumers are looking for minimally processed, and clean label products. Protein from pulses can be utilized to produce high protein nutrition bars. Functional properties like gelation, solubility, and browning should be considered to develop nutrition bars. Such properties were applied by companies like InnovoPro which developed high protein bars made from chickpea using CP-pro 70 ® combined with dates and coconut. The resulting bar is gluten-free and allergen-free with reportedly excellent taste (InnovoPro, 2017).

2.3 Composition, molecular characteristics and nutritional quality of pulse proteins

Chickpea, kidney bean, faba bean, green lentils, and yellow pea contain 20-32% proteins and around 60-70% carbohydrates. Pulse proteins are comprised of albumin and globulin as major fractions, accounting for about 10-20% and 70%, respectively, of total pulse proteins (Hall et al., 2017). Prolamin and glutelin are present in minor compositions. Albumins compose of enzymatic and structural proteins, protease inhibitors, and lectins and have a molecular mass ranging from 5-80 kDa. Albumin also has high methionine and cysteine than globulin (Boye et al., 2010). The storage protein globulin consists of legumin and vicilin in pulse seeds, which are as 11S-12S and 7S globulins, respectively, based on their sedimentation coefficient. Vicilin and legumin from pulses have oligomeric structures: legumin has hexameric structure with a molecular weight of 300-400 kDa and vicilin has trimeric structure with a molecular weight at 145-190 kDa (Boye et al., 2010; Shevkani et al., 2019).

Each legumin subunit consists of an acidic subunit (MW- 40 kDa) and a basic subunit (MW 20 kDa), which are bonded together by a disulfide bond (-S-S-). Acidic subunits are located on the surface of protein while basic subunits are present inside the hydrophobic core. Pulses like chickpea have legumin as its major globulin protein (Shevkani et al., 2019). Similarly, each vicilin structure consists of trimer that can be identical or non-identical with MW 50-70 kDa (Shevkani et al., 2019).

Vicilin is identical in structure in various pulses but varies in molecular weight and composition. Vicilin molecular weight of 136-150 kDa, 145-190 kDa, 150-250 kDa have been reported for kidney bean, chickpea, and soybean, respectively. Vicilin lacks cysteine residue and its structure is stabilized by non-covalent hydrophobic interaction, not by disulfide association. Pulses like a kidney bean, red bean, and mung bean have vicilin as its major globulin protein

accounting for around 88% of globulin (Tang & Ma, 2009; Meng & Ma, 2002). A storage protein that is different from legumin and vicilin was reported in pea called convicilin with a molecular mass of 71 kDa. The molecular conformation of 11S globulin is presented in Figure 1 (Adachi et al., 2003).

Soybean protein contains a mixture of proteins of 2S, 7S, 11S, and 15S proteins depending upon the sedimentation coefficient. The 7S is called β-conglycinin (vicilin) while 11S is known as glycinin (legumin) and is the major protein covering 80% of total proteins. β-conglycinin (MW-180 kDa) is trimeric in structure and consists of three subunits α (MW-67 kDa), α'(MW-71kDa), and β (MW-50 kDa) Glycinin is hexameric in the structure and comprised of an acidic (MW-35 kDa) and basic (MW-20 kDa) subunits that are linked together by disulfide (Nishinari et al., 2014). These structures are mainly stabilized by disulfide bonds along with hydrophobic interaction and, hydrogen bonding.

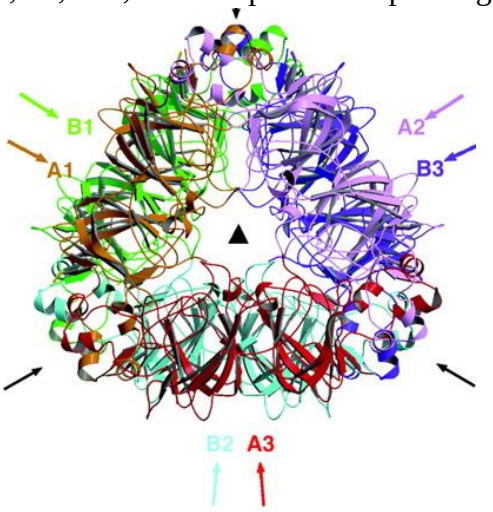


Figure 1 Molecular conformation of 11S globulin (Adachi et al., 2003)

Chickpea protein consists of 8-12% of albumin, 53-60% globulin, 19-24% glutelin and 3-6% prolamine (Dhawan et al., 1991). Similarly, kidney bean protein consists of 15% albumin and 60% globulin (Pusztai et al., 1979). On the other hand, faba bean protein consists of 1% albumin, 74% globulin, 18% glutelin, and 3% prolamine (El Fiel et al., 2003). Amino acid profile and protein digestibility are the major factors to determine the nutritional quality. Pulses, in general, are high in lysine, aspartic acid, leucine, glutamic acid, and arginine but lack sulfur-containing amino acids like methionine, cysteine, and tryptophan. Han et al (2007) have reported the protein digestibility of chickpea, yellow pea, lentils, green pea, and soybean. Yellow pea and green pea

showed the highest protein digestibility i.e. 82%, followed by lentil (79%), chickpea (74.3%) and lowest for soybeans (71.8%) (Singhal et al., 2016). With the knowledge of composition, the molecular structure of a protein, it would be easier to determine the changes that can be brought about by various modifications and hence leading to the changes in functionality as well as nutritional characteristics.

2.4 Functional properties of pulse proteins

Functional properties determine protein application in foods. The functional properties are influenced by size, shape, amino acid composition, hydrophobicity, hydrophilicity, structures of proteins (primary, secondary, tertiary, quaternary). The functionality of the proteins is highly affected by the molecular structure that regulates inter and intramolecular interactions (Boye et al., 2010). Properties such as solubility, water holding capacity (WHC), fat binding, gelation, foaming, and emulsification are employed for food applications. Some of the functional properties are discussed below.

2.4.1 Solubility

Pulse proteins are highly soluble at low pH and high alkaline pH while they are least soluble at isoelectric point (pH 4-6). In addition to pH, surface charge and salt concentration also affect the solubility of proteins. Protein obtained from pea, cowpea, and kidney bean follows a typical V-shaped protein solubility curve (Shevkani et al., 2019). Globulins are insoluble in water at pH range 4-5 but are soluble in dilute salt solutions (Barać et al., 2015). Functional properties such as gelation, emulsification, and foaming are dependent upon the solubility of the protein.

2.4.2 Emulsification

An emulsion is the dispersion of two immiscible liquids in which one type of liquid is dispersed in globules within another continuous phase liquid. Proteins are amphiphilic in nature and can be utilized as emulsifiers, maintaining emulsions by decreasing interfacial tensions.

Emulsifying properties of pulse proteins are measure by emulsion activity index and emulsion stability index (Shevkani et al., 2019). These properties vary for pulse proteins. Wani et al. (2015) reported the emulsifying properties of native and hydrolyzed kidney bean protein isolates (KPI) at pH 3, 5, and 7. The emulsifying activity and stability were higher for proteolyzed KPI at pH 7 compared to native proteins. Kimura et al. (2008) compared the emulsion properties of vicilin and legumin proteins from pea, cowpea, fava bean, and French bean and reported that vicilin has better emulsifying properties than legumin.

2.4.3 Water binding capacity (WBC)

Water binding capacity also called water absorption capacity or water holding capacity is the amount of water that can be retained per gram of protein materials. WBC of pulse proteins is due to the affinity of polar and charged side chains present in proteins to water molecules. Shevkani et al. (2019) reported the WBC of cowpea, kidney bean, and field pea of 2.1 g/g, 1.6-3.6 g/g, and 4.2 g/g respectively. Batista et al. (2010) also reported the WBC of common beans as 3.80 g/g.

2.4.4 Foaming

Foaming properties are required for foods such as cakes, ice-creams, fudges, and confectionery products. The foaming properties of pulse proteins depend upon the molecular weight, solubility, small net charge, surface hydrophobicity, and susceptibility to denaturation (Barać et al., 2015). They are categorized as foaming capacity and foaming stability. Aluko et al. (2009) reported that pea protein isolates (PPI) at pH 3 and 7 were better foaming agent than soy protein isolates (SPI-90% protein). The foaming capacity for hydrolyzed kidney bean was higher than native proteins (Wani et al., 2015).

2.4.5 Gelling properties

Gelling properties are important for foods such as puddings, desserts, and jellies. The ideal gelation is the least concentration required protein to form a self-supporting gel. The gelation capacity of albumin and globulin proteins in kidney bean was researched. Globulin has a higher gel formation capacity (least gel concentration- 6%) when compared to albumin (16%) (Mundi et al., 2012). This reduced gelation property for albumin might be due to less hydrophobic clusters, thus, limiting protein-protein interactions.

2.5 Extraction/ purification of pulse proteins

Several extraction methods have been established to extract the substantial proteins from the pulse flour/flakes for applications in the food industry as mentioned previously. Some of the established methods are discussed below.

2.5.1 Air classification

Air classification is a milling process that separates grains or seeds into high protein or high starch content flours. Two distinct sizes and density are prepared during the milling process that helps in the characterization of proteins. The lighter fraction is protein while heavy and coarse fraction is starch. The dehulled seeds are pin milled into flours and then the spiral air stream is used multiple times to separate proteins from starch (Boye et al., 2010). To obtain the optimal or higher protein, pulses are ground finely just enough to break the cells but without damaging the starch granules (Klupšaitė et al., 2015). The major limitation of air classification is its inability to produce high purity proteins; fractions generally contain 38-65% protein, which is lower than what can be obtained from alkaline extraction.

2.5.2 Alkaline extraction/Isoelectric precipitation

Alkaline extraction followed by isoelectric precipitation is a process that exploits the solubility of proteins and is usually done at higher pH (8-11) and then at pH values closer to isoelectric points (Fig. 2). The flour (with or without hulls) is dissolved in water at a ratio that ranges from 1:5-1:20 (w/v). Mixture pH is adjusted to 8-11 depending upon the substrate, allowed to solubilize for 30-180 min at 55-65ºC and then centrifuged. The supernatants are allowed to precipitate at pH 4.5 (isoelectric point) and centrifuged to recover protein. The precipitate is then washed, neutralized, and spray dried to obtain protein isolates (Boye et al., 2010). Karaca et al (2011) applied alkaline extraction to recover protein from chickpea, faba bean, lentil, and pea and obtained a product with a protein content of 85.4, 84.1, 81.9, and 88.76%, respectively. The major limitation of this method is its extraction yield as recovery can be just half of the available protein; the location of protein inside the plant matrix also plays a limiting role in the effective recovery of proteins (Karki et al., 2010).

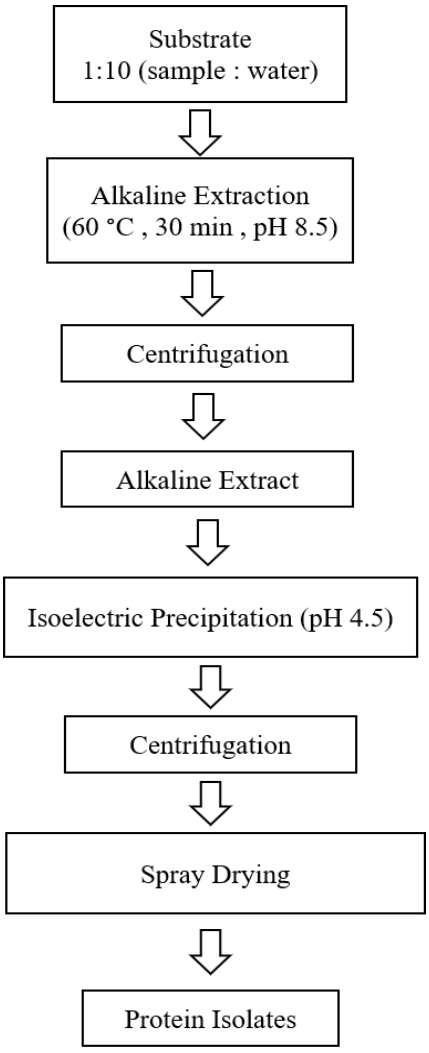


Figure 2 Alkaline extraction/Isoelectric precipitation of pulse protein

2.5.3 Acid extraction

Acid extraction works on the same principle as alkaline extraction except that its initial step is done under acidic conditions. Pulse proteins are also highly soluble under acidic conditions (pH<4) which is exploited to carry protein extraction and then further precipitate it, using

isoelectric precipitation or other filtration methods. Acid extraction and subsequent precipitation have obtained products from faba bean, kidney bean, pea, and lima bean with protein contents of 91.2, 95.7, 91.9, and 50% respectively (Boye et al., 2010; Klupšaitė et al., 2015).

2.5.4 Saline extraction

Salt extraction or micellization uses the salting-in and salting-out process to extract the proteins. The proteins are extracted using appropriate salt solutions at desired ionic strength. The solution is diluted and precipitation was induced and recovered by centrifugation or ultrafiltration technique. Stone et al., (2015) extracted pea protein using micellization with 1 N NaCl at 1:10 (w/v) ratio and obtained products with a protein content of 82% to 88% for three cultivars. Similarly, Karaca et al., (2011) extracted chickpea, faba bean, lentil, and pea and obtained products with 81.6, 82, 74.7, and 81% protein contents, respectively.

Several processing technologies are constantly focusing on improving the nutritional, sensory profile, and functionality to make products more acceptable to the consumers. The advanced technologies can be used as a pretreatment to enhance the extraction of proteins with better and desirable functional properties to expand their applications in the food industry. Some of the pretreatment that can be utilized are discussed below:

2.6 Physical modification

Any modification of products imparted by physical treatments is known as physical modification and divided into thermal and non-thermal treatments. Thermal treatments involve heat-moisture treatments, microwave heating, extrusion, and infrared heating while non-thermal treatment includes high-pressure processing, pulsed electric field, freezing, and sonication. We will be focusing on how physical processing is exploited to enhance the functionality, nutrition, and sensory profile of the food products.

2.6.1 Ultrasonication

Ultrasonication is an emerging processing technique in food industries as it is green chemistry, generally recognized as safe (GRAS), and ecofriendly. Ultrasound technology is based on the mechanical waves at a frequency that is above that of normal human hearing (>20 kHz). For classifying the ultrasound technology, power (W), sound intensity (W/m^2), and sound energy density (W/m^3) are the important parameters. Based on these factors ultrasonication can be either low energy or high energy sonication.

Low energy sonication uses any frequency in ranges above 100 kHz and intensities below 1 W/m^2. They are non-destructive and are used in medical imaging and diagnostic ultrasound. They are also used in quality control measures of some food systems like fruits, vegetables, meat, cereal products, fat and emulsion products, honey, food gels, and food proteins (Awad et al., 2012). Ultrasound provides a foundation for a non-destructive, fast and reliable technique for relating the quality of fruits and vegetables with various stages of development during growth, maturation, and storage (Awad et al., 2012). Bread making processes are characterized by empirical or rheological techniques. Ultrasound has been applied in the mixing of three different flour dough systems at various stages and a strong correlation between ultrasound conditions and rheology was noted which indicated the possibilities of using ultrasound for on-line dough quality control (Ross et al., 2004).

On the other hand, high energy sonication uses intensities higher than 1 W/m^2 (range of 10-1000 W/m^2) at frequencies between 18-100 kHz. High power sonication is relatively new and has not been fully explored in the food industry. However, high energy sonication has been explored in applications like extraction of bioactive compounds, proteins, sugars, filtration, homogenization, cutting, and degassing.

Mechanism of sonication

When sonication is applied to a liquid system it causes cavitation phenomenon which is generation, growth, and collapse of microbubbles. The sound waves propagate, the bubbles oscillate and eventually collapse which causes mechanical, thermal, and chemical effects on the medium. The effect of this cavitation phenomenon generates extremely high localized temperatures (5000 K) and high pressure (1000 atm) that produces high shear stress and turbulence in the cavitation zone (Fig. 3) (Sonotronic, 2020). Ultrasound also causes hydrolysis of water

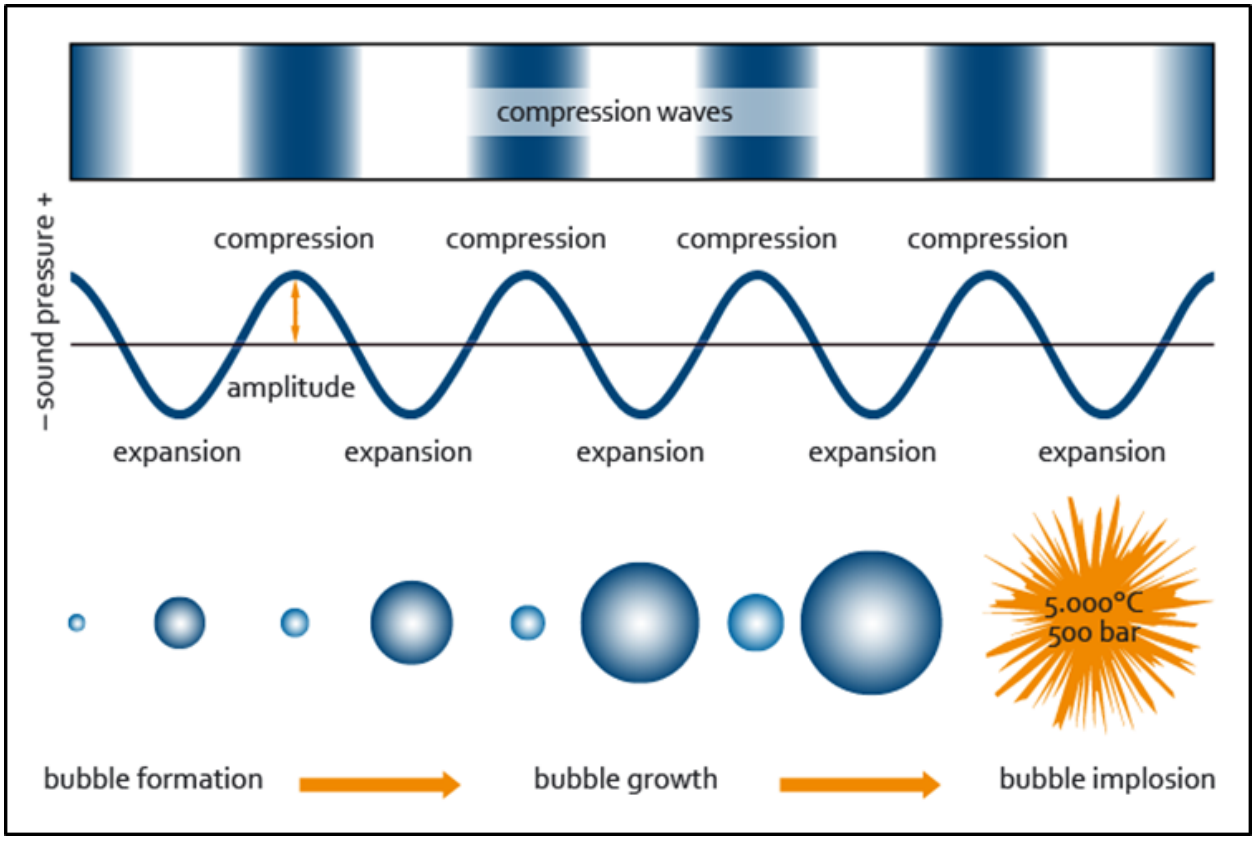


Figure 3 Mechanism of cavitation phenomenon induced during sonication (www.sonotronic.de)

leading to the formation of free radicals H^+ and OH^- and thus imparting chemical effects to the medium. These formations of OH^- radicals formed are highly reactive and can affect the quality of food.

2.6.1.1 Application of high-power ultrasound

High power sonication has been used not only for the extraction of bioactive compounds, proteins, carbohydrates, oils, and herbal extracts but also to modify the functional properties, which can be exploited for application in food industries. Some of the more widely used applications are as follows:

Extraction

High power sonication can be used as an inexpensive, reproducible, and efficient pretreatment method to improve the extraction of biological materials such as protein, carbohydrates, sugars, bioactive compounds (flavones, polyphenols), and oils (Vilkhu et al., 2008). Conventional methods of extraction of organic compounds from plants are based on a combination of solvents, heat, pH, and mixing. Ultrasonic assisted extraction (UAE) has been highly popular due to the increase in the extraction yield which can be attributed to the propagation of ultrasonic pressure and cavitation phenomenon. The energy that is released due to the cavitation disintegrates the cell, increase the hydration, and reduce particle size, thus facilitating the extraction.

There are several issues relating to UAE that need to be addressed for the successful extraction of desired compounds, i.e. nature of the tissue being extracted, and location of the components to be extracted, pretreatment of tissues before extraction. Living tissues where desired components are localized in surface glands can be stimulated to release the components by using ultrasonication thus, achieving complete or rapid extraction (Riera et al., 2004; Vinatoru, 2001). In using high power sonication as pretreatment, Karki et al. (2010) reported improved extraction yield of proteins (by 40-46%) from defatted soy flakes. Preece et al. (2017) used sonication conditions of 20 kHz and 400 W for 0, 0.5, 1, 5, and 15 min on soy slurry, and reported increased the extraction of protein, oil, and solids by 10% after 1 minute of sonication. Montalbo-Lomboy

et al. (2010) used 20 kHz frequency and amplitude of (192–320$_{\mu m}$ peak-to-peak) for a time interval of 5, 10, 15, 20 and 40 s on ground sugary-2 maize, which increased the sugar conversion rate by three-fold compared to unsonicated. The oil from chickpea was removed with the use of ultrasound-assisted extraction, oil extraction yield increased by 10.45% when sonicated for 20 min (230 W) (Lou et al., 2010). In addition to proteins, oils, sugars, isoflavones have also been extracted using sonication. Pananun et al. (2012) applied 20 kHz and varying amplitude (18-54$_{\mu m}$) for 1 and 3 min on soybean and reported 1.2-1.5 times more recovery of genistein compared to control. Table 1 provides some information on the utilization of ultrasonication on the extraction of various materials.

Table 1 Utilization of ultrasonication on extraction

Product	Ultrasound process	Remarks	Reference
Soy	20 kHz, 400 W for 0, 0.5, 1, 5 and 15 min	Increased extraction of protein, oil, solids by 10%	Preece et al., (2017)
Almond oil	20 kHz	Increased by 30% extraction yield	Riera et al., (2004)
Polyphenols (*Jatropa dioica, Flourensia cernua, Turnera diffusa, Eucalyptus camuldenesis*)	40 kHz, 40 min	Increased ability to extract polyphenols	Wong Paz et al.,(2015)
Carotenoids from guava pulp and waste powder	Ultrasonic bath (BUAE, 25°C for 30 min), and ultrasonic probe (PUAE, 25°C for 5 min	Efficient in extracting carotenoids, lycopene.	Silva Lima et al., (2019)
Herbal extracts (Fennel, hops, mint)	20-2400 kHz	Increased yield by 34%.	Vinatoru, (2001)

Emulsification

Emulsification is a simple process of dispersing one liquid in a second immiscible liquid. To produce an emulsion, an external source of energy is required which can be facilitated using high-power sonication. When the mixture of immiscible liquids is exposed to ultrasonic energy, the bubbles collapse near the surface of the phase boundary layers of two liquids, which produces extremely effective mixing. A great interest in ultrasonication assisted emulsion has been shown in the processing of food. Food products such as ketchup, mayonnaise, and fruit juices have been produced using ultrasonic-assisted emulsification (Wu et al., 2001; Patist and Bates, 2007).

Modification of functional properties of protein due to sonication

The free radicals and superoxide that are produced during the sonolysis of water can be used to induce crosslinking of protein molecules in an aqueous medium. High-intensity ultrasound has a wide variety of application in food-related industries which can alter the physicochemical properties and/or structural as well as functional properties on plant proteins like soybean protein (Jambrak et al., 2009; Karki et al., 2009; O'Sullivan., 2016) black bean protein (Jiang et al., 2014), and pea protein isolates (Xiong et al., 2018). Jambrak et al. (2009) reported the increase in solubility, foaming, and emulsion properties of soy protein isolate when treated with 20 kHz for 30 mins. This increase was attributed to cavitation that unfolds/breaks the protein, denaturation, decrease in droplet size changing the protein conformation and structure. Similarly, the solubility of SPI was increased by 34% at pH 7, and emulsification and foaming capacities decreased by 12 and 26%, respectively, when, sonication was exposed to 120 s at power density 2.56 W/mL (Karki et al., 2009).

Jiang et al. (2014) reported increased solubility and hydrophobicity of black bean protein isolates obtained from sonicated samples. The increase in surface hydrophobicity was due to the molecular unfolding of proteins, which exposed more of the hydrophobic groups that are inside the molecule. The effect of high-intensity sonication was evaluated on the structure and foaming properties of pea protein isolates (Xiong et al., 2018). The amplitude was varied to 30, 60, and 90% for 30 min which decreased the particle size and increased the hydrophobicity. Foaming ability increased from 145.6% to 200% and foaming stability increased from 58% to 73.3% with the increment of amplitude. Exposure to sonication has the potential benefit of modifying the foaming properties. Ultrasonication can be one of the potential processing tools to develop modified and new products with unique functionality.

Nazari et al. (2017) evaluated the effects of ultrasound treatments on functional and structural properties of millet protein concentrate. Solubility increased when sonication was done, while foaming capacity decreased when low intensity (18.4 W/cm^2) was used but increased when the intensity was higher. Poor solubility is a limiting factor for rice protein used in food industries. Zhang et al. (2017) used sonication-assisted alkali treatment in rice protein and solubility increased by 230-fold compared to untreated. The functional properties like solubility, foaming, emulsifying properties, WHC, oil holding capacity of tamarind seed protein isolates increased with intensity and time of the sonication (Biswas and Sit, 2020).

Change in molecular and structural properties

Ultrasonication modifies the functionality of protein by altering the physicochemical and molecular structure. The configuration of protein structure provides detailed information on how the functionality of protein might be altered. The change in the molecular structure of proteins is important because of its adverse effect on functionality like WHC, solubility, foaming,

emulsification, and hydrophobicity. The molecular structure has been categorized based on the primary, secondary, tertiary, and quaternary structure of proteins. Their effect of sonication in the primary structure of sonicated plant-based proteins can be accessed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). At the low-intensity native structure of protein undergoes unfolding and aggregation resulting in partial or complete unfolding which ultimately affects the secondary and tertiary structure but not able to alter the primary structure (Fig. 4) (Feng et al., 2013). It is difficult but not impossible to change the primary structure of a protein using high power sonication and extended sonication (Feng et al., 2013). For, example soy glycinin was sonicated at 60% power for 0, 5, 20, or 40 min. The protein bands were not significantly different for sonicated and control glycinin for both reducing and non-reducing SDS-PAGE.

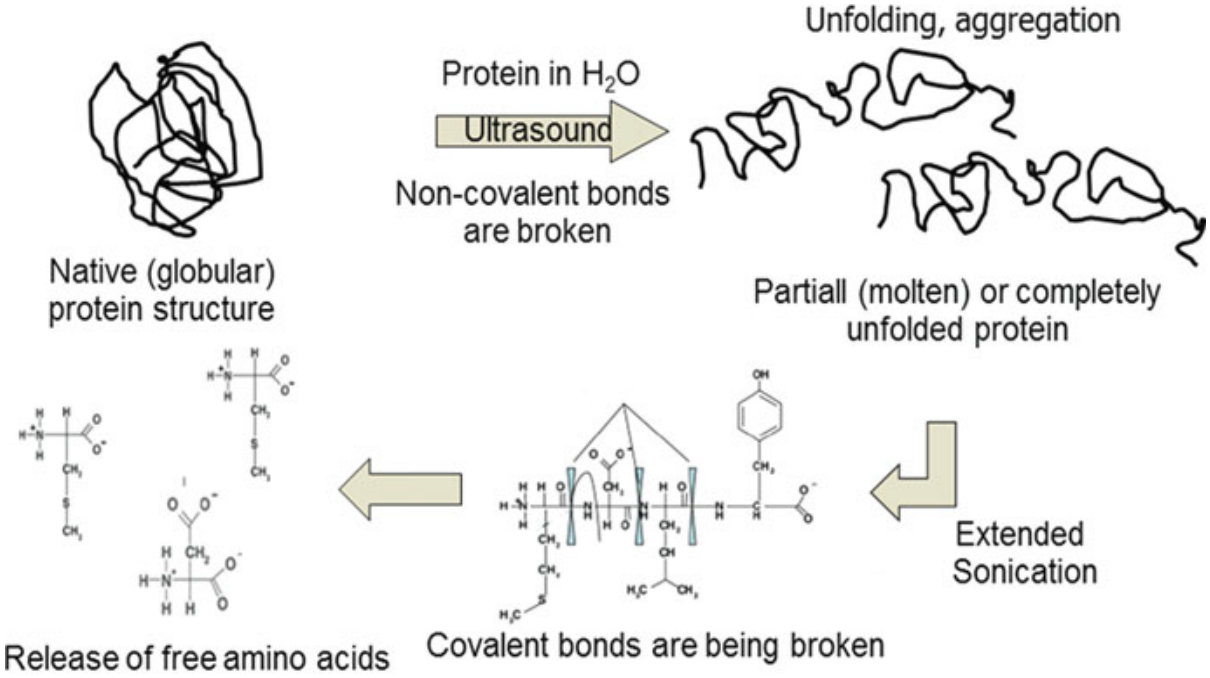


Figure 4 Impact of sonication on protein structure (Feng et al., 2013)

On the other hand, circular dichroism revealed that 40 min. sonication increased β-sheet and reduced β-turns in the secondary structure (Zhou et al., 2016). Similarly, sonication of rice protein decreased β-sheet and random coils but increased β-turns and α-sheet (Zhang et al., 2018). Millet protein concentrate (MPC) was sonicated with a high intensity of 73.95 W/cm^2 for 12.5 min,

and changes were observed in 40 kDa and 50 kDa bonds. Zeta potential (ζ) of amino acids present in sonicated MPC was higher than the control as a result of contact of charge residue in the surface protein to solvent molecules (Nazari et al., 2018). O'Sullivan et al. (2016) reported a similar case for vegetable proteins like soy protein isolate (SPI), pea protein isolate (PPI), and rice protein isolate (RPI) where no effect was seen in the sonicated sample and their respective controls. Jiang et al. (2014) also reported that sonication did not cause any major changes in protein profiles in black bean protein isolates (BBPI); however, CD spectroscopy of the secondary structure of native and sonicated BBPI at 300 W and 24 min indicated a reduction of the α-helix and increase of the β-sheet structure. Also, the zeta potential of sonicated BBPI was negative in charge. Tamarind seed protein isolates were also subjected to sonication and there was no effect on the primary structure of proteins (Biswas and Sit, 2020).

2.6.2 Extrusion

Extrusion has become one of the primary technologies for the processing of food products. It can be used as a pretreatment to physically modify the pulse-based ingredients to enhance the extraction, functionality, and nutritional profile. Extrusion is generally described as a process by which substrates are forced to flow through die under varying conditions. The food materials are released using extreme pressure during which the product becomes puffed into various shapes and dries out (Boye et al., 2010). Cereals, snack foods, crackers, and wafers are examples of popular extruded products. Extrusion cooking employs high temperature, short duration of time, moisture, pressure, and mechanical shear. The technology can be applied to convert agricultural commodities into a versatile range of products and modern foods that are fully cooked, shelf-stable with enhanced economic values and have qualities like texture, flavor, and desired shapes (Morales et al., 2015). Several combinations of an extruder, screw speed, and screw configuration have been

utilized by other researchers who reported several positive as well as negative effects on the nutritional attributes. With the increase in applications, there have been interests in the effect of extrusion conditions on the physicochemical, functional, and nutritionally properties. Pulses have been extruded to decrease cooking time and improve textural properties, nutritional profile, as well as sensory characteristics, which then facilitate the production of materials having high nutritional and economical value (Berrios, 2011). Also, the reduction of nutrient destruction and improvement in starch and protein digestibility are important benefits of extrusion. Furthermore, we will discuss the effect of extrusion on protein, carbohydrates, and ANFs.

2.6.2.1 Modification of functional properties due to extrusion

Alonso et al. (2000) extruded pea and kidney bean proteins with a moisture content of 250 g/kg, 100 rev/min at 156ºC outlet die temperature and addressed functional properties like protein solubility, WHC, and oil absorption capacity (OHC). Extrusion increased the WHC and water solubility of pea and kidney bean but decreased the OAC. The high temperature causes denaturation of protein, decreases in hydrophobicity and blocks possible hydrophobic sites reacting with the oil (Alonso et al., 2000). Batista et al. (2010) extruded hard-to-cook beans (pontal and grafite) and observed the decrease in emulsification properties which was reported due to alteration and aggregation of proteins. The extrusion also imparts severe changes in protein, carbohydrates, and anti-nutritional factors which are discussed below.

2.6.2.2 Nutritional changes due to extrusion

Protein and amino acids

Proteins are the complex molecules that are made up of combinations of amino acids. There are twenty-two different amino acids among which leucine, isoleucine, lysine, phenylalanine, methionine, threonine, valine, and tryptophan are designated as essential amino acids. The digestibility, availability, and quantity are the key factors that determine the nutritional value of protein. Extrusion cooking using twin-screw co-rotating extruder was done at 100ºC, 125ºC and 150ºC and moisture content were varied for each temperature at 15, 20 and 25 g/100 g for lentil and horsegram (Ghumman et al., 2016). Ghumman et al. (2016) reported the in vitro protein digestibility (IVPD) of lentil and horsegram as 83.3-87.8 g/100 g and 84-88.9 g/100 g, respectively. Extrusion at 150°C and moisture of 15 g/100 g resulted in the highest IVPD for lentil, i.e., 87.8 g/100 g. Nosworthy et al. (2018) utilized extrusion as one of the processing conditions to determine the in-vitro and in-vivo protein quality of *Phaseolus vulgaris* and *Vicia faba*. The IVPD was reported to be 79.42, 82.2, 79.51, 80.95, and 81.95% for extruded black bean, faba bean, navy bean, pinto bean, and red kidney bean, respectively. IVPD increased with extrusion temperature, likely due to protein denaturation and inactivation of enzyme inhibitors as well as several other anti-nutritional factors. Batista et al. (2010) also reported the improved IVPD for extruded carioca and black bean protein by 72.3% and 84.5%, respectively, which was attributed to the inactivation of trypsin inhibitors as well as denaturation of proteins. Extrusion of high protein content materials into appetizing foods is popular nowadays. With the use of a combination of several parameters in the extruders like die temperature, screw speed, and moisture, functional characteristics of protein can be enhanced (Tiwari & Jha, 2017).

Carbohydrates

Carbohydrates are a large group of organic compounds present in foods and living tissues that are used as an energy source and include starch and cellulose. They are categorized into simple monomeric sugars like glucose, fructose, and lactose and complex molecules like starch and fibers. During extrusion conditions, flatulence causing oligosaccharides like raffinose, stachyose are reduced. This reduction in oligosaccharides leads to an improvement in the nutritional quality of plant-based products. Morales et al. (2015) exploited extrusion cooking of fiber-rich lentil flours and observed the change in oligosaccharides. Raffinose and stachyose content increased while verbascose decreased when lentil flours were extruded. This increase in raffinose and stachyose contents during extrusion was possibly due to partial hydrolyzation of verbascose to raffinose and stachyose during the extreme temperature and pressure (Morales et al., 2015).

Anti-nutritional factors (ANFs)

ANFs are present in pulse seeds and prevent the absorption of nutrients inside the human body. ANFs reduces the nutritional profiles of the pulse by decreasing the bioavailability, digestibility of proteins, vitamins, and minerals. Some of the ANFs that are present naturally are phytic acid, trypsin inhibitors, phenolic compounds, tannins, and lectins. Extrusion is one of the excellent methods to reduce/eliminate such ANFs by time, temperature combination. Phytic acid (myo-inositol hexaphosphate) has six reactive phosphate groups that act as a strong chelator, forms complex reactions with several minerals like calcium, iron, zinc, and magnesium and reduces the mineral bioavailability (Shi et al., 2018). Tannins are ANFs that binds with proteins by multiple hydrogen bonding and are not easily digestible (Raes et al., 2014). Enzyme inhibitors are also considered as ANFs as they slow down or inhibit the catalytic actions of an enzyme. Enzyme inhibitors like trypsin inhibitors and α-amylase inhibitors are present in legumes and pulses; their

action leads to impaired growth, problems with protein digestion and metabolic interference in the utilization of amino acids. The sulfur-containing amino acids like methionine and cysteine are not utilized properly as these inhibitors restrict the catalytic activity, slow the digestibility and are excreted readily (Adeyemo & Onilude, 2013). Table 2 summarizes the effect of extrusion on the different types of ANFs in pulses.

Table 2 Effect of extrusion on the different types of anti-nutritional factors

Food type	Extrusion conditions	ANFs	Effects of extrusion	References
Lentil splits	Twin-screw extruder, Screw speed (150, 200 and 250 rpm), Four different temperatures zone. Conveying zone (95, 115 and 135ºC), Mixing zone (110, 130 and 150ºC), Cooking zone (125, 145 and 165ºC), High pressure zone (die) (140,160 and 180ºC)	Phytic acid, Tannins, Trypsin inhibitors (TIA), Polyphenols	Reduced phytic acid by 99.30%, tannins by 98.83%, and trypsin inhibitors by 98%.	Rathod & Annapure, 2016
Hard to cook beans *(Phaseolus vulgaris)*	Single screw extruder, central temperature (150ºC), Moisture content(20g/100g)	Phytic acid, Trypsin, α-Amylase inhibitors	Reduced phytic acid by 17% and 26% in pontal and grafite cultivar. 71% and 69% reduction of TI in pontal and grafite. Complete elimination of α-amylase	Batista et al., 2010
Red chief lentils *(Lens culinaris Medik)*	Twin-screw extruder, Screw speed (500 rpm), Barrel section and the die temperature (160 ± 1°C).	Phytic acid, TIA, Lectins	Reduction of phytic acid not significant. 96%reduction in TIA, 100% reduction in lectins.	Morales et al., 2015

Table 2.(continued)

Pea, Chickpea, Faba bean	Twin-screw extruder, Single-step conditioner (350 rpm), die temperature (108ºC-116ºC)	Phytic acid Trypsin inhibitors, Tannins,	Phytic acid reduced from 10.3-22.5% for faba, 4.5%-17.5% for chickpea, 2.5%-17.5% for pea. Trypsin inhibitors reduced by 30%-54%, 89%-91%, 48%-57% for faba, chickpea, and pea, Tannins reduced by 2.3-11%, 1.2%-14.66%, 1.12%-2.74% for faba, chickpea, and pea respectively.	Adamidou et al., 2011

2.7 Other methods of processing

Several traditional processes such as dehulling, germination, soaking, boiling, microwave-assisted cooking, and autoclaving have been utilized in modifying the physiochemical attributes of the pulse ingredients. Soaking is one of the conventional methods for the preparation of pulses. Soaking time, temperature and soaking solution (water, acidic or basic) are important parameters (Patterson et al., 2017). Soaking of mung beans for 12 h resulted in a reduction of tannin content and phytic acid content. Soaking caused a 42.8-48.9 % reduction of phytic acid in cowpea, kidney bean, and pea. Tannin contents were compared for Canadian and Egyptian cowpea, peas, and kidney beans (Khattab & Arntfield, 2009). In most of the nutritional factors cases, soaking reduced the phytate due to solubility in water and leaching, into the soaking water and activation of endogenous phytase (Patterson et al., 2017). Dehulling is the removal of the outer seed coat, similarly which leads to reduced cooking times as well as the removal of various ANFs.

2.7.1 Heat treatment

Heat treatment is another processing parameter that significantly improves the nutritional profile of the pulses by inactivating or eliminating heat-labile ANFs. Shi et al (2017) reported the changes in the level of enzyme inhibitors during heat-cooking of pulses in Canada. Enzyme inhibitors like α-amylase, trypsin and chymotrypsin inhibitors for 9 lentils, 4 peas, 3 chickpeas, 4 beans, and 2 faba beans were investigated, using soybean as a control. The α-amylase was reduced from 1369.75 to 143.87 AIU/g dry matter for dark red kidney beans, 1000.91 to 199.88 for pinto beans, 1079.83 to 71.32 for navy beans. Heat treatment brought a complete removal of trypsin inhibitors in split pea, green pea, lentils, and fava beans. Complete removal of trypsin inhibitor activity due to the boiling of whole seeds has been reported previously for peas (Shi et al., 2017) Similarly, oligosaccharides (verbascose, stachyose, raffinose) that produce flatulence have been reduced in pea due to boiling (Khattab & Arntfield, 2009). The effect of cooking on the nutritional quality of faba bean was evaluated by Osman et al. (2014), who found that cooking reduced the phytic acid content and increased the protein digestibility.

2.8 Biochemical modification

Biochemical modification refers to the treatment of substrates using enzymes, microorganisms to further produce value-added products.

2.8.1 Fermentation

Fermentation converts carbohydrates to alcohol and carbon dioxide or organic acid using yeast/bacteria under anaerobic conditions. Fermentation technology depends upon the type of microbes used and generates different compounds or products. For example, yeast performs fermentation by converting sugar into alcohol, while bacteria perform fermentation by converting carbohydrates into lactic acid. It is one of the oldest as well as widely used methods to add value and preserve products (Adebo et al., 2017; Liu et al., 2011). The fermentation process is carried in two ways i.e. solid-state fermentation (SSF) and submerged state fermentation (SmF).

SSF is a process in which microorganisms grow on solid materials without free liquid. The solid matrix consists of necessary moisture for the growth of microorganisms (Krishna, 2005). Filamentous fungi are the ideal group used in SSF as they have the tolerance to grow in lower water activity (a_w) and higher osmotic pressure conditions. The type of inoculum, moisture, water activity, temperature, pH, substrate, aeration, particle size, agitation, nutritional factors, oxygen, carbon dioxide, and biomass play a significant role in solid-state fermentation. *Cordyceps militaris* SN-18 was used for SSF of chickpea and its effect on the physicochemical and functional properties were determined. Fermentation improved WHC, fat absorption capacity, and emulsifying property of chickpea (Xiao et al., 2015). SSF of lupin and soybean using *L. sakei, P. acidilactici,* and *P. pentosaceus* successfully enhance the nutritional quality (Bartkiene et al., 2015). *B. amyloliquefaciens, L. acidophilus, L. plantarum, and S. cerevisiae* have also been used for SSF of soybean meal to improve the bioactivity (Chi & Cho, 2016).

Submerged state fermentation (SmF) is a process in which the cultivation of microorganisms is in liquid medium containing nutrients. The bioactive compounds are released into growth media and substrate in media is consumed vigorously. SmF is ideal for those microbes that have high water activity. It is advantageous over SSF as the desired bioactive compounds are easier to purify during the down streaming process (Hayes and García-Vaquero., 2016). SmF using lactic acid is widely used in the fermentation of milk, meat, and vegetables. *Lactobacillus plantarum*, Lactobacillus acidophilus, Bifidobacterium, and Streptococcus thermophilus have been used in SmF to ferment mung bean, faba bean, pea protein, and red bean respectively (Han Wu et al., 2015; Coda et al., 2015; Çabuk et al., 2018; Worku & Sahu, 2017).

Fermentation has been utilized to produce enzymes, metabolites, biomass, and biotransformation products and has enhanced the texture, shelf-life, flavor, appearance, and digestibility of products (Adebo et al., 2017). Mostly fermentation is carried out to enhance the nutritional profile of pulse-based ingredients. Several ANFs present in pulses like phytic acid, tannins, trypsin inhibitors, and flatulence-causing oligosaccharides (raffinose, stachyose) are dominant in pulses which diminish consumer acceptability. In vitro digestibility is another issue as most of the pulses have lower protein digestibility due to the presence of these ANFs. Therefore, fermentation has been carried out in numerous researches to reduce the ANFs, improve the nutritional quality and enhance in vitro protein digestibility. Microbes such as *L. plantarum, B. subtilis, B. stearothermophilus, S. thermophilus, L acidophilus,* and *A. orazyae* have been employed to ferment pulses and enhance nutritional quality.

2.8.1.1 Nutritional change due to fermentation

Carbohydrates

Pulses are rich sources of carbohydrates, which are present in varying amounts depending upon types of the pulse. Most carbohydrates in pulse are starch, which provides the most energy values. During fermentation, the microorganism produces hydrolytic enzymes that support the catalysis of the starch to form a product. Enzymes like α-amylase and maltase are produced, which converts starch to simple sugars (Osman, 2011). Kaczmarska et al. (2017) reported the effect of fermentation on the carbohydrate composition of soybean seeds and flour. There was a significant reduction of sucrose, i.e. from 28 mg/g to 13 mg/g, and oligosaccharides when fermented with YO-MIX yogurt *(Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus thermophilus*). Also, they observed a decrease in starch content and an increase of sugar content during fermentation as endogenous enzymes like amylase hydrolyze the starch into glucose.

Protein

Protein is a minor element in the fermentation of pulse-based foods. Microbes utilized during fermentation could produce enzymes that hydrolyze proteins and create smaller peptides, consequently modifying the chemical and structural properties that can then affect the functionality of food. Some reports have indicated the increase as well as a decrease in protein contents during fermentation (Adebo et al., 2017). Xiao et al. (2015) reported the increase in total crude protein and essential amino acids in *C. militaris* fermented chickpea, which was ascribed to the partial hydrolysis of proteins by endogenous and microbial enzymes while fermenting and also to the biomass of microbes. Additionally, the functional properties of fermented chickpea like emulsification capacity, fat absorption, water absorption were significantly improved. Wu et al. (2019) reported the increase of peptides by 26.88% when soybean meal was fermented by *B. stearothermophilus.* Worku & Sahu, (2017) reported an increase in protein digestibility of anger red beans by 92.5% during open fermentation due to denaturation or inactivation of enzymes. Coda et al. (2015) also reported the increase in protein digestibility (75.1-76.9%) of faba bean when fermented with *Lactobacillus plantarum.* In-vitro protein digestibility of fermented pea proteins showed no increasing trend as a function of fermentation time. At 0 h, digestibility was 80% and increased up to 87.4% when fermented for 5 h by *L. plantarum* (Çabuk et al., 2018). Chandra-Hioe et al. (2016) fermented chickpea, faba bean with yogurt culture containing *Lactobacillus delbrueckii subsp. bulgaricus and Streptococcus thermophilus* analyzed the in-vitro protein digestibility and reported the increase in digestibility for all the substrates. Digestibility improved for desi chickpea (from 70.5 to 77.25%) and Kabuli chickpea (from 74.5 to 76.2%), but not for faba bean.

Anti-nutritional factors

Fermentation has been utilized for various pulse-based substrates to reduce the anti-nutritional factors. For example, Khattab et al. (2009) fermented cowpea, kidney beans, and pea with *Saccharomyces cerevisiae* and reported a reduction in phytic acid contents of 67% for all three pulses. White beans were fermented by *L. plantarum* and *L. fermentum* had reported a reduction of TIA (27%) and phytic acid (85%) (Doblado et al., 2003). Chi et al. (2016) fermented soybean meal by using *Bacillus amyloliquefaciens* and *L. plantarum*. TIA was reduced significantly from 4.77 mg/g to 0.67 mg/gm and 0.53 mg/g when fermented with *Bacillus amyloliquefaciens* and *L. plantarum*. Some of the pulses-based fermentation and its effect on the ANFs and protein digestibility are summarized in Table 3.

Table 3 Effect of pulse-based fermentation on anti-nutritional factors

Substrate	Microorganism used for fermentation	Effect of fermentation on ANFs	References
Chickpea and faba bean	*Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus thermophillus*	9.5% increase in protein digestibility for chickpea, Trypsin inhibitor activity reduced by 2.7, 1.1 and 4.7 % for desi, Kabuli, and faba.	Chandra-Hioe et al., 2016
Faba bean	*Lactobacillus plantarum* VTT E-133328	Enhanced in-vitro protein digestibility, Trypsin inhibitors activity reduced.	Coda et al., 2015
Cowpeas (Vigna sinensis L)	*Lactobacillus plantarum* ATCC 14917	Increase in antioxidant activity	Dueñas et al., 2005

Table 3.(continued)

Pea protein concentrate	*Lactobacillus plantarum*	Trypsin inhibitors activity decreased; In-vitro protein digestibility increased.	Çabuk et al., 2018
Soybean flours	*A. oryzae* 2094T (ATCC 1011), *R. oryzae* CECT 2340 (ATCC 24563), *B. subtilis* CECT 39T (ATCC 6051) and *L. plantarum* CECT 748T (ATCC 14917)	Increased total phenolic contents	Fernandez-Orozco et al., 2007
Extruded and Fermented Soya Products	*Bacillus natto*	Trypsin inhibitor activity decreased, decrease in phytic acid content only in the fermented soy meal and low moisture extruded soya sample	Ojokoh & Yimin, 2011
Soybean meal	*Bacillus stearothermophilus*	74.05% decrease in trypsin inhibitor activity, DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging ability increased by 57.07%	Wu et al., 2019
Phaseolus vulgaris (red beans)	Thermophilic lactic culture (*Lactobacillus acidophilus, Bifidobacterium* and *Streptococcus thermophilus*)	Increased protein digestibility, decrease in phytic acid	Worku & Sahu, 2017

2.9 Conclusion

Pulses are a rich source of proteins, carbohydrates, fibers, and minerals but their consumption is lower due to the presence of several antinutritional factors and lower digestibility. This review has focused on the extraction of proteins and processing techniques as a pretreatment to modify the pulse-protein to enhance nutritional quality. It is also clear from the review that not every processing technology can enhance nutrition and eliminate the anti-nutritional factors that limit the usage of plant-based ingredients. As these modifications alter functionality, pulse-based plant proteins offer a wide range of applications to food industries as well as provide various health benefits.

2.10 References

Adachi, M., Takenaka, Y., Gidamis, A. B., Mikami, B., & Utsumi, S. (2001). Crystal structure of soybean proglycinin A1aB1b homotrimer. *Journal of Molecular Biology*, *305*(2), 291–305. https://doi.org/10.1006/jmbi.2000.4310

Adamidou, S., Nengas, I., Grigorakis, K., Nikolopoulou, D., & Jauncey, K. (2011). Chemical composition and antinutritional factors of field peas (*Pisum sativum*), chickpeas (*Cicer arietinum*), and faba beans (*Vicia faba*) as affected by extrusion preconditioning and drying temperatures. *Cereal Chemistry Journal*, *88*(1), 80–86. https://doi.org/10.1094/cchem-05-10-0077

Adebo, O. A., Njobeh, P. B., Adebiyi, J. A., Gbashi, S., Phoku, J. Z., & Kayitesi, E. (2017). Fermented pulse-based food products in developing nations as functional foods and ingredients. Johannesburg, Gauteng, South Africa. https://doi.org/10.5772/intechopen.69170

Adeyemo, S. M., & Onilude, A. A. (2013). Enzymatic reduction of anti-nutritional factors in fermenting soybeans by *Lactobacillus plantarum* isolates from fermenting cereals. *Nigerian Food Journal*, *31*(2), 84–90. https://doi.org/10.1016/s0189-7241(15)30080-1

Alonso, R., Aguirre, A., & Marzo, F. (2000). Effects of extrusion and traditional processing methods on antinutrients and in vitro digestibility of protein and starch in faba and kidney beans. *Food Chemistry*, *68*(2), 159–165. https://doi.org/10.1016/S0308-8146(99)00169-7

Aluko, R. E., Mofolasayo, O. A., & Watts, B. M. (2009). Emulsifying and foaming properties of commercial yellow pea (*Pisum sativum l.*) Seed flours. *Journal of Agriculture and Food Chemistry*, *57*, 9793–9800. https://doi.org/10.1021/jf902199x

Awad, T. S., Moharram, H. A., Shaltout, O. E., Asker, D., & Youssef, M. M. (2012). Applications of ultrasound in analysis, processing and quality control of food: A review. *Food Research International*, *48*, 410–427. https://doi.org/10.1016/j.foodres.2012.05.004

Barać, M. B., Pešić, M. B., Stanojević, S. P., Kostić, A. Z., & Čabrilo, S. B. (2015). Techno-functional properties of pea (*Pisum sativum*) protein isolates-a review. *Acta Periodica Technologica*, *46*(January), 1–18. https://doi.org/10.2298/apt1546001b

Bartkiene, E., Krungleviciute, V., Juodeikiene, G., Vidmantiene, D., & Maknickiene, Z. (2015). Solid state fermentation with lactic acid bacteria to improve the nutritional quality of lupin and soya bean. *Journal of the Science of Food and Agriculture*, *95*(6), 1336–1342. https://doi.org/10.1002/jsfa.6827

Batista, K. A., Pereira, W. J., Moreira, B. R., Silva, C. N., & Fernandes, K. F. (2020.). Effect of autoclaving on the nutritional quality of hard-to-cook common beans (*Phaseolus vulgaris*). *International Journal of Environment, Agriculture and Biotechnology*, *5*(1). https://doi.org/10.22161/ijeab.51.4

Batista, K. A., Prudêncio, S. H., & Fernandes, K. F. (2010). Changes in the functional properties and antinutritional factors of extruded hard-to-cook common beans (*Phaseolus vulgaris*). *Journal of Food Science*, *75*(3). https://doi.org/10.1111/j.1750-3841.2010.01557.x

Biswas, B., & Sit, N. (2020). Effect of ultrasonication on functional properties of tamarind seed protein isolates. *Journal of Food Science and Technology*. https://doi.org/10.1007/s13197-020-04241-8

Bourré, L., McMillin, K., Borsuk, Y., Boyd, L., Lagassé, S., Sopiwnyk, E., Jones, S., Dyck, A., Malcolmson, L. (2019). Effect of adding fermented split yellow pea flour as a partial replacement of wheat flour in bread. *Legume Science*, *1*(1). https://doi.org/10.1002/leg3.2

Boye, J. I., Aksay, S., Roufik, S., Ribéreau, S., Mondor, M., Farnworth, E., & Rajamohamed, S. H. (2010). Comparison of the functional properties of pea, chickpea and lentil protein concentrates processed using ultrafiltration and isoelectric precipitation techniques. *Food Research International*, *43*, 537–546. https://doi.org/10.1016/j.foodres.2009.07.021

Boye, J., Zare, F., & Pletch, A. (2010). Pulse proteins: Processing, characterization, functional properties and applications in food and feed. *Food Research International*. https://doi.org/10.1016/j.foodres.2009.09.003

Çabuk, B., Nosworthy, M. G., Stone, A. K., Korber, D. R., Tanaka, T., House, J. D., & Nickerson, M. T. (2018). Effect of fermentation on the protein digestibility and levels of non-nutritive compounds of pea protein concentrate. *Food Technology and Biotechnology*, *56*(2), 257. https://doi.org/10.17113/ftb.56.02.18.5450

Chandra-Hioe, M. V., Wong, C. H. M., & Arcot, J. (2016). The potential use of fermented chickpea and faba bean flour as food ingredients. *Plant Foods for Human Nutrition*, *71*(1), 90–95. https://doi.org/10.1007/s11130-016-0532-y

Chi, C. H., & Cho, S. J. (2016). Improvement of bioactivity of soybean meal by solid-state fermentation with Bacillus amyloliquefaciens versus *Lactobacillus spp*. and *Saccharomyces cerevisiae*. *LWT - Food Science and Technology*, *68*, 619–625. https://doi.org/10.1016/j.lwt.2015.12.002

Coda, R., Melama, L., Rizzello, C. G., Curiel, J. A., Sibakov, J., Holopainen, U., Pulkkinen, M., Sozer, N. (2015). Effect of air classification and fermentation by *Lactobacillus plantarum* VTT E-133328 on faba bean (*Vicia faba L.*) flour nutritional properties. *International Journal of Food Microbiology*, *193*, 34–42. https://doi.org/10.1016/j.ijfoodmicro.2014.10.012

De J, J. (2011). Extrusion processing of main commercial legume pulses, 209–236. https://doi.org/10.1201/b11286-9

Dhawan, K., Malhotra, S., Dahiya, B. S., & Singh, D. (1991). Seed protein fractions and amino acid composition in gram (*Cicer arietinum*). *Plant Foods for Human Nutrition*, *41*, 225–232.

Doblado, R., Frisa, J., Munoz, R., & Vidal-Valverde, C. (2003). Fermentation of *Vigna sinensis var. Carilla* flours by natural microflora and lactobacillus species. *Journal of Food Protection*, *66*(12), 2313–2320.

El Fiel, H. E., El Tinay, A. H., & Elsheikh, E. A. (2003). Effect of cooking on protein solubility profiles of faba beans (*Vicia faba L.*) grown using different nutritional regimes. *Plant Foods for Human Nutrition*, *58*, 63–74.

Feng, H., Barbosa-Canovas, G. V, & Weiss, J. (2013). Ultrasound Technologies for Food Bioprocessing (Vol. 53). https://doi.org/10.1017/cbo9781107415324.004

Fernandez-Orozco, R., Frias, J., Muñoz, R., Zielinski, H., Piskula, M. K., Kozlowska, H., & Vidal-Valverde, C. (2007). Fermentation as a bio-process to obtain functional soybean flours. *Journal of Agricluture and Food Chemistry*, *55*, 8972–8979. https://doi.org/10.1021/jf071823b

Ghribi, A. M., Amira, A. Ben, Gafsi, I. M., Lahiani, M., Bejar, M., Triki, M., Zouari, A., Attia, H., Besbes, S. (2018). Toward the enhancement of sensory profile of sausage "Merguez" with chickpea protein concentrate. https://doi.org/10.1016/j.meatsci.2018.04.025

Ghumman, A., Kaur, A., Singh, N., & Singh, B. (2016). Effect of feed moisture and extrusion temperature on protein digestibility and extrusion behaviour of lentil and horsegram. *LWT - Food Science and Technology*, *70*, 349–357. https://doi.org/10.1016/j.lwt.2016.02.032

Han, I. H., Swanson, B. G., & Baik, B.-K. (2007). Protein digestibility of selected legumes treated with ultrasound and high hydrostatic pressure during soaking. *Cereal Chemistry Journal*, *84*(5), 518–521. https://doi.org/10.1094/cchem-84-5-0518

Han Wu, Xin Rui , Wei Li, Xiaohong Chen, Mei Jiang, M. D. (2015). Mung bean (*Vigna radiata*) as probiotic food through fermentation with *Lactobacillus plantarum* B1-6. *LWT - Food Science and Technology*, *63*(1), 445–451. https://doi.org/10.1016/j.lwt.2015.03.011

Impossible™ Foods: Meat made from plants. (2020). Retrieved March 15, 2020, from https://impossiblefoods.com/

Jambrak, A. R., Lelas, V., Mason, T. J., Krešic, G., & Marija, B. (2009). Physical properties of ultrasound treated soy proteins. *Journal of Food Engineering*, *93*, 386–393.

Jiang, L., Wang, J., Li, Y., Wang, Z., Liang, J., Wang, R., Chen, Y., Ma, W., Qi, B., Zhang, M. (2014). Effects of ultrasound on the structure and physical properties of black bean protein isolates. *Food Research International*. https://doi.org/10.1016/j.foodres.2014.04.022

Joshi, V., & Kumar, S. (2015). Meat analogues: Plant based alternatives to meat products- A review. *International Journal of Food and Fermentation Technology*, *5*(2), 107. https://doi.org/10.5958/2277-9396.2016.00001.5

Kaczmarska, K. T., Chandra-Hioe, M. V, Zabaras, D., Frank, D., & Arcot, J. (2017). Effect of germination and fermentation on carbohydrate composition of australian sweet lupin and soybean seeds and flours. https://doi.org/10.1021/acs.jafc.7b02986

Karaca, A. C., Low, N., & Nickerson, M. (2011). Emulsifying properties of chickpea, faba bean, lentil and pea proteins produced by isoelectric precipitation and salt extraction. https://doi.org/10.1016/j.foodres.2011.06.012

Karki, B., Lamsal, B. P., Grewell, D., Pometto, A. L., Van Leeuwen, J., Khanal, S. K., & Jung, S. (2009). Functional properties of soy protein isolates produced from ultrasonicated defatted soy flakes. *Journal of the American Oil Chemists' Society*, *86*, 1021–1028. https://doi.org/10.1007/s11746-009-1433-0

Karki, B., Lamsal, B. P., Jung, S., van Leeuwen, J. (Hans), Pometto, A. L., Grewell, D., & Khanal, S. K. (2010). Enhancing protein and sugar release from defatted soy flakes using ultrasound technology. *Journal of Food Engineering*, *96*(2), 270–278. https://doi.org/10.1016/J.Jfoodeng.2009.07.023

Khattab, R. Y., & Arntfield, S. D. (2009). Nutritional quality of legume seeds as affected by some physical treatments 2.Antinutritional factors. *LWT - Food Science and Technology*, *42*(6), 1113–1118. https://doi.org/10.1016/j.lwt.2009.02.004

Klupšaitė, D., & Juodeikienė, G. (2015). Legume: composition, protein extraction and functional properties. A review. *Chemical Technology*, *66*(1), 5–12. https://doi.org/10.5755/j01.ct.66.1.12355

Krishna, C. (2005). Solid-State Fermentation Systems-An Overview. *Critical Reviews in Biotechnology*, *25*(30), 1–30. https://doi.org/10.1080/07388550590925383

Lou, Z., Wang, H., Zhang, M., & Wang, Z. (2010). Improved extraction of oil from chickpea under ultrasound in a dynamic system. *Journal of Food Engineering*, *98*(1), 13–18. https://doi.org/10.1016/j.jfoodeng.2009.11.015

Meng, G., & Ma, C. Y. (2002). Characterization of globulin from *Phaseolus angularis* (red bean). *International Journal of Food Science and Technology*, *37*(6), 687–695. https://doi.org/10.1046/j.1365-2621.2002.00601.x

Millar, K. A., Barry-Ryan, C., Burke, R., Hussey, K., McCarthy, S., & Gallagher, E. (2017). Effect of pulse flours on the physiochemical characteristics and sensory acceptance of baked crackers. *International Journal of Food Science and Technology*, *52*(5), 1155–1163. https://doi.org/10.1111/ijfs.13388

Montalbo-Lomboy, M., Johnson, L., Khanal, S. K., (Hans) van Leeuwen, J., & Grewell, D. (2010). Sonication of sugary-2 corn: A potential pretreatment to enhance sugar release. *Bioresource Technology*, *101*(1), 351–358. https://doi.org/10.1016/j.biortech.2009.07.075

Morales, P., Berrios, J. D. J., Varela, A., Burbano, C., Cuadrado, C., Muzquiz, M., & Pedrosa, M. M. (2015). Novel fiber-rich lentil flours as snack-type functional foods: An extrusion cooking effect on bioactive compounds. *Food and Function*, *6*(9), 3135–3143. https://doi.org/10.1039/c5fo00729a

Mundi, S., & Aluko, R. E. (2012). Physicochemical and functional properties of kidney bean albumin and globulin protein fractions. *Food Research International*, *48*(1), 299–306. https://doi.org/10.1016/j.foodres.2012.04.006

Nazari, B., Amin Mohammadifar, M., Shojaee-Aliabadi, S., Feizollahi, E., & Mirmoghtadaie, L. (2017). Effect of ultrasound treatments on functional properties and structure of millet protein concentrate. https://doi.org/10.1016/j.ultsonch.2017.10.002

Nishinari, K., Fang, Y., Guo, S., & Phillips, G. O. (2014). Soy proteins: A review on composition, aggregation and emulsification. *Food Hydrocolloids*, *392*, 301–318. https://doi.org/10.1016/j.foodhyd.2014.01.013

Nosworthy, M. G., Medina, G., Franczyk, A. J., Neufeld, J., Appah, P., Utioh, A., Frohlich, P., House, J. D. (2018). Effect of processing on the in vitro and in vivo protein quality of beans (*Phaseolus vulgaris* and *Vicia Faba*). *Nutrients*, *10*(6). https://doi.org/10.3390/nu10060671

O'Sullivan, J., Murray, B., Flynn, C., & Norton, I. (2016). The effect of ultrasound treatment on the structural, physical and emulsifying properties of animal and vegetable proteins. *Food Hydrocolloids*, *53*, 141–154. https://doi.org/10.1016/j.foodhyd.2015.02.009

Ojokoh, A. O., & Yimin, W. (2011). Effect of fermentation on chemical composition and nutritional quality of extruded and fermented soya products. *International Journal of Food Engineering Recommended Citation*, *7*. https://doi.org/10.2202/1556-3758.1857

Osman, A. M. A., Hassan, A. B., Osman, G. A. M., Mohammed, N., Rushdi, M. A. H., Diab, E. E., & Babiker, E. E. (2014). Effects of gamma irradiation and/or cooking on nutritional quality of faba bean (*Vicia faba L.*) cultivars seeds. *Journal of Food Science and Technology*, *51*(8), 1554–1560. https://doi.org/10.1007/s13197-012-0662-7

Osman, M. A. (2011). Effect of traditional fermentation process on the nutrient and antinutrient contents of pearl millet during preparation of Lohoh. *Journal of the Saudi Society of Agricultural Sciences*, *10*(1), 1–6. https://doi.org/10.1016/j.jssas.2010.06.001

Our Products – InnovoPro. (2020). Retrieved March 28, 2020, from https://innovopro.com/products/

Pananun, T., Montalbo-Lomboy, M., Noomhorm, A., Grewell, D., & Lamsal, B. (2012). High-power ultrasonication-assisted extraction of soybean isoflavones and effect of toasting. *LWT - Food Science and Technology*, *47*(1), 199–207. https://doi.org/10.1016/j.lwt.2011.12.003

Patist, A., & Bates, D. (2007). Ultrasonic innovations in the food industry: From the laboratory to commercial production. *Innovative Food Science & Emerging Technologies*, *9*, 147–154. https://doi.org/10.1016/j.ifset.2007.07.004

Patterson, C. A., Curran, J., & Der, T. (2017). Effect of processing on antinutrient compounds in pulses. *Cereal Chemistry Journal*, *94*(1), 2–10. https://doi.org/10.1094/cchem-05-16-0144-fi

Preece, K. E., Hooshyar, N., Krijgsman, A., Fryer, P. J., & Zuidam, J. (2017). Intensified soy protein extraction by ultrasound. *Chemical Engineering & Processing: Process Intensification*, *113*, 94–101. https://doi.org/10.1016/j.cep.2016.09.003

Pusztai, A., Clarke, E. M. W., King, T. P., & Stewart, J. C. (1979). Nutritional evaluation of kidney beans (*Phaseolus vulgaris*): Chemical composition, lectin content and nutritional value of selected cultivars. *Journal of the Science of Food and Agriculture*, *30*(9), 843–848. https://doi.org/10.1002/jsfa.2740300902

Raes, K., Knockaert, D., Struijs, K., & Van Camp, J. (2014). Role of processing on bioaccessibility of minerals: Influence of localization of minerals and anti-nutritional factors in the plant. *Trends in Food Science and Technology*, *37*(1), 32–41. https://doi.org/10.1016/j.tifs.2014.02.002

Rathod, R. P., & Annapure, U. S. (2015). Effect of extrusion process on antinutritional factors and protein and starch digestibility of lentil splits. https://doi.org/10.1016/j.lwt.2015.10.028

Riera, E., Gol, Y., Blanco, A., Gallego, J. A., Blasco, M., & Mulet, A. (2004). Mass transfer enhancement in supercritical fluids extraction by means of power ultrasound. *Ultrasonics - Sonochemistry*, *11*, 241–244. https://doi.org/10.1016/j.ultsonch.2004.01.019

Ross, K. A., Pyrak-Nolte, L. J., & Campanella, O. H. (2004). The use of ultrasound and shear oscillatory tests to characterize the effect of mixing time on the rheological properties of dough. *Food Research International*, *37*(6), 567–577. https://doi.org/10.1016/j.foodres.2004.02.013

Sethi, S., Tyagi, S. K., & Anurag, R. K. (2016). Plant-based milk alternatives an emerging segment of functional beverages: a review. *Journal of Food Science and Technology*. Springer India. https://doi.org/10.1007/s13197-016-2328-3

Shevkani, K., Singh, Narpinder, Chen, Ying, Kaur, A., & Yu, Long. (2019). Pulse proteins: secondary structure, functionality and applications. *Journal of Food Science and Technology*, *56*. https://doi.org/10.1007/s13197-019-03723-8

Shi, L., Mu, K., Arntfield, S. D., & Nickerson, M. T. (2017). Changes in levels of enzyme inhibitors during soaking and cooking for pulses available in Canada. *Journal of Food Science and Technology*, *54*(4), 1014–1022. https://doi.org/10.1007/s13197-017-2519-6

Silva Lima, D. R., Nunes, I. L., & Block, J. M. (2019). Ultrasound-assisted extraction for the recovery of carotenoids from guava's pulp and waste powders. *Plant Foods for Human Nutrition*. https://doi.org/10.1007/s11130-019-00784-0

Simons, C. W., & Hall, C. (2018). Consumer acceptability of gluten-free cookies containing raw cooked and germinated pinto bean flours. *Food Science & Nutrition*, *6*(1), 77–84. https://doi.org/10.1002/fsn3.531

Singhal, A., Karaca, A. C., Tyler, R., & Nickerson, M. (2016). Pulse proteins: from processing to structure-function relationships. In *Grain Legumes*. InTech. https://doi.org/10.5772/64020

Stone, A. K., Karalash, A., Tyler, R. T., Warkentin, T. D., & Nickerson, M. T. (2014). Functional attributes of pea protein isolates prepared using different extraction methods and cultivars. https://doi.org/10.1016/j.foodres.2014.11.017

Tang, C.-H., & Ma, C.-Y. (2009). Effect of high pressure treatment on aggregation and structural properties of soy protein isolate. *LWT - Food Science and Technology*, *42*(2), 606–611. https://doi.org/10.1016/j.lwt.2008.07.012

Tang, C. H., & Sun, X. (2010). Physicochemical and structural properties of 8S and/or 11S globulins from mungbean [*Vigna radiata (L.) Wilczek*] with various polypeptide constituents. *Journal of Agricultural and Food Chemistry*, *58*(10), 6395–6402. https://doi.org/10.1021/jf904254f

Tharanathan, R. N., & Mahadevamma, S. (2003). Grain legumes - A boon to human nutrition. *Trends in Food Science and Technology*. Elsevier Ltd. https://doi.org/10.1016/j.tifs.2003.07.002

Thushan Sanjeewa, W. G., Wanasundara, J. P. D., Pietrasik, Z., & Shand, P. J. (2010). Characterization of chickpea (*Cicer arietinum L.*) flours and application in low-fat pork bologna as a model system. *Food Research International*, *43*, 617–626. https://doi.org/10.1016/j.foodres.2009.07.024

Vilkhu, K., Mawson, R., Simons, L., & Bates, D. (2008). Applications and opportunities for ultrasound assisted extraction in the food industry — A review. *Innovative Food Science & Emerging Technologies*, *9*(2), 161–169. https://doi.org/10.1016/j.ifset.2007.04.014

Vinatoru, M. (2001). An overview of the ultrasonically assisted extraction of bioactive principles from herbs. In *Ultrasonics Sonochemistry* (Vol. 8, pp. 303–313). https://doi.org/10.1016/S1350-4177(01)00071-2

Wani, I. A., Singh Sogi, D., Shivhare, U. S., & Gill, B. S. (2015). Physico-chemical and functional properties of native and hydrolyzed kidney bean (Phaseolus vulgaris L.) protein isolates. https://doi.org/10.1016/j.foodres.2014.08.027

What is a pea protein beverage? - Nutrilicious. (2020). Retrieved March 28, 2020, from http://www.nutrilicious.ca/pea-protein-beverage/

Wong Paz, J. E., Muñiz Márquez, D. B., Martínez Ávila, G. C. G., Belmares Cerda, R. E., & Aguilar, C. N. (2015). Ultrasound-assisted extraction of polyphenols from native plants in the Mexican desert. *Ultrasonics Sonochemistry*, *22*, 474–481. https://doi.org/10.1016/j.ultsonch.2014.06.001

Worku, A., & Sahu, O. (2017). Significance of fermentation process on biochemical properties of *Phaseolus vulgaris* (red beans). *Biotechnology Reports*, *16*, 5. https://doi.org/10.1016/j.btre.2017.09.001

Wu, H., Hulbert, G. J., & Mount, J. R. (2001). Effects of ultrasound on milk homogenization and fermentation with yogurt starter. *Innovative Food Science & Emerging Technologies*, *1*, 211–218.

Wu, P., Guo, Y., Golly, M. K., Ma, H., He, R., Luo, S., Zhang, C., Zhang, L., Zhu, J. (2019). Feasibility study on direct fermentation of soybean meal by *Bacillus stearothermophilus* under non-sterile conditions. *Journal of the Science of Food and Agriculture*, *99*(7), 3291–3298. https://doi.org/10.1002/jsfa.9542

Xiao, Y., Xing, G., Rui, X., Li, W., Chen, X., Jiang, M., & Dong, M. (2015). Effect of solid-state fermentation with *Cordyceps militaris SN-18* on physicochemical and functional properties of chickpea (*Cicer arietinum L.*) flour. *LWT - Food Science and Technology*, *63*(2), 1317–1324. https://doi.org/10.1016/j.lwt.2015.04.046

Xiong, T., Xiong, W., Ge, M., Xia, J., Li, B., & Chen, Y. (2018). Effect of high intensity ultrasound on structure and foaming properties of pea protein isolate. *Food Research International*, *109*, 260–267. https://doi.org/10.1016/j.foodres.2018.04.044

Zhang, L., Pan, Z., Shen, K., Cai, X., Zheng, B., & Miao, S. (2017). Influence of ultrasound-assisted alkali treatment on the structural properties and functionalities of rice protein. https://doi.org/10.1016/j.jcs.2017.10.013

Zhou, M., Liu, J., Zhou, Y., Huang, X., Liu, F., Pan, S., & Hu, H. (2016). Effect of high intensity ultrasound on physicochemical and functional properties of soybean glycinin at different ionic strengths. *Innovative Food Science and Emerging Technologies*, *34*, 205–213. https://doi.org/10.1016/j.ifset.2016.02.007

CHAPTER 3: EFFECT OF HIGH-POWER SONICATION PRETREATMENT ON EXTRACTION AND SOME PHYSICOCHEMICAL PROPERTIES OF PROTEINS FROM CHICKPEA, KIDNEY BEAN, AND SOYBEAN.

Adapted from a paper published in *International Journal of Biological Macromolecules.*

[1]Bibek Byanju, [1]Md Mahfuzur Rahman, [2]Milagros P. Hojilla-Evangelista, and [1]Buddhi P. Lamsal[*]

[1] Department of Food Science and Human Nutrition, Iowa State University, Ames, IA, 50011

[2] Plant Polymer Research Unit, USDA ARS National Center for Agricultural Utilization Research, Peoria, IL, 61604

* Corresponding author:

2312 Food Sciences Building

536 Farm House Lane

Department of Food Science and Human Nutrition

Iowa State University

Ames, Iowa 50011-1057

Phone: (515) 294-8681.

E-mail: lamsal@iastate.edu

Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture (USDA) and Iowa State University (ISU). USDA and ISU are equal opportunity providers and employers.

3.1 Abstract

Impact of high-power sonication (HPS) as pretreatment in the extraction and some physicochemical properties of proteins from soybean flakes and flours of soybean, chickpea, and kidney bean was evaluated. Soybean flakes and flours from soybean, chickpea, and kidney bean were dispersed in distilled water (1:10 w/v) and sonicated at two power densities (PD) of 2.5 and 4.5 W/mL for 5 min continuously. Proteins were extracted at pH range 8-8.5. PD 2.5 and 4.5 W/mL significantly increased protein extraction yields from soy flakes to 29.03% and 25.87%, respectively, compared to 15.28% for unsonicated controls, but did not increase for flours. Freeze-dried spent substrates at higher PD sonication aggregated in size. Free sulfhydryl content for both sonicated and unsonicated soy flakes and flour were similar but increased in chickpea and kidney bean when HPS of 4.5 W/mL was applied, indicating the unfolding of protein structure. The protein band patterns for sonicated and unsonicated legumes proteins were found to be similar, indicating no peptide profile alterations by HPS. However, circular dichroism analysis showed changes in secondary structure composition in extracted kidney bean protein, causing unfolding and destabilizing the native structure. The secondary structure composition for soy flakes and flour protein and chickpea protein remained unchanged.

Keywords: Legume proteins, High power sonication, Ultrasonic-assisted extraction, Protein extraction, Protein secondary structure.

3.2 Introduction

Plant-based food proteins are studied for its better and cheaper source of nutrients including essential amino acids. Legumes, for example, peas, lentils, soybeans, and dry beans are a rich source of proteins, fibers, and carbohydrates making them valuable as food ingredients. They can also be incorporated as meat replacers to lower the energy density while providing important nutrients (Rebello et al., 2014). Extraction, isolation, and purification of proteins is the first step in utilizing them as ingredients; however, the presence of cell wall polysaccharides and other cell polymers and the location of proteins inside the cell-matrix limit their extraction (Taiz & Zeiger, 2006).

Conventional extraction methods for plant proteins include alkali extraction with pH modulation in the range of 8.5-9, where the solubility of proteins plays a major role in extraction. This method extracts approximately half of the available proteins from defatted soy flakes (Karki et al., 2010). Several other extraction methods like, microwave heating, enzymatic modifications and chemical modifications of soy substrate were carried out to improve protein extractability from plants (Jung et al., 2006; Kasai & Ikehara, 2005). In addition to conventional methods, enzyme-assisted extraction (Endoprotease Protex 5L) at pH 9.5 of soybean and rapeseed resulted in the increased protein extraction yields by 10% and 40%, respectively but both enzymes and process are expensive (Sari et al., 2013). On the other hand, Mu et al. (2010) reported that ultrasonication as pretreatment substantially increased the protein yield from soy flakes and reduced the cost of producing the proteins, which demonstrated a potential alternative to existing conventional methods. Karki et al. (2010) also reported the use of high-power sonication as one of the pretreatment methods with improved protein extraction yield of 40-46% from defatted soy meal.

Ultrasound with low frequency (16-100 kHz) and power intensity of 10-1000 W/mL are known as high power sonication (HPS). When HPS is applied to the aqueous medium, cavitation bubbles form and collapse violently leading to extreme temperatures (5000 K) and pressures (1000 atm) that produce high shear energy and turbulence in the cavitation zone (Suslick et al., 1986). Cavitation disintegrates cellular matrices and aids in the extraction of protein, sugar, oils, isoflavones, polyphenols, and saponins from plant cells (Karki et al., 2010; Vilkhu et al., 2008). HPS can decrease particle size by approximately 10-fold resulting in more protein extraction from the substrate due to increased surface area (Karki et al., 2010).

Apart from enhanced extraction, HPS, however, can alter protein molecular configuration by breaking hydrogen bonds, and hydrophobic interactions; HPS may also induce the dissociation and/ or aggregation of subunits. The interaction among polar, non-polar, acidic, and basic groups within the polypeptide chain create a complex three-dimensional structure. As sonication can break down the interaction between proteins, structural integrity is lost, and hence the altered functionality. Thus, the secondary structure of proteins is important in evaluating the changes in functionality. Functional properties of protein obtained from sonicated soy flakes like solubility and emulsion capacity are reported in the literature: the solubility for sonicated soy flakes increased by 34% and emulsion capacity decreased by 12% (Karki et al., 2009). Ultrasound has also been used to alter structural, physical, chemical, and functional properties of protein isolates prepared from various sources, for example, rice, soybean, pea, black bean, and sunflower (Arzeni et al., 2012; Jiang et al., 2014; O'Sullivan et al., 2016; Xiong et al., 2018). High-intensity ultrasonication induced changes in free sulfhydryl content, particle size and secondary structure, which in turn resulted in alteration of surface hydrophobicity and activity (Gülseren et al., 2007). Hu et al. (2013)

reported partial unfolding and reduction in intermolecular interactions of soybean protein isolate based on increased free sulfhydryl groups and surface hydrophobicity, which in turn improved the solubility of soy protein isolates (SPI) dispersion.

HPS has been used in a limited way as a pretreatment for the extraction of proteins from legumes. In the sonic-assisted extraction of oil from chickpea, extraction yield increased by 10.45% when using ultrasonic power of 230 W was used (Lou et al., 2010). Extraction of oil from soybean (Li et al., 2004) and glucose release from corn slurry obtained from dry-ground ethanol plants was done using ultrasonication (Khanal et al., 2007). Use of high-power sonication as a pretreatment in maximizing legume protein extraction can benefit the industry but has not been reported for various types of legumes, for example, chickpeas and kidney beans, nor has its effect on their protein secondary structure been evaluated. This study investigated the effects of HPS on the extraction yields and physical and structural properties of proteins from important legumes like chickpea and kidney bean and compared with soybean. The specific objectives of this study were to 1) evaluate the effect of sonication power densities on extraction and yield of plant-based protein preparations, and 2) evaluate the comparative changes in the secondary molecular structure of extracted plant proteins as affected by high power sonication.

3.3 Materials and methods

3.3.1 Materials and reagents

Defatted soy flakes (20 PDI, protein dispersibility index) were obtained from Cargill Inc., (Cedar Rapids, IA). Defatted soy flour (80-90 PDI) was obtained from Archer Daniels Midland Company, (Decatur, IL). Chickpea and red kidney beans were obtained from Dr. Chibuike Udenigwe, (University of Ottawa, Ottawa, Canada and were prepared into flours as described in

Sec 3.3.2. The Pierce BCA protein assay kit, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), urea, ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), and β-mercaptoethanol were purchased from Fisher Scientific (Waltham, MA, USA). Bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St Louis, MO, USA). Milli-Q water (Millipore, Billerica, MA, USA) had a resistivity of 18.2 MΩ- at 25ºC. All chemicals used were of analytical grade.

3.3.2 Preparation of legume flours

Chickpea and kidney beans were soaked in water for 12 h and manually de-hulled followed by oven drying at 50°C for 24 h. Milling was done by passing beans through 0.03" gap with corrugation of 1/8" followed by 0.02" gap and 1/16" corrugation using a Witt corrugated roller mill (Witt Corrugating Inc., Wichita, KS) then ground using Nutri mill (Pleasant Hill Grain, NE, USA). The particle size of ground soy flakes (100 g) was analyzed using a Ro-Tap sieve shaker (W.S. Tyler Industrial Group, Mentor, OH) fitted with graded U.S standard mesh sieves 12 (1.7 mm), 16 (1.18 mm), 25 (0.71 mm), 30 (0.6 mm), 35 (0.5 mm), and 60 (0.25 mm). Sieve used for chickpea flour, kidney bean flour, and soy flour were mesh 25 (0.71 mm), 35 (0.5 mm), 60 (0.25 mm), 120 (0.125 mm), 170 (0.09 mm), and 200 (0.075 mm).

3.3.3 Proximate analyses

The proximate analysis of all legume samples was carried out using standard methods in the Plant Polymer Research Unit Lab (USDA-ARS, Peoria, IL). Moisture, crude protein (Dumas combustion % N x 6.25), crude oil, and crude fiber contents were analyzed according to AOCS standard methods Ba 2a-38, Ba 4e-93, Am 5-04, and Ba 6-05, respectively (Brühl, 1997). Ash contents were analyzed according to AOAC method 942.05 (Horwitz & AOAC International., 2000) and carbohydrate content was calculated by difference (100 - sum of other components). Soluble or insoluble carbohydrates were not identified.

3.3.4 High-power sonication-based extraction of legume proteins

Ultrasonic treatments (model VCX 750, Sonics & Materials, Inc., CT, USA) was carried out in temperature-controlled centrifuge tubes at a frequency of 20 kHz with 750 W maximum power output. A 13-mm (1/2") probe of titanium alloy threaded to a 3 mm tapered micro tip generated ultra-high intensity by sonication for 5 min at 160μm peak-to-peak. The ultrasonic power density (PD) was defined as the input power of the ultrasound per volume of the slurry and varied at 2.5 W/mL and 4.5 W/mL by adjusting amplitude (20-40%) and volume of the samples. 40% was the highest amplitude supported by the horn. Power density 2.5 W/mL (intensity ~315 W/cm^2) and 4.5 W/mL (intensity ~390 W/cm^2) were designated as lower and higher PD in this research. These two PD was determined as PD 2.5 is the power that is relevant to the industrial application (Montalbo-Lomboy et al., 2010) which was compared to PD 4.5 to see how the protein will behave. Also, the highest PD limitation of the sonication unit at the given volume was PD 4.5.

Protein products were prepared according to modified protocols of the standard methods (H. Wang, Johnson, & Wang, 2004). The schematic diagram of the process is shown in Figure 5. Sample to water ratio of 1 g: 10 mL were treated at PD 2.5 W/mL and 4.5 W/mL for 5 min by placing the centrifuge tubes in a temperature-controlled ice bath (below 45°C). These sonicated samples were then used for the extraction of the proteins at pH 8.5, 60°C, and 30 min stirring using a magnetic bar on a stir plate. The samples were centrifuged at 14,000 x *g* at 15°C for 10 min. The supernatants were collected to measure total volume with a graduated cylinder and protein content quantified for extraction yield. The supernatant pH was adjusted to 4.5 by adding 2 N HCl and then refrigerated at 4°C for 1 h to facilitate the formation of larger and stronger curds. Centrifugation was done again at 14,000 x *g* at 15°C for 10 min to segregate curd and whey where

curd was neutralized using distilled water of pH 7.0 and then freeze-dried at -20°C. The mass of the freeze-dried sample was measured, and the percent yield of protein preparation was calculated. Besides, spent solids were also collected and freeze-dried to measure particle size distribution.

3.3.5 Protein content of extracted supernatant

The protein content of extracted supernatant was quantified using bicinchoninic acid (BCA) protein assay kit (Pierce^{TM} Rockford, IL 61105, USA). An aliquot (0.1 mL) of supernatant was mixed with 2 mL of working reagent, incubated at 37°C for 30 min, and then cooled down for 10 min. The absorbance was measured using a spectrophotometer (Shimadzu UV 160) at 562 nm and converted to protein concentration using a BSA standard curve.

3.3.6 Scanning electron microscope (SEM)

Freeze-dried spent solids were placed onto aluminum stubs with metallic backed adhesive tape and sputter-coated with platinum (30 nm) using a Cressington HR208 sputter coater. Images were captured using a Hitachi SU-4800 field emission scanning electron microscope (Hitachi High Technologies in America, Schaumburg, IL) at 10kV.

3.3.7 Particle size distribution after sonication

The freeze-dried spent solids from protein extraction were passed through a 1-mm pore size then analyzed for particle size distribution using particle size analyzer (Mastersizer 2000 S, Malvern Inc., Worcestershire, UK). The particle size distribution was measured using a refractive index ratio of 1.520. The sonicated and unsonicated spent solids were then dispersed into distilled water to obtain the obscuration of 12-16%. Analyses were done in triplicate and averaged.

3.3.8 Effect of sonication on protein secondary structure

3.3.8.1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The sonicated and unsonicated protein solutions were subjected to SDS-PAGE with slight modification (Banach, Clark, & Lamsal, 2013). Thirteen percent resolution gel (Acryl-Bisacrylamide) at the bottom and 4% percent stacking gel at the top were prepared. The protein concentration of 2 mg/mL was prepared in sample buffer (15.1 g/L Tris, 300 g/L urea, 2 g/L SDS, 20 mL/L glycerol, and 0.1 g/L bromophenol blue) and incubated at 80°C for 5 min. The protein standard (6,500 – 66,000 Da, Product number M3913-SigmaMarker™) and samples were loaded onto gel at equal volume (10µL) and electrophoresed at a constant voltage of 200V for 45 min using standard SDS buffer (25mM Tris, 191 mM glycine and 1 g SDS per liter). The gels were stained with Coomassie blue for 1 h and de-stained with methanol: acetic acid: deionized water in ratio 10:2:8 until the gels were clear and transparent.

3.3.8.2 Free sulfhydryl content of final protein products

The free sulfhydryl (SH) content of the soluble fraction of freeze-dried samples was determined using Ellman's Reagent DNTB (5, 5'-dithio-bis- [2-nitrobenzoic acid]) (Shimada & Cheftel, 1988). Reaction buffer containing 6 M guanidine hydrochloric acid and 1.27 mm ethylenediaminetetraacetic acid (EDTA) of pH 8.6 was prepared. Two hundred fifty mg of protein product was dissolved in 25 mL of reaction buffer overnight followed by centrifugation at 12,000 x *g* for 10 min. The supernatant was collected and 250 µL of native and sonicated protein solutions from the supernatant was added to 2.5 ml of reaction buffer, followed by the addition of 50 µL of Ellman's reagent. Samples were then vortexed and incubated at room temperature for 15 min. Absorbance at 412 nm was measured using a UV–visible spectrophotometer. The free SH is presented in µmol/g protein.

3.3.8.3 Secondary structure analysis by circular dichroism

Circular dichroism (CD) spectra were scanned at the far- UV range (260-180) with a CD spectropolarimeter (Jasco 715, Jasco Corp) in a 0.1 cm quartz CD cuvette at 25°C. Freeze-dried protein samples (0.03 mg/mL) were dissolved in 0.01 M sodium phosphate buffer (pH 7.0) and centrifuged at 12,000 x g for 10 min to remove any insoluble residue. The values of scan rate, response, and bandwidth were 50 nm min^{-1}, 0.25 s, and 1.0 nm, respectively (Home-Jer & Chang, 2004) Three scans were averaged to obtain one spectrum. The mean residue ellipticity (θ) was expressed as $deg \cdot cm^2 \cdot dmol^{-1}$. The protein concentration was determined by BCA protein assay kit as mentioned in section 2.5. The secondary structure was estimated using computer program SELCON3 originated by the method of Sreerama & Woody, (2000) and reference dataset 6 was used. Four secondary structures were estimated, i.e., α-helix, β-sheet, β-turn, and unordered using the method of Whitmore & Wallace, (2004).

3.3.9 Protein extraction yield and protein yield in product

The extraction yield and protein preparation (product) yield are calculated by the equations given below:

$$Protein\ extraction\ yield\ (\%) = \frac{Total\ protein\ in\ extracted\ supernatant}{Total\ protein\ in\ initial\ substrate} * 100$$

$$Protein\ yield\ in\ product\ (\%) = \frac{Total\ protein\ in\ freeze\ dried\ product}{Total\ \ protein\ in\ initial\ substrate} * 100$$

3.3.10 Statistical analyses

The experimental design was a randomized complete block design (RCBD) with two replications. Statistical analyses were performed using the JMP® statistical methods (100 SAS Campus Drive; Cary, NC). A two-way analysis of variance (ANOVA) was performed to assess

the effect of sonication on extraction yield and structural characteristics. Treatment means were compared within each substrate for sonication effect on protein extraction yield, Protein yield in a product, free sulfhydryl content, and secondary structures. Results having different superscript letters within each substrate group show a significant difference ($p < 0.05$). Graphs were prepared using GraphPad Prism software (GraphPad Software, San Diego, CA, USA) and OriginPro 8.5® (Northampton, MA, USA).

4 Results and discussion

4.1.1 Proximate analyses

The final particle size of soy flakes, soy flour, chickpea flour, and kidney bean flour used for further analyses was 0.85 mm, 0.07 mm, 0.22 mm, and 0.10 mm respectively. The composition of legumes for proximate analysis is given in Table 4. Soy flakes and flours had similar proximate contents and are a rich source of proteins, as flours are prepared by milling the flakes. These legumes are an excellent source of proteins that are essential for the synthesis and repair of body tissues. The proximate composition of defatted soy flour in our research agrees with (Rosset et al., 2014) with a slight variation in protein content (47.79%) which might be due to the defatting and processing conditions of soy flour and flakes preparations. The proximate composition for kidney beans is comparable except for the carbohydrate composition which is 70.9% for our sample and 53.02% for Hayat et al. (2014) which might be due to the different varieties of kidney beans. Similarly, the composition of chickpea is comparable to values reported by Boye et al. (2010). Crude fibers observed were less than 2%, as dehulling or seed coat removal might be a possible factor for the reduced crude fiber content (Ndife et al., 2011). The carbohydrate contents observed were higher in the samples, which were not differentiated between soluble and insoluble fractions.

4.1.2 Protein extraction yield

The ultrasound-assisted extraction yields of the proteins for different legume substrates are provided in Fig. 6. The protein extraction yield was greater for soy flakes when exposed to high power sonication both at higher and lower PD, compared to unsonicated soy flakes. The extraction yield of proteins from sonicated soy flakes increased significantly ($p < 0.05$) by 90% and 68.5% for higher and lower PD respectively compared to unsonicated soy flakes. This increase in protein extraction yield was also observed in Karki et al. (2010) where two minutes sonication at 84 μm_{pp} gave 46% greater yield. This sonication induced increase in protein extraction yield might be due to the structural damage, as was corroborated by the extensive cellular disruptions seen in SEM analysis (Section 3.4.4) and resulting in the release of cell constituents into the aqueous system (Dolatowski et al., 2007). However, the increase was not linear with the increase in power density. Similarly, there was no significant change in protein extraction yield for soy flour after sonication possibly due to the smaller particle size than the flakes.

The yield increased significantly ($p<0.05$) by 16.39% when higher PD sonication was applied to kidney bean flour. On the other hand protein extraction yield for chickpea reduced after sonication. This decrease may have been due to the higher fat content in chickpea (7.03%), which can form protein-lipid interaction that inhibits the dissolution of proteins and limits the isolation of proteins (Johnston et al., 2015). Also, the carbohydrates which are present at higher levels of ~66% contain cellulose and non-cellulosic polymers in chickpea which lowered the free water to extract proteins; made the gel viscous preventing the accessibility of proteins to be extracted. Overall, our finding indicates that HPS could be used efficiently as extraction pretreatment when substrate particle size is bigger, leading to a decrease in particle size and facilitating the extraction of proteins as in soy flakes.

4.1.3 Protein yield in product

The protein product yields for various legumes are presented in Fig. 7. Soy flakes protein product yield increased significantly ($p < 0.05$) from 8.4% (unsonicated) to 33.45% for lower PD and up to 30.6% for higher PD respectively. For soy flour, protein yield increased slightly from 43% to 50% with sonication but the values were not significantly different. Similarly, the protein product yield of kidney beans increased from 44.5% (unsonicated) to 51.4% (lower PD), but the change was not significant. Chickpea, when exposed to the two PD, showed a reduction in the protein product yield, which was also seen in protein extraction yields, possibly due to protein-lipid interactions forming viscous slurry-like appearance. The protein content of the final product is shown in Table 5; the purity of the proteins in product for all legumes decreased as the sonication power density increased. The highest protein content is that of unsonicated soy flakes followed by soy flour, kidney bean, and chickpea. Due to sonication, several other compounds like oils, sugar, and iso-flavones might have been extracted along with the protein, lowering the purity of protein (Lou et al., 2010).

4.1.4 Scanning electron microscopy of spent substrate

SEM for unsonicated flakes and flour showed intact cells for all the samples and presence of intracellular materials (Fig. 8 A, B, C, D left column), which are comparable to SEM studies by other researchers (Karki et al., 2010; Pananun et al., 2012). Several micro-fractures appeared in sonicated soy flakes and soy flour samples. There was a deposition of debris on the surface (Fig. 8 middle and right column) which suggested cell breakdown and layer formation. The sonicated samples looked like an aggregation of fragmented parts. The lower and higher PD seemed to disintegrate the cell (Fig. 8A and 8B middle & right column) and caused the deposition of cell-matrix in both soy flakes and flour. Formation of larger aggregates was observed in a dry state after freeze-drying of ultra-sonicated legumes which was also observed by Hu et al. (2013).

SEM examination for chickpea and kidney beans without sonication (Fig. 8C and 8D left column) showed the presence of starch granules that are embedded in the matrix of protein bodies and surrounded by the fiber-rich cell wall (Pelgrom et al., 2015). Large oval and small spherical granules of starch having a smooth surface without cracks were observed in unsonicated chickpea (Polesi et al., 2010) and kidney beans (Figure 8C and 8D left column). On the other hand, the sonicated chickpea (Fig. 8C, 8D middle and right column) had an irregular structure with cracked granules along with deposition of cell debris and aggregation of cell-matrix with embedded starch granules. The cracks/fissures that are due to sonication helps in the release of the proteins and several other biological components. Similarly, in the unsonicated kidney bean, starch granules were regular in shape. At lower PD, there was protein layer embedding starch granules, whereas higher PD sonication degraded the cell-matrix resulting in the aggregation of the fragmented cell materials.

4.1.5 Sonication effect on the particle size of spent substrate

All the samples after treatment were freeze-dried, which led to the aggregation of particles. The samples were gently ground with a mortar pestle. The particle size distribution is shown in Figure 9. Soy flour treated at lower PD showed a bimodal distribution with major and minor peaks and reduced particle size upon sonication. Also, because of the aggregation of the cell-matrix and starch granules as seen in SEM (Fig. 8B middle and right column), there was an apparent enlargement particle size. The particle size of chickpea apparently increased from approximately 20 µm to 110 µm at higher PD which is supported by SEM studies (Fig. 8C right column) where there is an aggregation of proteins and cellular matrix that was fragmented during the sonication process. Similar aggregation was seen in ultrasonicated soy protein isolates (Hu et al., 2013). Similarly, Jiang et al. (2014) reported the larger particle size of sonicated (150W - 450W, 12-24

min) black bean protein compared to untreated samples which might be due to the formation of unstable aggregate. The particle size for kidney bean spent substrate decreased from approximately 120 µm to 105 µm for lower PD and increased from approximately 20 µm to 130 µm at higher PD compared to unsonicated sample, which had been attributed to the re-polymerization of aggregates through noncovalent interaction such as hydrophobic interactions (Tang et al., 2009).

4.1.6 Effect of sonication on protein secondary structure

4.1.6.1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Figure 10 depicts the electrophoretic pattern of protein subunits obtained from flakes and flours of soybean, kidney beans, and chickpeas. Soybean protein consists of two major proteins namely 7S (β-conglycinin) and 11S (glycinin). β-conglycinin is a trimeric protein and consists of three subunits (namely, α'~72, α ~68, and β- ~52 kDa) (Fig. 10 Left). Glycinin consists of acidic and basic subunits with ~ 35 and ~20 kDa, respectively (Hidayat et al., 2011). The unsonicated soy proteins and ultra-sonicated proteins at higher and lower PD had generally similar protein subunit electrophoretic patterns, suggesting that sonication did not change the protein profiles for soy protein regardless of sonication conditions. Similar results were observed by Wang et al. (2011) and Karki et al. (2009) who also reported no modification in soy protein profiles.

Kidney bean protein consists of phaseolin and chickpea protein consists of legumin and vicilin as a major protein subunit; these can be seen in the gel as major bands (Fig. 6 Right). These gels show that sonication conditions did not alter the primary structure of protein subunits in kidney bean and chickpea protein, however, secondary structures may have been altered (Sec 3.4.6.3). Comparing protein bands with the standard (6.5 kDa-66 kDa), suggested no changes in the molecular weight of the protein after sonication at both PD.

4.1.6.2 Free sulfhydryl content of final protein products

The free sulfhydryl content of protein product had no significant changes between unsonicated and sonicated soy flakes and flour (Table 6). This result is similar to Arzeni et al. (2012), who studied the effect of high-intensity ultrasound on free and total sulfhydryl content of SPI and reported no significant modification. However, Hu et al. (2013) reported an increase in free sulfhydryl content of soluble SPI from 9.13 ± 0.44 μmol g^{-1} soluble protein to 18.08 ± 0.39 μmol g^{-1} upon sonication at 600 W for 30 min. Such differences in free sulfhydryl content might be due to the sonication conditions and preparation methods of protein products.

Higher and lower PD increased the free SH in kidney beans protein products. Free SH increased significantly ($p < 0.05$) from 3.95 ± 0.87 μmol g^{-1} (unsonicated) to 11.81 ± 1.46 μmol g^{-1} at lower PD and 13.67 ± 3.85 μmol g^{-1} at higher PD. There was significant ($p < 0.05$) increase of free SH in chickpea protein from 6.76 ± 0.55 μmol g^{-1} to 19.30 ± 2.00 μmol g^{-1} at higher PD sonication. This finding suggests that HPS could break the disulfide bonds (i.e., can cause the reduction of S-S linkage to form free -SH groups), which exposes the sulfhydryl group to the surface of proteins (Hu et al., 2013; Jianga et al., 2017). The unfolding of the buried sulfhydryl group in proteins when exposed to high pressure and sheer force of cavitation phenomenon might lead to an increase in the free sulfhydryl group.

4.1.6.3 Secondary structure composition of legume by circular dichroism

The contents of α-helix, β-strands, β-turns, and unordered groups are shown in Table 7. The distribution of these attributes of protein secondary structure from both unsonicated soy flour protein and soy flakes protein seems to be similar. The results suggested that α-helical structure is not the main structure in protein; instead, the β-structure (strands and turns) is the main secondary structure for all the unsonicated legumes (Hu et al., 2013; Yin et al., 2011). Hu et al. (2013)

reported that HPS (400 W and 600W) combined with a longer time decreased the β-strands in soy protein isolates. Soy flakes protein at higher PD tended to an increase in β-strands, and a decrease in α-helix which was also reported by Stathopulos et al. (2004) for BSA, myoglobin, lysozyme, and black bean protein (Jiang et al., 2014).

The α-helical structure seemed to decrease for proteins from sonicated chickpea and kidney beans. The β-strands decreased significantly in kidney beans when high and low PD sonication was applied, and unordered form increased significantly from 33.22% to 52.35 % and 50.73% for kidney beans during sonication (Table 7). The unordered structure for lower and higher PD was 52.3% and 50.7%, respectively. CD spectra of kidney bean protein are shown in Figure 11. As the secondary structure of proteins is stabilized by hydrogen bonding and electrostatic interactions, it is reasonable to infer that sonication might disrupt these interactions leading to changes in secondary structure (Saleem & Ahmad, 2016). In the current work, ultrasonication likely destabilized the native structure of proteins, and therefore, changed the secondary structure composition by increasing the unordered structure. HPS induced partial unfolding and intermolecular interactions as indicated by an increase in free sulfhydryl content in chickpea and kidney beans. The variation among the literature for the secondary structure may be due to the various reference spectra, algorithms, and software used for analyses. Furthermore, the protein isolation technique also contributes to differences in the conformation of proteins.

4.2 Conclusions

High power sonication of defatted soy flakes resulted in higher protein extraction yields when exposed to higher power sonication compared to unsonicated soy flakes, for example, 90% and 68.7% increment with lower and higher PD sonication in the study. However, sonicated chickpea flours resulted in a decrease in the protein extraction yield, possibly due to high carbohydrate and fat contents reducing the access to proteins in cell matrices. Protein subunit bands for all the substrate were not altered by sonication; significant changes in the secondary structure of kidney bean protein were observed and indicated by circular dichroism analyses. Also, an increase of the free sulfhydryl contents in sonicated kidney bean protein and chickpea protein suggests an alteration in the structure of native protein due to partial unfolding. Our study indicated that HPS has the potential to improve the extraction of various plant proteins with altered molecular structure. This will have an impact on how these proteins will be utilized in various food applications.

4.3 Acknowledgments

This paper is a product of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa; Project No. IOW05447 is sponsored by Hatch Act and State of Iowa funds. The project was also funded partially by USDA/ NIFA, Award no. 2018-67017-27560. Special thanks to Dr. David Grewell for initial help with sonication equipment.

4.4 References

Arzeni, C., Martínez, K., Zema, P., Arias, A., Pérez, O. E., & Pilosof, A. M. R. (2012). Comparative study of high intensity ultrasound effects on food proteins functionality. *Journal of Food Engineering*, *108*(3), 463–472. https://doi.org/10.1016/j.jfoodeng.2011.08.018

Banach, J. C., Clark, S., & Lamsal, B. P. (2013). Characterization of extruded and toasted milk protein concentrates. *Journal of Food Science*, *78*(6), E861–E867. https://doi.org/10.1111/1750-3841.12122

Brühl, L. (1997). Official methods and recommended practices of the American Oil Chemist's Society, physical and chemical characteristics of oils, fats and waxes, section I. Ed. The AOCS Methods Editor and the AOCS Technical Department. 54 pages. AOCS Press, Champaign, 199. *Fett/Lipid*, *99*(5), 197–197. https://doi.org/10.1002/lipi.19970990510

Dolatowski, Z. J., Stadnik, J., & Stasiak, D. (2007). Applications of ultrasound in food technology. *Acta Sci. Pol Technol. Aliment*, *6*(3), 89–99.

Gülseren, İ., Güzey, D., Bruce, B. D., & Weiss, J. (2007). Structural and functional changes in ultrasonicated bovine serum albumin solutions. *Ultrasonics Sonochemistry*, *14*(2), 173–183. https://doi.org/10.1016/j.ultsonch.2005.07.006

Hayat, I., Ahmad, A., Ahmed, A., Khalil, S., Gulfraz, M., & Kashmir, A. (2014). Exploring the potential of red kidney beans (*Phaseolus vulgaris l.*) to develop protein-based product for food applications. *The Journal of Animal & Plant Sciences*, *24*(3), 860–868.

Hidayat, M., Sujatno, M., Sutadipura, N., Setiawan, & Faried, A. (2011). β-conglycinin content obtained from two soybean varieties using different preparation and extraction methods. *HAYATI Journal of Biosciences*, *18*(1), 37–42. https://doi.org/10.4308/hjb.18.1.37

Home-Jer, D., & Chang, S. K.-C. (2004). Structural characteristics of purified glycinin from soybeans stored under various conditions. *Journal of Agricluture and Food Chemistry*, *52*, 3792–3800. https://doi.org/10.1021/jf035072z

Horwitz, W., & AOAC International. (2000). *Official methods of analysis of AOAC International. Volume 1, Agricultural chemicals ; contaminants ; drugs*. AOAC International.

Hu, H., Wu, J., Li-Chan, E. C. Y., Zhu, L., Zhang, F., Xu, X., Fan, G., Wang, L., Huang, X., Pan, S. (2013). Effects of ultrasound on structural and physical properties of soy protein isolate (SPI) dispersions. *Food Hydrocolloids*, *30*, 647–655. https://doi.org/10.1016/j.foodhyd.2012.08.001

Boye, J. I., Aksay, S., Roufik, S., Ribéreau, S., Mondor, M., Farnworth, E., & Rajamohamed, S. H. (2010). Comparison of the functional properties of pea, chickpea and lentil protein concentrates processed using ultrafiltration and isoelectric precipitation techniques. *Food Research International*, *43*, 537–546. https://doi.org/10.1016/j.foodres.2009.07.021

Jiang, L., Wang, J., Li, Y., Wang, Z., Liang, J., Wang, R., Chen, Y., Ma, W., Qi, B., Zhang, M. (2014). Effects of ultrasound on the structure and physical properties of black bean protein isolates. *Food Research International*. https://doi.org/10.1016/j.foodres.2014.04.022

Jianga, S., Dinga, J., Andradea, J., Rababahb, T. M., Almajwalc, A., Abulmeatyc, M. M., & Feng, H. (2017). Modifying the physicochemical properties of pea protein by pH-shifting and ultrasound combined treatments. *Ultrasonics - Sonochemistry*, *38*, 835–842. https://doi.org/http://dx.doi.org/10.1016/j.ultsonch.2017.03.046

Johnston, S. P., Nickerson, M. T., & Low, N. H. (2015). The physicochemical properties of legume protein isolates and their ability to stabilize oil-in-water emulsions with and without genipin. *Journal of Food Science and Technology*, *52*(7), 4135–4145. https://doi.org/10.1007/s13197-014-1523-3

Jung, S., Lamsal, B., Stepien, V., Johnson, L. ., & Murphy, P. A. (2006). Functionality of soy protein produced by enzyme-assisted extraction. *Journal of the American Oil Chemists' Society*, *83*(1), 71–78.

Karki, B., Lamsal, B. P., Grewell, D., Pometto, A. L., Van Leeuwen, J., Khanal, S. K., & Jung, S. (2009). Functional properties of soy protein isolates produced from ultrasonicated defatted soy flakes. *Journal of the American Oil Chemists' Society*, *86*, 1021–1028. https://doi.org/10.1007/s11746-009-1433-0

Karki, B., Lamsal, B. P., Jung, S., van Leeuwen, J. (Hans), Pometto, A. L., Grewell, D., & Khanal, S. K. (2010). Enhancing protein and sugar release from defatted soy flakes using ultrasound technology. *Journal of Food Engineering*, *96*(2), 270–278. https://doi.org/10.1016/J.Jfoodeng.2009.07.023

Kasai, N., & Ikehara, H. (2005). Stepwise extraction of proteins and carbohydrates from soybean seed. *Journal of Agricultural and Food Chemistry*, *53*(10), 4245–4252. https://doi.org/10.1021/jf048337y

Khanal, S. K., Montalbo, M., van Leeuwen, J. (Hans), Srinivasan, G., & Grewell, D. (2007). Ultrasound enhanced glucose release from corn in ethanol plants. *Biotechnology and Bioengineering*, *98*(5), 978–985. https://doi.org/10.1002/bit.21497

Li, H., Pordesimo, L., & Weiss, J. (2004). High intensity ultrasound-assisted extraction of oil from soybeans. *Food Research International*, *37*(7), 731–738. https://doi.org/10.1016/j.foodres.2004.02.016

Lou, Z., Wang, H., Zhang, M., & Wang, Z. (2010). Improved extraction of oil from chickpea under ultrasound in a dynamic system. *Journal of Food Engineering*, *98*(1), 13–18. https://doi.org/10.1016/j.jfoodeng.2009.11.015

Montalbo-Lomboy, M., Johnson, L., Khanal, S. K., (Hans) van Leeuwen, J., & Grewell, D. (2010). Sonication of sugary-2 corn: A potential pretreatment to enhance sugar release. *Bioresource Technology*, *101*(1), 351–358. https://doi.org/10.1016/j.biortech.2009.07.075

Mu, L., Zhao, M., Yang, B., Zhao, H., Cui, C., & Zhao, Q. (2010). Effect of ultrasonic treatment on the graft reaction between soy protein isolate and gum acacia and on the physicochemical properties of conjugates. *J. Agric. Food Chem*, *58*, 4494–4499. https://doi.org/10.1021/jf904109d

Ndife, J., Abdulraheem, L. ., & Zakari, U. M. (2011). Evaluation of the nutritional and sensory quality of functional breads produced from whole wheat and soya bean flour blends. *African Journal of Food Science*, *5*(8), 466–472.

O'Sullivan, J., Murray, B., Flynn, C., & Norton, I. (2016). The effect of ultrasound treatment on the structural, physical and emulsifying properties of animal and vegetable proteins. *Food Hydrocolloids*, *53*, 141–154. https://doi.org/10.1016/j.foodhyd.2015.02.009

Pananun, T., Montalbo-Lomboy, M., Noomhorm, A., Grewell, D., & Lamsal, B. (2012). High-power ultrasonication-assisted extraction of soybean isoflavones and effect of toasting. *LWT - Food Science and Technology*, *47*(1), 199–207. https://doi.org/10.1016/j.lwt.2011.12.003

Pelgrom, P. J. M., Boom, R. M., & Schutyser, M. A. I. (2015). Method development to increase protein enrichment during dry fractionation of starch-rich legumes. *Food and Bioprocess Technology*, *8*(7), 1495–1502. https://doi.org/10.1007/s11947-015-1513-0

Polesi, L. F., Sarmento, S. B. S., & Anjos, C. B. P. dos. (2010). Composition and characterization of pea and chickpea starches. *Brazilian Journal of Food Technology*, *14*(1), 74–81. https://doi.org/10.4260/bjft2011140100010

Rebello, C. J., Greenway, F. L., & Finley, J. W. (2014). A review of the nutritional value of legumes and their effects on obesity and its related co-morbidities. *Obesity Reviews*, *15*(5), 392–407. https://doi.org/10.1111/obr.12144

Rosset, M., Acquaro, V. R., & Beléia, A. D. P. (2014). Protein extraction from defatted soybean flour with viscozyme l pretreatment. *Journal of Food Processing and Preservation*, *38*(3), 784–790. https://doi.org/10.1111/jfpp.12030

Saleem, R., & Ahmad, R. (2016). Effect of ultrasonication on secondary structure and heat induced gelation of chicken myofibrils. *Journal of Food Science and Technology*, *53*(8), 3340–3348. https://doi.org/10.1007/s13197-016-2311-z

Sari, Y. W., Bruins, M. E., & Sanders, J. P. M. (2013). Enzyme assisted protein extraction from rapeseed, soybean, and microalgae meals. *Industrial Crops and Products*, *43*, 78–83. https://doi.org/10.1016/j.indcrop.2012.07.014

Shimada, K., & Cheftel, J. C. (1988). Determination of sulfhydryl groups and disulfide bonds in heat-induced gels of soy protein isolate. *Journal of Agricultural and Food Chemistry*, *36*(1), 147–153. https://doi.org/10.1021/jf00079a038

Sreerama, N., & Woody, R. W. (2000). Estimation of protein secondary structure from circular dichroism spectra: comparison of CONTIN, SELCON, and CDSSTR methods with an expanded reference set, *287*, 252–260. https://doi.org/10.1006/abio.2000.4880

Stathopulos, P. B., Scholz, G. A., Hwang, Y.-M., Rumfeldt, J. A. O., Lepock, J. R., & Meiering, E. M. (2004). Sonication of proteins causes formation of aggregates that resemble amyloid. *Protein Science*, *13*(11), 3017–3027. https://doi.org/10.1110/ps.04831804

Suslick, K. S., Hammerton, D. A., & Cline, R. E. (1986). Sonochemical hot spot. *Journal of the American Chemical Society*, *108*(18), 5641–5642. https://doi.org/10.1021/ja00278a055

Taiz, L., & Zeiger, E. (2006). Cell Walls: Structure, Biogenesis, and Expansion. In *Plant physiology*. Retrieved from https://www.psu.edu/dept/cellwall/cellwall.pdf

Tang, C. H., Wang, X. Y., Yang, X. Q., & Li, L. (2009). Formation of soluble aggregates from insoluble commercial soy protein isolate by means of ultrasonic treatment and their gelling properties. *Journal of Food Engineering*, *92*(4), 432–437. https://doi.org/10.1016/j.jfoodeng.2008.12.017

Vilkhu, K., Mawson, R., Simons, L., & Bates, D. (2008). Applications and opportunities for ultrasound assisted extraction in the food industry-A review. *Innovative Food Science and Emerging Technologies*, *9*, 161–169. https://doi.org/10.1016/j.ifset.2007.04.014

Wang, C., Jiang, L., Wei, D., Li, Y., Sui, X., Wang, Z., & Li, D. (2011). Effect of secondary structure determined by ftir spectra on surface hydrophobicity of soybean protein isolate. *Procedia Engineering*, *15*, 4819–4827. https://doi.org/10.1016/j.proeng.2011.08.900

Wang, H., Johnson, L. A., & Wang, T. (2004). Preparation of soy protein concentrate and isolate from extruded-expelled soybean meals. *Journal of the American Oil Chemists' Society*, *81*(7), 713–717. https://doi.org/10.1007/s11746-004-966-8

Whitmore, L., & Wallace, B. A. (2004). DICHROWEB, an online server for protein secondary structure analyses from circular dichroism spectroscopic data. *Nucleic Acids Research*, *32*(Web Server), W668–W673. https://doi.org/10.1093/nar/gkh371

Xiong, T., Xiong, W., Ge, M., Xia, J., Li, B., & Chen, Y. (2018). Effect of high intensity ultrasound on structure and foaming properties of pea protein isolate. *Food Research International*, *109*, 260–267. https://doi.org/10.1016/j.foodres.2018.04.044

Yin, S.-W., Huang, K.-L., Tang, C.-H., Yang, X.-Q., Wen, Q.-B., & Qi, J.-R. (2011). Surface charge and conformational properties of phaseolin, the major globulin in red kidney bean (*Phaseolus vulgaris L*): effect of pH. *International Journal of Food Science and Tenchnology*, *46*, 1628–1635. https://doi.org/10.1111/j.1365-2621.2011.02662.x

Figures

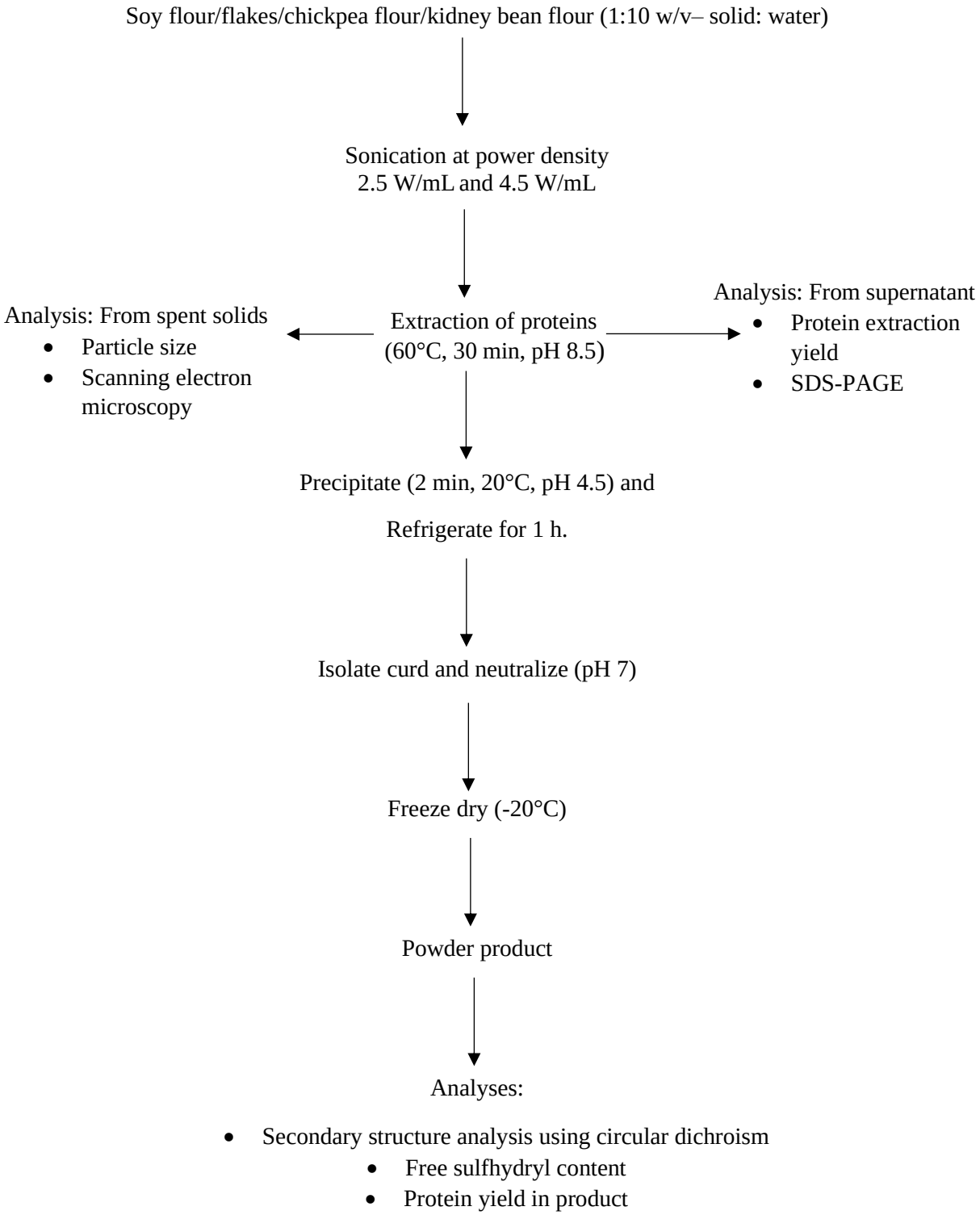
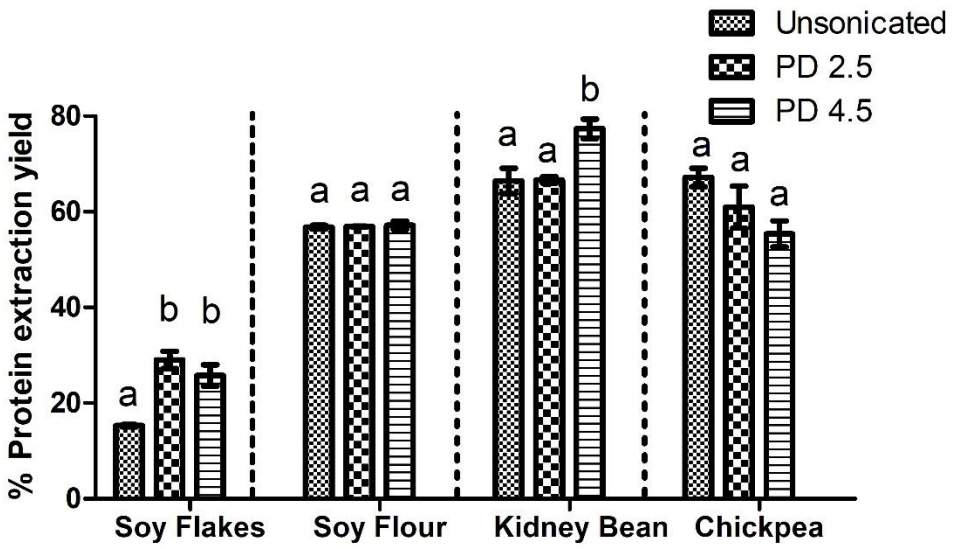
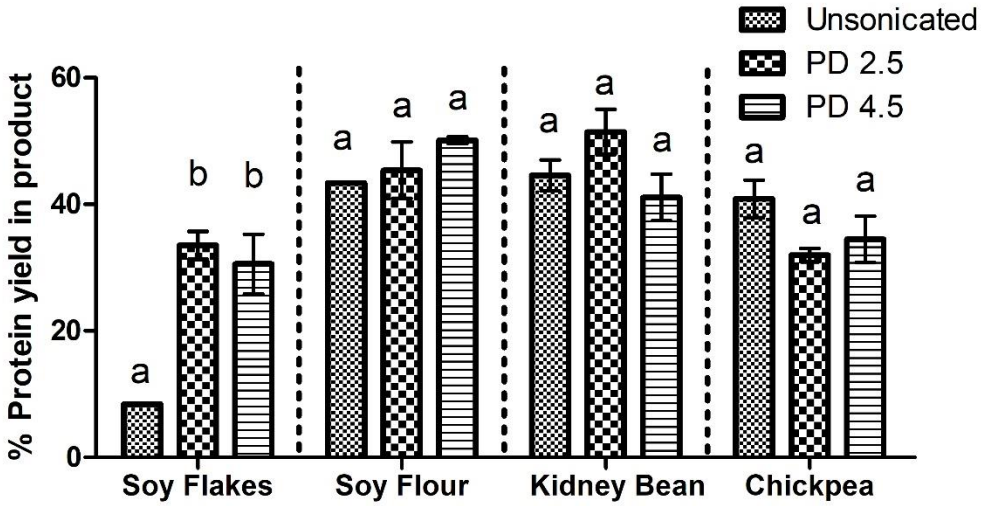


Figure 5 Schematic diagram and analysis of sonication as pretreatment for protein extraction



Means with the same letters on top of each column are not significantly different at $p<0.05$. Means were compared between control and treatments within the same substrate.

Figure 6 Effect of high-power sonication on protein extraction yield of some plant-based proteins at two power densities



Means with the same letters on top of each column are not significantly different at $p<0.05$. Means were compared between control and treatments within the same substrate.

Figure 7 Effect of sonication on protein yield in product of some plant-based proteins at two power densities

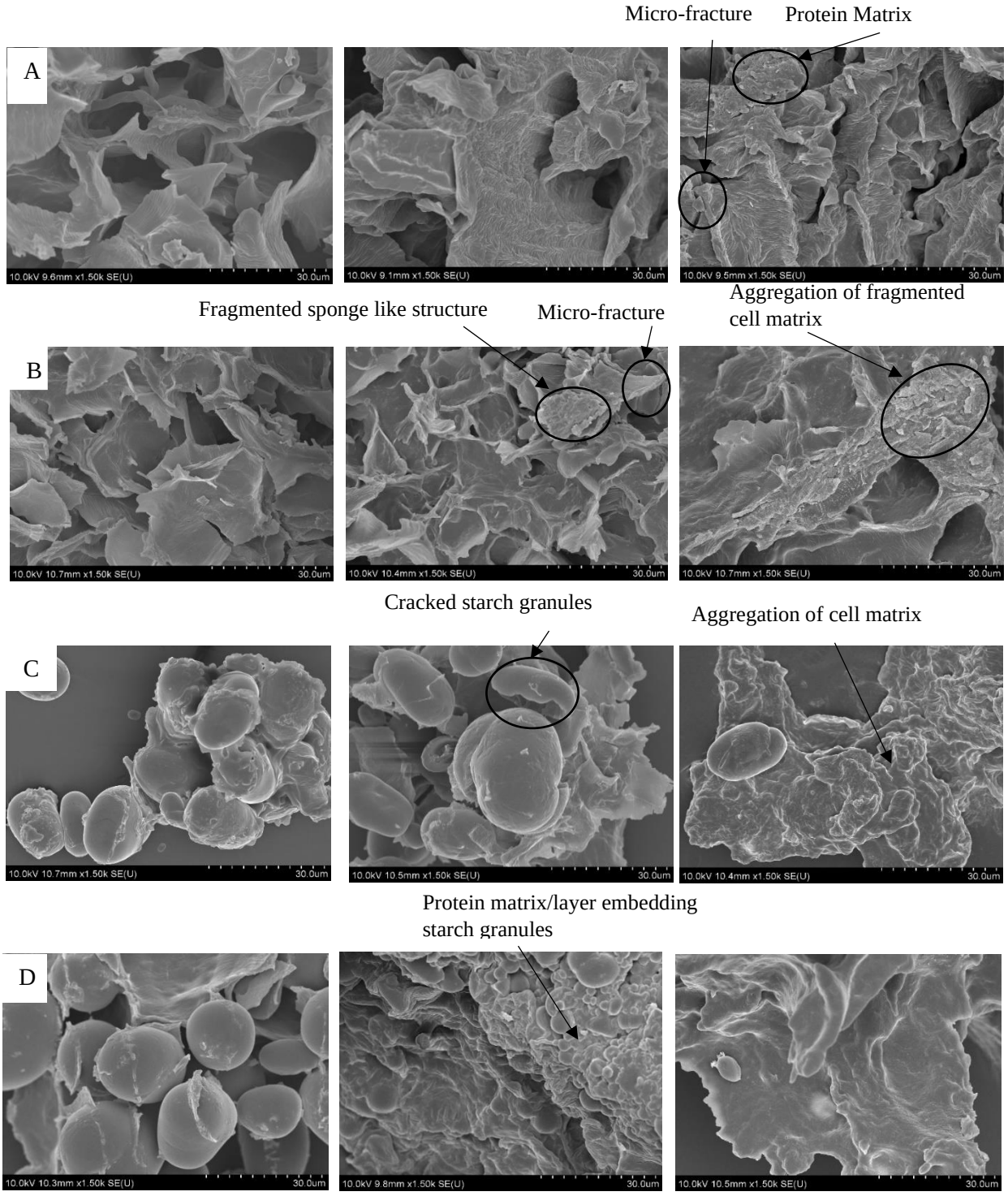


Figure 8 Scanning electron microscopy of spent substrate before and after sonication of A: soy flakes, B: soy flour, C: chickpea, and D: kidney bean. Left (Unsonicated), Middle (Lower PD) and Right (Higher PD)

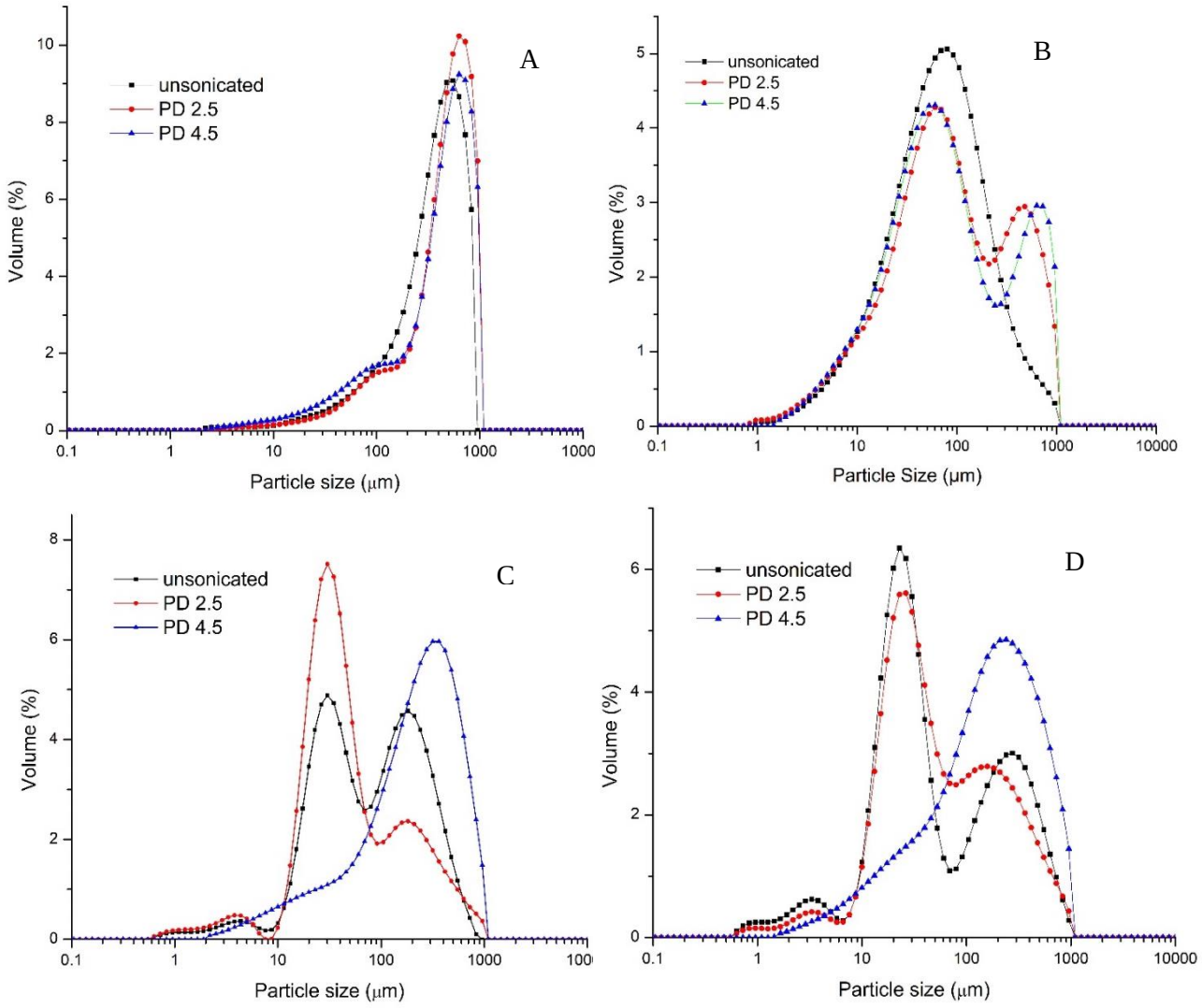
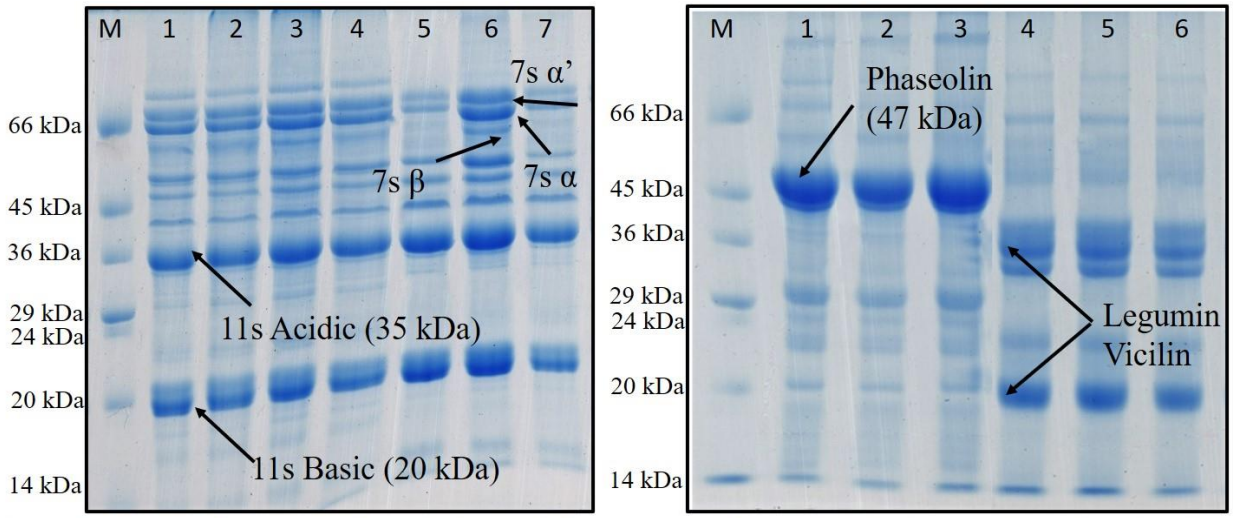


Figure 9 Particle size distribution of (A) Soy flakes; (B) Soy flour; (C) Kidney bean; (D) Chickpea at Unsonicated, Lower PD and Higher PD



Soy flour protein 1: unsonicated, 2: PD 2.5, 3: PD 4.5, 4: soy flour protein- control 5: soy flakes protein control, 6: PD 2.5, 7: PD 4.5.

1: Unsonicated, 2: PD 2.5, 3: PD 4.5, 4: control, 5: PD 2.5, 6: PD 4.5

Figure 10 Gel electrophoretic pattern of sonicated legume-proteins

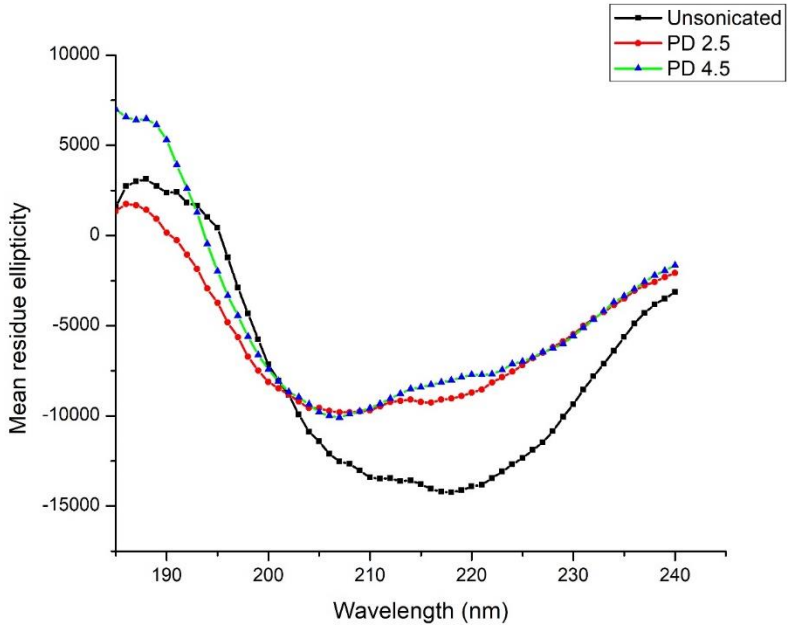


Figure 11 Representative CD spectra of unsonicated and sonicated kidney bean proteins measured at 185-240 nm

Tables

Table 4 Proximate analysis (% dry basis) of legumes before sonication treatment

Sample	Moisture	Protein	Fat	Crude Fiber	Ash	Carbohydrate
Soy flakes	8.52 ± 0.06	54.95 ± 1.28	0.79 ± 0.09	1.77 ± 0.15	6.29 ± 0.05	36.21
Soy flour	8.99 ± 0.18	53.11 ± 0.95	0.62 ± 0.19	1.24 ±0.26	6.18 ± 0.02	38.86
Kidney bean	7.34 ± 0.12	23.84 ± 0.72	1.31 ± 0.12	0.35 ± 0.07	3.60 ± 0.03	70.90
Chickpea	5.89 ± 0.09	23.66 ± 0.64	7.03 ± 0.17	0.57 ± 0.06	2.74 ± 0.01	65.99

Table 5 Percent protein in powder product prepared after sonication at two power densities (W/mL)

Substrate	Treatment	% Protein in product
Soy flakes	Unsonicated	65.76 ± 0.46[a]
	PD 2.5	64.08 ± 0.47[a]
	PD 4.5	57.00 ± 1.21[b]
Soy flour	Unsonicated	60.04 ± 1.28[a]
	PD 2.5	59.32 ± 1.65[a]
	PD 4.5	59.48 ± 3.90[a]
Kidney bean	Unsonicated	59.55 ± 2.66[a]
	PD 2.5	48.66 ± 4.28 [a]
	PD 4.5	39.91 ± 2.47[b]
Chickpea	Unsonicated	54.17 ± 4.77[a]
	PD 2.5	34.17 ± 0.94[b]
	PD 4.5	35.44 ± 1.62[b]

Mean % protein in the product was compared within each substrate for the sonication effect. Results having different superscript letters within each substrate group show a significant sonication effect ($p<0.05$).

Table 6 Free sulfhydryl in unsonicated and sonicated plant-based protein preparations at two power densities (W/mL)

Substrate	Treatment	Free SH (µmole/gram of protein)
Soy flakes	Unsonicated	4.31 ± 0.21[a]
	PD 2.5	4.54 ± 0.02[a]
	PD 4.5	4.85 ± 0.94[a]
Soy flour	Unsonicated	6.41 ± 0.43[a]
	PD 2.5	5.50 ± 0.60[a]
	PD 4.5	5.89 ± 0.26[a]
Kidney bean	Unsonicated	3.95 ± 0.87[a]
	PD 2.5	11.81 ± 1.44[b]
	PD 4.5	13.67 ± 3.85[b]
Chickpea	Unsonicated	6.76 ± 0.55[a]
	PD 2.5	8.38 ± 3.52[a]
	PD 4.5	19.30 ± 2.0[b]

Mean free sulfhydryl content were compared within each substrate for sonication effects. Results having different superscript letters within each substrate group show a significant sonication effect ($p<0.05$).

Table 7 Secondary structure composition of unsonicated and sonicated legume from CD in far UV region (180-260 nm)

Substrate	Treatment (W/mL)	α-Helix	β-Strands	β-Turns	Unordered
Soy flakes protein	Unsonicated	25.2 ± 2.2[a]	21.8 ± 4.4[a]	20.5 ± 0.8[a]	32.4 ± 2.9[a]
	PD 2.5	26.0 ± 1.9[a]	20.1 ± 1.4[a]	20.3 ± 0.4[a]	33.3 ± 0.9[a]
	PD 4.5	13.0 ± 5.2[a]	37.1 ± 5.0[b]	25.7 ± 5.4[a]	24.0 ± 4.7[a]
Soy flour protein	Unsonicated	26.2 ± 0.6[a]	19.9 ± 0.6[a]	19.9 ± 2.0[a]	33.8 ± 3.3[a]
	PD 2.5	28.1 ± 1.0[a]	20.7 ± 0.0[a]	21.8 ± 0.4[a]	29.2 ± 0.6[a]
	PD 4.5	25.4 ±4.9[a]	25.1 ± 4.5[a]	20.4 ± 1.5[a]	29.8 ± 1.8[a]
Kidney bean protein	Unsonicated	23.5 ± 0.8[a]	22.4 ± 1.1[a]	20.5 ± 1.6[a]	33.2 ± 3.5[a]
	PD 2.5	16.2 ± 1.8[a]	16.6 ± 1.06[b]	14.7 ± 1.5[a]	52.3 ± 4.5[b]
	PD 4.5	14.3 ± 2.8[a]	19.5 ± 0.4[b]	15.3 ± 2.1[a]	50.7 ± 5.1[b]
Chickpea protein	Unsonicated	21.6 ± 1.4[a]	17.6 ±1.3[a]	17.1±2.[a]	43.6 ± 4.8[a]
	PD 2.5	18.4 ± 4.4[a]	20.7± 6.3[a]	18.5 ±1.0[a]	42.1 ± 2.9[a]
	PD 4.5	16.4 ± 1.8[a]	18.8 ±1.7[a]	16.0 ±1.7[a]	48.6 ±1.7[a]

Mean secondary structure composition was compared within each substrate for the sonication effect. Results having different superscript letters within each substrate group show a significant sonication effect ($p<0.05$).

CHAPTER 4: FERMENTATION PERFORMANCE AND NUTRITIONAL ASSESSMENT OF PHYSICALLY PROCESSED LENTIL AND GREEN PEA FLOUR.

Bibek Byanju and Buddhi P. Lamsal, Iowa State University

Modified from a manuscript to be submitted to LWT-Food Science and Technology

4.1 Abstract

A significant amount of nutrients, including dietary fibers, proteins, minerals, and vitamins are present in legumes, but the presence of anti-nutritional factors (ANFs) like phytic acid, tannins, and enzyme inhibitors impact their availability. These ANFs could be reduced or modified with physical/ biochemical processes, for example, extrusion, sonication, and fermentation. In this research, the effect of a combination of a physical process (sonication or precooking) and fermentation on some legume ANFs was evaluated. Flours of soybean, lentil, and green peas were sonicated for 2 and 4 min (power density ~ 2.5 W/mL) at a 1:8 ratio (substrate: water) and fermented. Physically modified flours were inoculated with *Lactobacillus plantarum* and *Pediococcus acidilactici* at 10^8 CFU/mL and fermented in shake flasks for 72 h at 37°C, and 200 rpm. The microbial growth and pH were measured at 6, 12, 24, 48, and 72 h. The pH dropped from 6.5 to 4.5 during the initial 24 h and microbial growth reached around 10^{13} CFU/mL at 24 h. The population doubling time for *L. plantarum* was shortest on precooked green pea flour (0.94 h) and longest on 4 min sonicated green pea flour (2.46 h). Similarly, the doubling time for *P. acidilactici* was the least for precooked lentil (0.9 h) and greatest for 2 min sonicated green pea flour (1.36 h). Total phenolic contents were significantly ($p<0.05$) reduced for all physically modified and fermented substrates compared to non-fermented controls. Trypsin inhibitory activity (TIA) was

reduced significantly for all the substrates except for unsonicated soybean and lentil fermented with *L. plantarum* and *P. acidilactici*. When physical processing was done, there was a decrease in TIA. Phytic acid content decreased for physically modified soybean and lentil but not significant for green pea. Even though there was a decrease in ANFs, there was no significant change in in vitro protein digestibility for all substrates except for unsonicated *L. plantarum* fermented soybean flour and precooked *L. plantarum* fermented lentil.

Keywords: Fermentation, high-power sonication, anti-nutritional factors (ANFs), plant proteins, and digestibility

4.2 Introduction

Legumes are plants in the Leguminosae family that includes beans, peas, lentils, chickpea, and soybean, and are grown worldwide. Around 73 million metric tons (MMT) of pulses are produced globally with dry beans (*Phaseolus vulgaris*), chickpea (*Cicer arietinum*), lentil (*Len culinaris*), and dry pea (*Pisum sativum*) accounting for about 52 MMT (Patterson et al., 2017). There has been growing interest in the use of whole pulse, pulse flour, protein, starch, dietary fibers, and bioactive compounds for food and non-food applications. Cheaper pulse proteins and their derivatives can be substituted for animal-based protein and other essential nutritional components (Adebo et al., 2017). Even though pulses contain a high amount of proteins, dietary fiber, minerals, and vitamins, their use in food products is still not prevalent due to presence of off-flavor and several ANFs like tannins, trypsin inhibitors, phytic acid, and flatulence causing compounds (e.g., stachyose, raffinose, and verbascose). Phytic acid chelates essential dietary minerals, protein, and starch, which then reduces their bioavailability in humans. Tannins and trypsin inhibitors inhibit the digestive enzymes, thus reducing the digestion and absorption of dietary proteins and carbohydrates (Khattab & Arntfield, 2009).

Physical and biochemical processing generally modify favorably some physicochemical attributes of plant-based food ingredients, including pulses. Many traditional processes such as soaking, dehulling, boiling, germination, autoclaving, and microwave-assisted cooking are reported to impact the nutritional composition and anti-nutritional factors in pulses, for example, mung beans, white kidney beans, and cowpea (Mubarak, 2005). Thermal treatment at high temperatures has the potential to enrich the nutritional quality of legumes. ANFs like trypsin inhibitors, phytic acid, phenolics, and tannins are sensitive to heat and are reduced during processing. Chickpea, dry beans, faba beans, dry peas, and lentils, when exposed to thermal treatments, reduced ANFs; thus, increasing the digestibility and enhancing the nutritional profile (Patterson et al., 2017).

Similarly, sonication is a relatively newer application in the food processing industry and mostly used for its disruption of cell matrices at higher intensities. Ultrasound with low frequency (16-100 kHz) and power intensity of 10-1000 W/cm^2 are known as high power sonication (HPS). When HPS is applied to the aqueous medium, cavitation bubbles are formed and collapse leading to extreme temperatures (5000 K) and pressures (1000 atm) that produce high shear and turbulence in localized cavitation zones (Suslick et al., 1986). Cavitation disintegrates cellular matrices and facilitates the extraction of protein, sugar, polyphenols, isoflavones, oils, and saponins from the plant cell. The use of high-power sonication increased the sugar release of defatted soy flakes by 50% compared to untreated (Karki et al., 2010), which can be utilized in fermentation by microbes to modify and improve substrate characteristics.

Fermentation is another simple and low-cost bioprocessing technology that has been used to enhance nutritional and quality aspects of food ingredients, reduce undesirable compounds and enrich with essential amino acids and vitamins (Liu et al., 2011). Controlled fermentation is preferred to enhance the nutritional profile, texture, color, appearance, flavor, shelf life, and protein digestibility of ingredients, including pulses, as opposed to natural fermentation depending on naturally occurring microbes (Chandra-Hioe et al., 2016). Fermentation also has the added benefit of providing probiotic effects, if carried with food-grade probiotic bacteria. *Lactobacillus, Lactococcus, Streptococcus, Leuconostoc,* and *Pediococcus* are common lactic acid bacteria used during fermentation due to their favorable acidic and organoleptic properties (Liu et al., 2011). Fermentation of chickpea with *Rhizopus oligosporous* for 72 h increased protein content by 21.7% (Reyes-Moreno et al., 2004). Fermentation caused a 72% reduction of oligosaccharides that causes flatulence like stachyose, raffinose, and verbascose. These reductions may be attributed to the secretion of hydrolytic enzymes like α-galactosidase. While there are several other processing techniques for food modification, fermentation is important because it improves sensory qualities, reduces pathogenic microorganisms, and enhance functional and health beneficial effects of food. Soy flour, lentil flour, and green pea flour containing a higher amount of carbohydrates (~ 30-70%) can be used as a substrate for probiotic microorganisms like *Lactobacillus plantarum* and *Pediococcus acidilactici*. Functional properties of proteins are also modified by physicochemical modifications brought about by processing, for example, extrusion, high power sonication, and fermentation. Fermentation makes hydrolysis of proteins easier and alters functionality. Fermentation has also been shown to modify the functionality of fava bean and chickpea (Chandra-Hioe et al., 2016). Xiao et al. (2015) reported the significant increase of water holding capacity by fermentation of chickpea flour with *Cordyceps militaris*.

There has been limited research on the modification of ANFs in pulse flours through a physical process (heat treatment or high-power sonication) and successive fermentation by common probiotic bacteria. In this study, precooked or sonicated lentil, green pea flour was fermented with *L. plantarum* and *P. acidilactici*, and evaluated for resulting protein and nutritional changes. The specific objectives of this study were to 1) compare fermentation performance by *L. plantarum* and *P. acidilactici* in some physically modified pulse flours, and 2) evaluate the impact of physical modification and fermentation on the nutritional and anti-nutritional factors in the substrate.

4.3 Material and methods

4.3.1 Flours and reagents

Green pea seeds, lentil seeds, precooked lentil, and pea flours were provided by Dr. Donna Winham, Iowa State University (Ames, IA). Soy flour (80-90 PDI) was obtained from Archer Daniels Midland Company (Decatur, IL). De Man, Rogosa and Sharpe (MRS) media, ferric chloride hexahydrate, pancreatin, gallic acid, sulfosalicylic acid, polyvinyl-polypirrolidone (PVPP), folin ciocalteu reagents were purchased from Fisher Scientific (Waltham, MA, USA). Benzoyl-DL,-arginine-p-nitoanalide hydrochloric (BAPA) and trypsin porcine pancreas were purchased from VWR (Chicago, IL). All the chemicals used were of analytical grade.

4.3.2 Preparation of initial substrate

Pea and lentil samples were processed into flours at North Dakota State University (Fargo, ND) by Dr. Clifford Hall. Each step was applied individually to the pulses (the peas and lentils were not combined). First, pulses were soaked overnight at 25C in water (10-parts water 1-part pulse). The pulses were not dehulled. Second, pulses were drained over a 40-mesh sieve (Gilson Inc., Lewis OH), with any material passing through the screen discarded. Then, pulses were placed

on perforated baking pans in single layers (approximately 0.45 kg per tray). Heat treatment was completed in a Baxter OV300G Mini Rotating Rack Convection Oven (Baxter Manufacturing Co., Orting WA) set at 149ºC for 18 minutes (lentil) or 33 minutes (peas). Next, the pulses were mixed at five-minute intervals until the end of their heating time. After the mixing step, the heat-treated pulses were milled with a roller mill, then sifted through an 80 mesh and 100 mesh sieves.

Whole seeds were prepared into flour as described below. Milling was done by passing beans through a Witt corrugated roller mill (Witt Corrugating Inc., Wichita, KS) with 0.03" gap roller corrugation of 1/8" followed by 0.02" gap and 1/16" corrugation. They were then ground using a Nutri mill (Pleasant Hill Grain, NE, USA). Each flour slurry at a (1:8 w/v substrate: water) of raw green pea, lentil, and soybean was sonicated for 2 and 4 min at 100% amplitude (power density~ 2.5 W/mL) with a 2.2 kW sonicator (Branson 2000 Series, Branson Ultrasonics Corporation, Danbury, CT, USA). The schematic diagram of the entire process is shown in Fig.12.

4.3.3 Proximate analyses

The proximate analyses for all the unmodified samples were carried out using standard methods by Eurofins Scientific Inc., (Des Moines, IA). Moisture, protein, ash, fat, and crude fiber contents were analyzed according to AACC international methods 44-15A, 46-30, 08-01, Ba 3-38, 32-50.01, and 76-13, respectively. Carbohydrate content was calculated by difference (100 − sum of other components).

4.3.4 Microorganisms and fermentation

Lactobacillus plantarum and *Pediococcus acidilactici* were provided by Lallemand Animal Nutrition-North America (Milwaukee, WI, USA). The microbes were stated to have a viable count of 2.5 x 10^{11} CFU per gram of dry carrier. Substrate slurries (precooked, sonicated, or control) were prepared with 1:8 w/v ratio (substrate: water), adjusted to pH 6.5 and inoculated

with *Lactobacillus plantarum* and *Pediococcus acidilactici* at 10^8 CFU/mL and fermented in shake flasks for 72 h at 37°C and 200 rpm. The inoculation (powder) was done directly without preparing seed media. The microbial growths and pH were measured at 6, 12, 24, 48, and 72 h. The microbial viable count was calculated by serial dilution plate count method under a biosafety cabinet (Sanders, 2012). The microbial growth rate was compared based on the specific growth rate (SGR) parameter, μ, during the exponential phase. The logarithm of cell count during the exponential phase was plotted against time. The resulting plot was fitted with a linear equation (Eq. 1). The slope of this line is the specific growth rate of a microorganism, μ.

$$\text{Ln}(X) = \mu\, t + \text{Ln}(X_0) \ldots\ldots\ldots \quad (1)$$

where X is a number of cells at a given time t during the log phase, X_0 is the initial number of cells at the beginning of the exponential phase. Doubling times of microorganisms (t_d) were calculated by dividing 0.693 by μ. All the treatments were performed in duplicate.

4.3.5 Evaluation of modified flours

4.3.5.1 Total phenolic content

The total phenolic content (TPC) was determined using the Folin-Ciocalteu assay with slight modification (Chandra et al., 2014). Flour samples (0.5 g) were extracted with 7.5 mL 1% HCl in methanol for 2 h and centrifuged at 2000 x *g* and 25°C for 10 min. The supernatant extract (0.2 mL) was mixed with 0.6 mL of distilled water and 0.2 mL of Folin-Ciocalteu's phenol reagent (1: 1 v/v reagent: distilled water). One mL of saturated sodium carbonate solution (8% w/v in water) was added after 5 min and the volume was made up to 3 mL with distilled water. They were stored in dark for 30 min and absorbance was measured at 765 nm using UV-visible spectrophotometer (Shimadzu UV 160). The phenolic content was calculated as gallic acid equivalents GAE/g of dry flour. All assay determinations were carried out in duplicate.

4.3.5.2 Trypsin inhibitor assay

Trypsin inhibitor assay (TIA) was carried using colorimetric assay using a UV-visible spectrophotometer (Shimadzu UV 160) with a slight modification to Liu and Markakis (1989) and Çabuk et al. (2018). Briefly, 0.25 g of raw/ fermented sample was placed in a 50-mL centrifuge tube and 25 mL of 0.01 M NaOH was added. Tubes were then vortexed for 1 min and stirred on a mechanical stirrer at 500 rpm for 3 h. The mixture was centrifuged (Thermo Sorvall legend XT, Thermo Fisher Scientific, MA, USA) at 14000 x *g* for 10 min at 4°C. One mL of supernatant was used for TIA assay where 2 mL of BAPA and 0.5 mL of trypsin were also added and mixed. The reaction was stopped by adding 1 mL of acetic acid after 10 min. The absorbance of the reaction mix was measured at 410 nm in a spectrophotometer (Shimadzu UV 160). One trypsin inhibitory unit (TIU) was equivalent to an increase of 0.01 absorbance unit at 410 nm per 10 mL of reaction mixture compared to the blank sample that had a trypsin solution added after acetic acid. TIA was defined as the number of trypsin units inhibited per mg of dry flours.

4.3.5.3 In vitro protein digestibility of modified substrates

The in vitro protein digestibility (IVPD) was evaluated based on a method described by Akeson & Stahmann, (1964), with modifications (Almeida et a., 2015). Briefly, 0.25 g of each raw/ fermented flour or 250 mL of deionized water (for the blank) was suspended in 15 mL of 0.1 N HCl containing 1.5 mg/mL pepsin and incubated for 3 h at 37ºC in a water bath. The pepsin hydrolysis was neutralized with the addition of 7.5 mL of 0.5 N of NaOH. Then, the pancreatic digestion was started with the addition of 10 mL of 0.2 mol/L phosphate buffer (pH 8.0), containing 10 mg of pancreatin with 1 mL of 0.005 mol/L sodium azide and incubated at 37ºC overnight. After the pancreatic digestion, 1 mL of 10 g/100 mL of trichloroacetic acid (TCA) was

added, followed by centrifugation at 503 x *g* for 20 min. The supernatant was collected, and the total protein content was estimated by BCA (Bicinchoninic acid) assay. The IVPD values were calculated according to the equation:

$$\% \text{ Digestibility} = (Ns-Nb)/Ns * 100$$

Where, Ns and Nb represent the nitrogen content in supernatants of the sample and the blank, respectively.

4.3.5.4 Phytic acid determination

Phytic acid was determined using the method of Gao et al. (2007). Samples of 500 mg fermented modified flours were mixed with 10 mL of 2.4% HCl, mixed for 16 h and then centrifuged at 2000 x *g* and 10ºC for 20 min. The supernatants were transferred to 14-mL Falcon tubes containing 1 g NaCl, shaken at 350 rpm for 20 min to dissolve the salt and were settled at 4°C for 60 min. The mixtures were centrifuged at 2000 x *g* and 10ºC for 20 min, and clear NaCl treated supernatants were collected for color development. This treatment precipitated matrix components that could interfere with the colorimetric reaction. The clear supernatant (1 mL) was diluted 25-fold by mixing with 24 mL of distilled water. Three milliliters of this diluted sample were combined with 1 mL of modified Wade reagent (0.03% $FeCl_3 \cdot 6H_2O$ + 0.3% sulfosalicylic acid), vortexed, and centrifuged at 2000 x *g* at 10°C for 10 min. A series of calibration standards containing 0, 0.224, 0.448, 0.896, and 1.12 µg/mL PA-P (phytic acid phosphorous) were prepared from phytic acid dodeca-sodium salt hydrate the phosphorous content of which was determined as 20.11%. The absorbance of color reaction products for both samples and standards was read at 500 nm and phytic acid was calculated in g/100 g of flour.

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

Protein solutions extracted from the physically processed and fermented substrates were subjected to SDS-PAGE with slight modification (Banach et al., 2013). Two types of gel, 13% resolution gel (Acryl-Bisacrylamide) at the bottom and 4% percent stacking gel at the top were prepared. The protein concentration of 1.5 mg/mL was prepared in sample buffer (15.1 g/L Tris, 300 g/L urea, 2 g/L SDS, 20 mL/L glycerol, and 0.1 g/L bromophenol blue) and incubated at 80°C for 5 min. The protein standard (6,500 – 66,000 Da, Product number M3913-SigmaMarker™) and physically processed/ fermented samples were loaded onto gel at equal volume (15 µL) and electrophoresed at a constant voltage of 200V for 50 min using standard SDS buffer (25mM Tris, 191 mM glycine and 1 g SDS per liter). The gels were stained with Coomassie blue for 1 h and de-stained with methanol: acetic acid: deionized water in ratio 10:2:8 until the gels were clear and transparent.

4.3.6 Statistical analyses

The experimental design was a randomized complete block design (RCBD) with two replications. Statistical analyses were performed using the JMP® statistical methods (100 SAS Campus Drive, Cary, NC). Two-way analysis of variance (ANOVA) and Tukey tests were performed to assess the effect of physical modification/fermentation. Treatment means were compared within each substrate. Results having different superscript letters within each substrate group show a significant difference ($p < 0.05$). Graphs were prepared using GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

4.4 Results and discussion

4.4.1 Proximate composition

The proximate composition of the initial substrate before fermentation is given in Table 8. Green pea and precooked green pea have similar composition of protein, ash, carbohydrates, moisture, fat, and calories. These pulses are an excellent source of protein and carbohydrates. Proteins in precooked and raw green pea were 18.84% and 19.75%, respectively, and are in close agreement with Millar et al. (2019). Similarly, lentil and precooked lentil have 24.31% and 24.78% protein, respectively, and high carbohydrate contents. The composition of green pea is comparable to the report by Han & Baik, (2008). Due to the inclusion of a seed coat, while milling, the fiber contents in all the substrates were higher.

4.4.2 Microorganism growth performance on physically modified substrates

The viable microbial population and change in pH during fermentation of modified substrates by *L. plantarum* and *P. acidilactici* are presented in Fig. 13 and Fig. 14, respectively. The exponential growth for *L. plantarum* for all the precooked and raw substrates was observed between 6 and 24 h, except for precooked green pea flour for which it was 6-48 h. Similarly, the exponential growth of *L. plantarum* for sonicated substrates was observed between 6 and 48 h (Fig. 13A). The pH was adjusted initially to 6.5 before fermentation as it was an optimal pH for microbial growth. The pH decreased significantly during the first 24 h fermentation for *L. plantarum* as the microbial population was the highest during this time (Fig. 13B). After 24 h, the pH slightly increased to a pH range of 4.2- 4.8.

For *P. acidilactici*, the exponential growth was observed between 6 and 24 h for all physically processed flours (Fig. 14A). The pH decreased significantly during the first 24 h fermentation for *P. acidilactici*, as the microbial population was the highest during this time (Fig. 14B). As the microorganism used were facultative hetero-fermentative, there is a production of lactic acid as well as acetic acid which reduces the pH.

The specific growth rates (μ), and population doubling times ($t_d = 0.693/\mu$) for *L. plantarum* and *P. acidilactici* for physically modified substrates are presented in Table 9. *L. plantarum* had the highest growth rates on 2- and 4-min sonicated soybean flours at 0.95±0.03 h^{-1} and 0.76±0.02 h^{-1} respectively, compared to unsonicated flour, followed by precooked green pea (0.74±0.03 h^{-1}), precooked lentil (0.73±0.01 h^{-1}), unsonicated lentil (0.72±0.02 h^{-1}), and unsonicated green pea (0.70±0.03 h^{-1}). Similarly, *P. acidilactici* had the highest growth rate of 0.78±0.05 h^{-1}, 0.77±0.03 h^{-1}, 0.77±0.03 h^{-1}, and 0.76±0.20 h^{-1} for 2 min sonicated lentil, precooked lentil, 4 min, and 2 min sonicated soybean flour, followed by 4 min sonicated lentil (0.76±0.00 h^{-1}), respectively. Compared to *L. plantarum, P. acidilactici* had a lower population doubling time for most of the substrate, resulting in the highest growth rate.

4.4.3 Impact of fermentation on modified flours

4.4.3.1 In vitro protein digestibility

In vitro protein digestibility of physically processed fermented substrates is presented in Fig. 15. The protein digestibility of physically processed substrates fermented with *L. plantarum* and *P. acidilactici* was generally above 85%. Also, the IVPD was higher for precooked lentil and green pea flours, compared to raw counterparts, possibly due to high temperature which causes denaturation of proteins as well as inactivation of enzyme inhibitors and other anti-nutritional factors (Nosworthy et al., 2018). Similarly, the IVPD of raw green pea (89.12%), lentil (91.87%),

and soybean (96.72%) from our research were higher than reported i.e. 82.60, 79, and 71.80%, respectively, by Han et al. (2007), Baik & Han (2012), which might be due to different processing conditions and cultivar. It could also be due to autoclaving (121ºC, 30 min) before fermentation. Autoclaving potentially leads to a decrease in anti-nutritional factors and expose protein to greater denaturation and enzymatic hydrolysis (Batista et al., 2020). The highest protein digestibility was seen in soybean, irrespective of the sonication and fermentation conditions. There was a significant increase in digestibility when unsonicated soybean flour was fermented with *L. plantarum*. When flours were sonicated for 2 min and 4 min, there was no significant change in protein digestibility of soybean flour. Also, there were no significant changes in protein digestibility for green pea and lentil when sonicated and fermented with *L. plantarum* and *P. acidilactici*. There was a significant increase in IVPD when substrates were precooked and fermented for green pea (Fig. 15 middle). Similarly, IVPD improved for lentil when precooked and fermented by *L. plantarum* (Fig. 15 bottom). Ogodo et al. (2018) reported an increase in IVPD of soybean meal fermented with lactic acid bacteria (LAB) consortium from 85% to 93.5% which was due to the pH reduction, thus enhancing proteolytic enzyme activity and breaking proteins into small peptides.

4.4.3.2 Total phenolic content

The total phenolic contents for raw, modified, and fermented soybean, green pea, and lentil are given in Table 10. The highest phenolic content was observed in raw soybean flour (4.6±0.22 mg GAE/ dry g). After soybean flours were sonicated (2 and 4 min) and fermented with *L. plantarum*, and *P. acidilactici*, there was a significant decrease in phenolic contents. Georgetti et al. (2009) and Juan et al. (2009) have reported the total phenolic contents of 15.4 mg GAE/g and 15.94 mg GAE/g for soybean flour which is higher than what we obtained. The reduced phenolic content we observed is likely due to the thermal treatment before fermentation. Xu et al. (2008) reported total phenolic content of pressure boiled (15 psi, 15 min) green pea (0.66 mg GAE/g) that

is lower than our values (Table 10). Torino et al. (2013) also reported the higher total phenolic content for lentil (32 mg GAE/ g) compared to our results i.e. 1.9 mg GAE/ g. Green pea and lentil flours when sonicated and fermented also showed a significant decrease in phenolic contents irrespective of microorganisms used. The precooked green pea and lentil also showed the same decreasing trend when fermented. Chi et al. (2016) also reported the decrease in total phenolic contents when soybean meal was fermented with *L. plantarum* and *L. acidophilus*, which was reportedly due to lower pH activity. The acidic environment results in abstraction of hydride ions which rearranges the structure of phenolic compounds, hence, unable to be detected by Folin ciocalteu reagents. This loss of phenolic compounds can be attributed to the chemical transformation, formation of protein-phenolic complex, and decomposition during thermal treatments (Kalpanadevi et al., 2013).

4.4.3.3 Trypsin inhibitor activity

Trypsin inhibitors are a type of ANFs that hinder pancreatic protease activity and absorption of dietary proteins. TIA expressed in the trypsin unit inhibited in a dry sample was lower for most of the physically processed fermented soybean, green pea, and lentil flour (Figure 16). For unsonicated soybean, fermentation by *L. plantarum* did not significantly reduce TIA, on the other hand, TIA was reduced significantly by 49.8% and 52.7% when sonicated for 2 min and 4 min, respectively, and fermented by *L. plantarum*. Similarly, fermentation of unsonicated soybean by *P. acidilactici* did not reduce TIA significantly ($p > 0.05$) but reduced the value by 34.5% and 46.7% when sonicated for 2 min and 4 min, respectively.

For unsonicated green pea, fermentation with *L. plantarum* significantly reduced TIA by 47.3% compared to raw sample, while sonication pretreatment for 2- and 4-min. reduced TIA by 48.1 and 48.9% when fermented by *L. plantarum*. Similarly, fermentation of unsonicated green

pea by *P. acidilactici* reduced TIA significantly by 46.9% and the combination with sonication for 2 min and 4 min reduced TIA further by 48.9% and 46.9%, respectively. The reduction of TIA for precooked green pea was 78% when compared to its raw counterpart. Çabuk et al. (2018) also reported the decrease in TIA when pea protein concentrate was fermented by *L. plantarum*. This reduction of TIA was due to heat treatment as well as fermentation, which degrades or modifies trypsin inhibitors resulting in losing its activity to bind to trypsin (Chen et al., 2013). The highest reduction, 83%, was seen in precooked green pea followed by fermentation with *L. plantarum* or *P. acidilactici*.

For unsonicated lentil, fermentation with *L. plantarum* and *P. acidilactici* did not significantly reduce the TIA. When lentil was sonicated for 2 min or 4 min, and then fermented by *L. plantarum*, the TIA was reduced significantly by 21.9 and 24.4%, respectively, compared to raw lentil. Also, TIA was reduced by 21.4 and 27.6% when sonicated for 2 and 4 min and fermented by *P. acidilactici*. Precooked lentil followed by fermentation using *L. plantarum* and *P. acidilactici* showed the highest reductions, i.e. 80.6 and 91.6%, respectively. Physical processing and subsequent fermentation by these probiotic microbes reduced the trypsin inhibitor activity and enhanced the nutritional profiles of these substrates.

4.4.3.4 Phytic acid

Table 11 shows the phytic acid (PA) content of raw and physically processed then fermented substrates. The phytic acid content of soybean flour was the highest among all substrates, particularly with raw flour at 41.22 mg/100 g. Ojokoh et al. (2011) and Shi et al. (2018) reported phytic acid content of around 275 mg/100g to 2.29 g/100 for soybean meal, which is higher than that obtained in our results (Table 11). Our lower values could be attributed to all the substrates being autoclaved for 30 min at 121ºC prior to fermentation and PA assays. Avanza et

al. (2013) and Khattab et al. (2009), reported that phytic acid is heat-labile and it forms insoluble complexes between phytate and other components like calcium and magnesium, thus decreasing the phytic acid content. Sonication followed by fermentation was effective in reducing the phytic acid content for soybean flour. Compared to 2 min sonication of soybean flour, 4 min sonication significantly reduced phytic acid in soybean flour fermented by *L. plantarum* and *P. acidilactici* (by 42 and 41%, respectively). During fermentation, phytases are produced, which catalyzes the conversion of phytate to inorganic orthophosphate, thus reducing the phytic acid content as was observed during physical processing and fermentation of soybean flour (Adeyemo et al., 2013).

The Phytic acid content of raw green pea has been reported in the range of 543 mg/100 g to 855 mg/ 100 g (Millar et al., 2019, Shi et al., 2018). For raw and precooked green pea flour, there was only a minor reduction in PA content when physically processed and fermented. For lentil, phytic acid content was reported in the range of 0.86-1.71 g/100 g for various cultivar (Shi et al., 2018). Phytic acid content was reduced greatly in unsonicated lentil, from 7.20 mg/100 g (raw) to 0.91 mg/100 g and 2.92 mg/100 g, when fermented by *L. plantarum* and *P. acidilactici*, respectively, compared with that of the sonicated then fermented samples. Also, 2 min sonication and fermentation were optimal for reducing phytic acid in lentil. For precooked and then fermented lentil, both microorganisms were able to reduce the phytic acid contents significantly.

4.4.3.5 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Figure 17 presents the electrophoretic pattern of protein subunits obtained from green pea, lentil, and soybean. In unmodified (raw) substrates (lane A1, B1, and C1), and precooked substrate (lane A6 and B6), there were high molecular weight (MW) bands as well as higher intensity (darker in color) at MW>36 kDa. Similarly, physical processing and then fermentation by *L. plantarum* and *P. acidilactici* led to considerably modified, as indicated by reduced band intensity (lighter in

color) for all the substrates (green pea: A2-A5, A7-A8, lentil: B2-B5, B7-B8, soybean: C2-C5). This is possibly due to extensive proteolytic activity on the protein during fermentation. Di Stefano et al. (2019) reported that fermentation of green lentils and yellow pea with *L. plantarum* decreased the subunit band intensity due to proteolytic enzymes that hydrolyzed the proteins, which formed fractions with MW less than 10 kDa. Kiers et al. (2000) also fermented soybean with *Bacillus subtilis* and reported that the protein bands virtually disappeared after fermentation.

4.5 Conclusions

Both modified and unmodified soybean flour, green pea flour, and lentil flour supported the growth of *L. plantarum* and *P. acidilactici*. The fermentation of this physically processed legume and pulse flours influenced the non-nutritive compounds. The phytic acid contents were significantly reduced for soybean flour and lentil flour when sonicated or precooked and fermented. Similarly, trypsin inhibitors were also reduced for most of the physically processed and fermented substrates. Total phenolic content was reduced significantly when physically processed substrates were fermented. Physical modification along with fermentation did not affect the protein digestibility for nearly all the substrates, except for the extruded green pea. This study demonstrated the impacts of fermentation and some physical processing such as sonication and precooking on reducing the ANFs in pulse-based ingredients, thereby potentially leading to enhanced nutritional quality.

4.6 Acknowledgments

This paper is a product of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa; Project No. IOW05447 is sponsored by Hatch Act and State of Iowa Funds. The project was also funded partially by USDA/NIFA, Award no. 2018-67017-27560. Special thanks to Dr. David Grewell for initial help with sonication equipment.

4.7 References

Adebo, O. A., Njobeh, P. B., Adebiyi, J. A., Gbashi, S., Phoku, J. Z., & Kayitesi, E. (2017). fermented pulse-based food products in developing nations as functional foods and ingredients. Johannesburg, Gauteng, South Africa. https://doi.org/10.5772/intechopen.69170

Adeyemo, S. M., & Onilude, A. A. (2013). Enzymatic reduction of anti-nutritional factors in fermenting soybeans by *Lactobacillus plantarum* isolates from fermenting cereals. *Nigerian Food Journal*, *31*(2), 84–90. https://doi.org/10.1016/s0189-7241 (15)30080-1

Akeson, W. R., & Stahmann, M. A. (1964). A pepsin pancreatin digest index of protein quality evaluation. *Journal of Nutrition*, *83*, 257–261.

Almeida, C. C., Monteiro, M. L. G., Costa-Lima, B. R. C. da, Alvares, T. S., & Conte-Junior, C. A. (2015). In vitro digestibility of commercial whey protein supplements. *LWT - Food Science and Technology*, *61*(1), 7–11. https://doi.org/10.1016/J.LWT.2014.11.038

Avanza, M., Acevedo, B., Chaves, M., & Añón, M. (2013). Nutritional and anti-nutritional components of four cowpea varieties under thermal treatments: Principal component analysis. *LWT - Food Science and Technology*, *51*, 148–157. https://doi.org/10.1016/j.lwt.2012.09.010

Baik, B. K., & Han, I. H. (2012). Cooking, roasting, and fermentation of chickpeas, lentils, peas, and soybeans for fortification of leavened bread. *Cereal Chemistry*, *89*(6), 269–275. https://doi.org/10.1094/cchem-04-12-0047-r

Batista, K. A., Pereira, W. J., Moreira, B. R., Silva, C. N., & Fernandes, K. F. (2020). Effect of autoclaving on the nutritional quality of hard-to-cook common beans (*Phaseolus vulgaris*). *International Journal of Environment, Agriculture and Biotechnology (IJEAB)*, *5*(1), 22–30. https://doi.org/10.22161/ijeab.51.4

Çabuk, B., Nosworthy, M. G., Stone, A. K., Korber, D. R., Tanaka, T., House, J. D., & Nickerson, M. T. (2018). Effect of fermentation on the protein digestibility and levels of non-nutritive compounds of pea protein concentrate. *Food Technology and Biotechnology*, *56*(2), 257–264. https://doi.org/10.17113/ftb.56.02.18.5450

Chandra-Hioe, M. V., Wong, C. H. M., & Arcot, J. (2016). The potential use of fermented chickpea and faba bean flour as food ingredients. *Plant Foods for Human Nutrition*, *71*(1), 90–95. https://doi.org/10.1007/s11130-016-0532-y

Chandra, S., Khan, S., Avula, B., Lata, H., Yang, M. H., Elsohly, M. A., & Khan, I. A. (2014). Assessment of total phenolic and flavonoid content, antioxidant properties, and yield of aeroponically and conventionally grown leafy vegetables and fruit crops: A comparative study. *Evidence-Based Complementary and Alternative Medicine*, *2014*. https://doi.org/10.1155/2014/253875

Chen, L., L. Madl, R., V. Vadlani, P., Li, L., & Wang, W. (2013). Value - added products from soybean: removal of anti- nutritional factors via bioprocessing. *Soybean - Bio-Active Compounds* (pp. 161–179). https://doi.org/10.5772/52993

Di Stefano, E., Tsopmo, A., Oliviero, T., Fogliano, V., & Udenigwe, C. C. (2019). Bioprocessing of common pulses changed seed microstructures, and improved dipeptidyl peptidase-IV and α-glucosidase inhibitory activities. *Scientific Reports*, *9*(1), 1–13. https://doi.org/10.1038/s41598-019-51547-5

Gao, Y., Shang, C., Saghai Maroof, M. A., Biyashev, R. M., Grabau, E. A., Kwanyuen, P., Burton, J. W., Buss, G. R. (2007). A modified colorimetric method for phytic acid analysis in soybean. *Crop Science*, *47*(5), 1797–1803. https://doi.org/10.2135/cropsci2007.03.0122

Georgetti, S. R., Vicentini, F. T. M. C., Yokoyama, C. Y., Borin, M. F., Spadaro, A. C. C., & Fonseca, M. J. V. (2009). Enhanced in vitro and in vivo antioxidant activity and mobilization of free phenolic compounds of soybean flour fermented with different β-glucosidase-producing fungi. *Journal of Applied Microbiology*, *106*(2), 459–466. https://doi.org/10.1111/j.1365-2672.2008.03978.x

Han, H., & Baik, B.-K. (2008). Antioxidant activity and phenolic content of lentils (*Lens culinaris*), chickpeas (*Cicer arietinum L*), peas (*Pisum sativum L*) and soybeans (*Glycine max*), and their quantitative changes during processing. *International Journal of Food Science & Technology*, *43*(11), 1971–1978. https://doi.org/10.1111/j.1365-2621.2008.01800.x

Han, I. H., Swanson, B. G., & Baik, B.-K. (2007). Protein digestibility of selected legumes treated with ultrasound and high hydrostatic pressure during soaking. *Cereal Chemistry*, *84*(5), 518–521. https://doi.org/10.1094/cchem-84-5-0518

Juan, M.-Y., & Chou, C.-C. (2010). Enhancement of antioxidant activity, total phenolic and flavonoid content of black soybeans by solid state fermentation with *Bacillus subtilis BCRC 14715*. *Food Microbiology*, *27*, 586–591. https://doi.org/10.1016/j.fm.2009.11.002

Karki, B., Lamsal, B. P., Jung, S., van Leeuwen, J. (Hans), Pometto, A. L., Grewell, D., & Khanal, S. K. (2010). Enhancing protein and sugar release from defatted soy flakes using ultrasound technology. *Journal of Food Engineering*, *96*(2), 270–278. https://doi.org/10.1016/J.Jfoodeng.2009.07.023

Khattab, R. Y., & Arntfield, S. D. (2009). Nutritional quality of legume seeds as affected by some physical treatments 2.Antinutritional factors. *LWT - Food Science and Technology*, *42*(6), 1113–1118. https://doi.org/10.1016/j.lwt.2009.02.004

Kiers, J. L., Van Laeken, A. E. A., Rombouts, F. M., & Nout, M. J. R. (2000). In vitro digestibility of Bacillus fermented soya bean. *International Journal of Food Microbiology*, *60*, 163–169.

Liu, S., Han, Y., & Zhou, Z. (2011). Lactic acid bacteria in traditional fermented Chinese foods. *Food Research International*, *44*(3), 643–651. https://doi.org/10.1016/j.foodres.2010.12.034

Millar, K. A., Gallagher, E., Burke, R., McCarthy, S., & Barry-Ryan, C. (2019). Proximate composition and anti-nutritional factors of fava-bean (*Vicia faba*), green-pea and yellow-pea (*Pisum sativum*) flour. *Journal of Food Composition and Analysis*, *82*, 103233. https://doi.org/10.1016/j.jfca.2019.103233

Mubarak, A. E. (2005). Nutritional composition and antinutritional factors of mung bean seeds (*Phaseolus aureus*) as affected by some home traditional processes. *Food Chemistry*, *89*(4), 489–495. https://doi.org/10.1016/j.foodchem.2004.01.007

Nosworthy, M. G., Medina, G., Franczyk, A. J., Neufeld, J., Appah, P., Utioh, A., Frohlich, P., House, J. D. (2018). Effect of processing on the in vitro and in vivo protein quality of beans (*Phaseolus vulgaris* and *Vicia Faba*). *Nutrients*, *10*(6). https://doi.org/10.3390/nu10060671

Ogodo, A. C., Ugbogu, O. C., Onyeagba, R. A., & Okereke, H. C. (2018). In-vitro starch and protein digestibility and proximate composition of soybean flour fermented with lactic acid bacteria (LAB) consortia. *Agriculture and Natural Resources*, *52*(5), 503–509. https://doi.org/10.1016/j.anres.2018.10.001

Ojokoh, A. O., & Yimin, W. (2011). Effect of fermentation on chemical composition and nutritional quality of extruded and fermented soya products. *International Journal of Food Engineering*, *7*(4), 1–16. https://doi.org/10.2202/1556-3758.1857

Patterson, C. A., Curran, J., & Der, T. (2017). Effect of processing on antinutrient compounds in pulses. *Cereal Chemistry*, *94*(1), 2–10. https://doi.org/10.1094/cchem-05-16-0144-fi

Reyes-Moreno, C., Cuevas-Rodríguez, E., Milán-Carrillo, J., Cárdenas-Valenzuela, O., & Barrón-Hoyos, J. (2004). Solid state fermentation process for producing chickpea(*Cicer arietinum L*) tempeh flour. Physicochemical and nutritional characteristics of the product. *Journal of the Science of Food and Agriculture*, *84*(3), 271–278. https://doi.org/10.1002/jsfa.1637

Sanders, E. R. (2012). Aseptic laboratory techniques: Plating methods. *Journal of Visualized Experiments*, (63), 1–18. https://doi.org/10.3791/3064

Shi, L., Arntfield, S. D., & Nickerson, M. (2018). Changes in levels of phytic acid, lectins and oxalates during soaking and cooking of Canadian pulses. *Food Research International Journal*, *107*, 660–668. https://doi.org/10.1016/j.foodres.2018.02.056

Singh, S., Gamlath, S., & Wakeling, L. (2007). Nutritional aspects of food extrusion: A review. *International Journal of Food Science and Technology*, *42*(8), 916–929. https://doi.org/10.1111/j.1365-2621.2006.01309.x

Torino, M. I., Limón, R. I., Martínez-Villaluenga, C., Mäkinen, S., Pihlanto, A., Vidal-Valverde, C., & Frias, J. (2013). Antioxidant and antihypertensive properties of liquid and solid-state fermented lentils. *Food Chemistry*, *136*(2), 1030–1037. https://doi.org/10.1016/j.foodchem.2012.09.015

Towo, E., Matuschek, E., & Svanberg, U. (2006). Fermentation and enzyme treatment of tannin sorghum gruels: Effects on phenolic compounds, phytate and in vitro accessible iron. *Food Chemistry*, *94*(3), 369–376. https://doi.org/10.1016/j.foodchem.2004.11.027

Xiao, Y., Xing, G., Rui, X., Li, W., Chen, X., Jiang, M., & Dong, M. (2015). Effect of solid-state fermentation with *Cordyceps militaris SN-18* on physicochemical and functional properties of chickpea (*Cicer arietinum L*) flour. *LWT - Food Science and Technology*, *63*(2), 1317–1324. https://doi.org/10.1016/j.lwt.2015.04.046

Xu, B., & Chang, S. K. C. (2008). Effect of soaking, boiling, and steaming on total phenolic content and antioxidant activities of cool season food legumes. *Food Chemistry*, *110*, 1–13. https://doi.org/10.1016/j.foodchem.2008.01.045

Figures

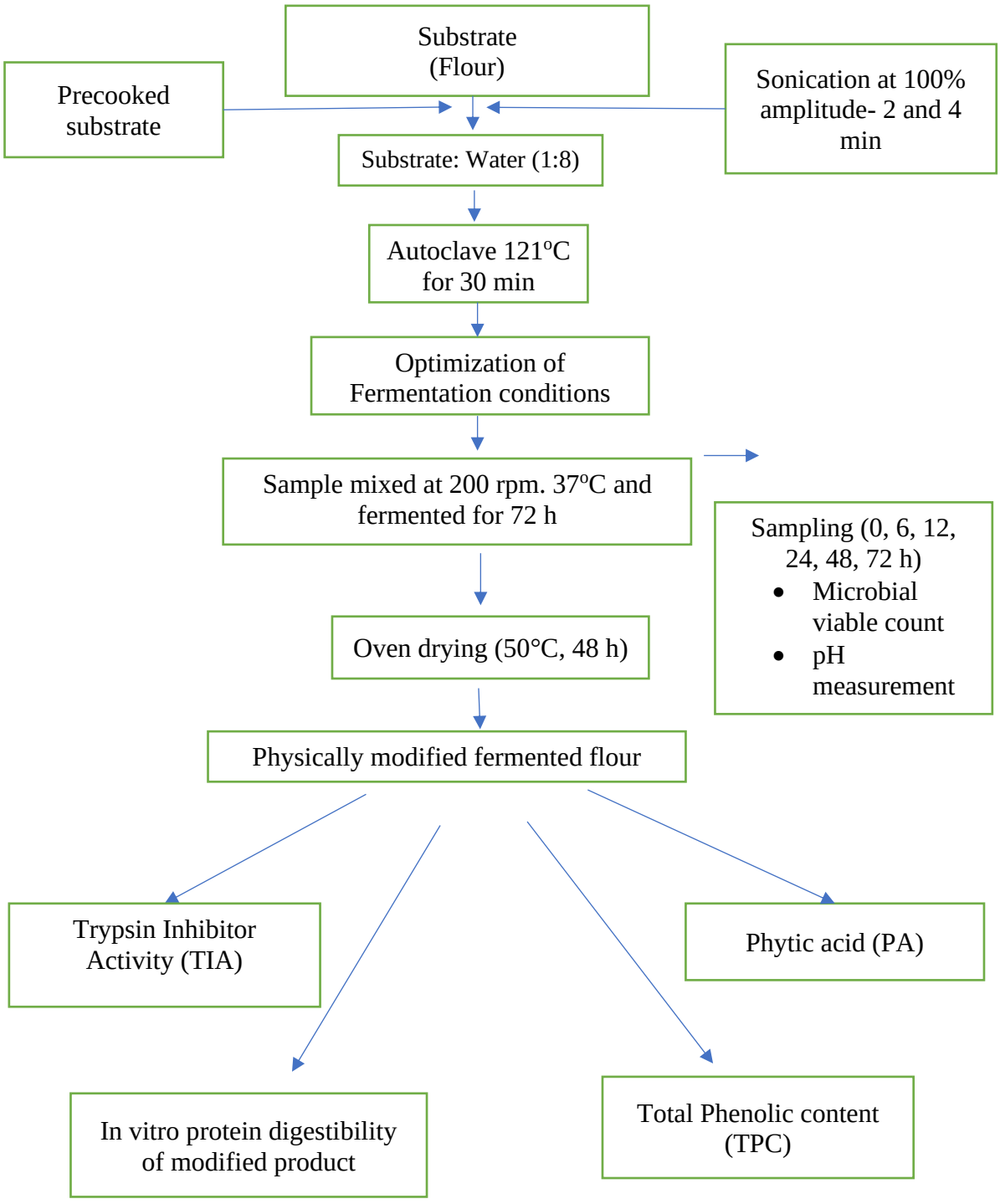


Figure 12 Conceptual framework: flour modification with two processing options and evaluation of resulting ingredients

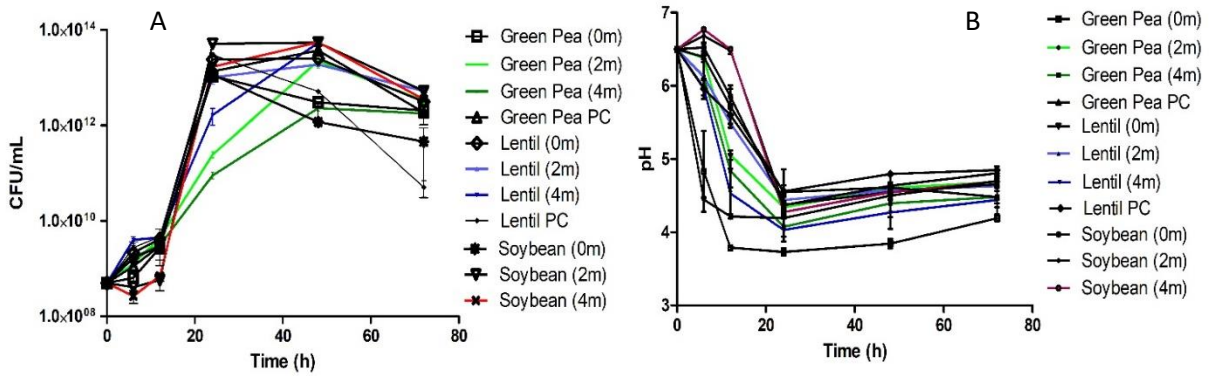


Figure 13 Microbial viable population (A) and pH (B) of *Lactobacillus plantarum* for physically modified substrates

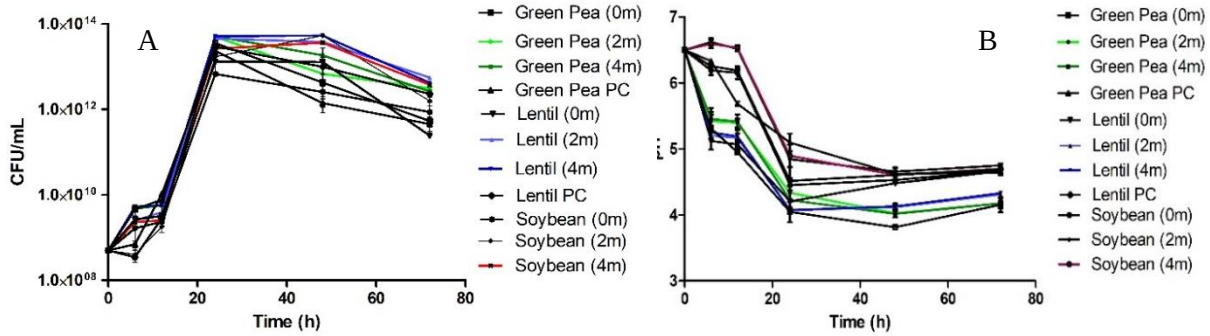


Figure 14 Microbial viable population (A) and pH (B) of *Pediococcus acidilactici* for physically modified substrates

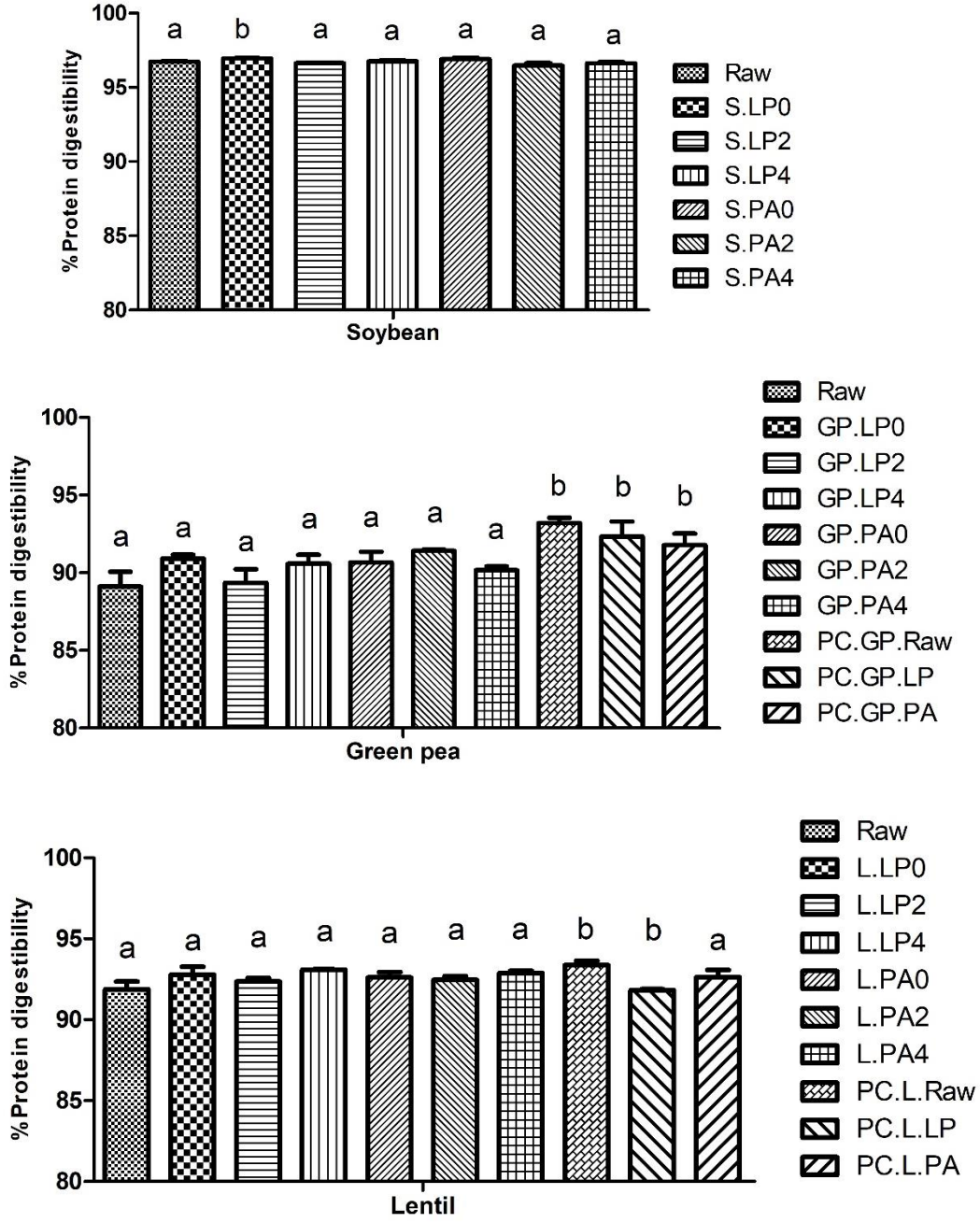


Figure 15 In vitro protein digestibility of physically modified fermented substrates: Top: Soybean; Middle: Green pea; Bottom: Lentil

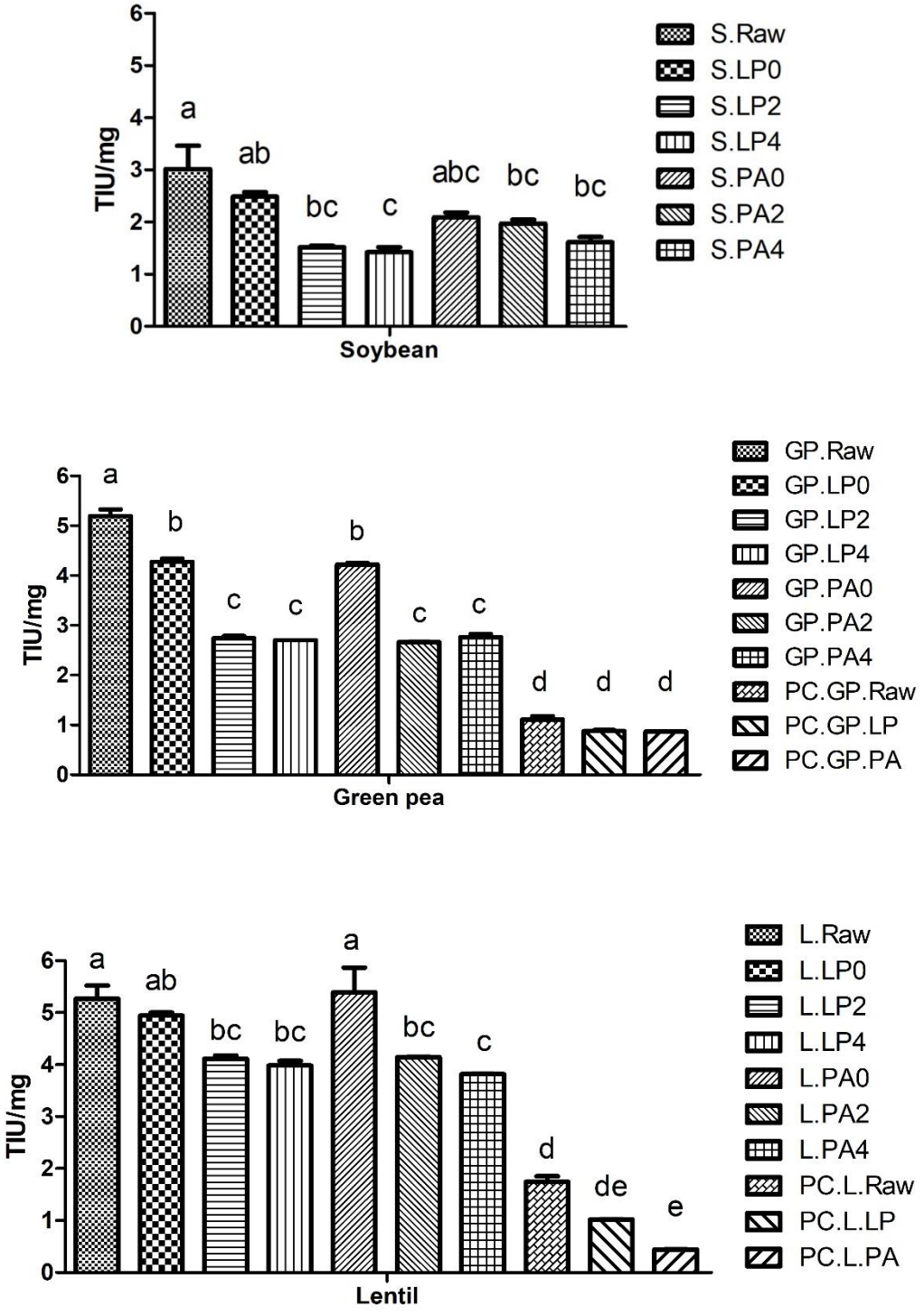
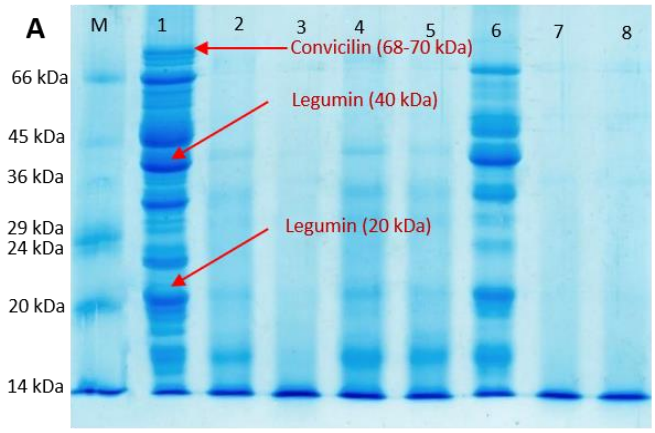
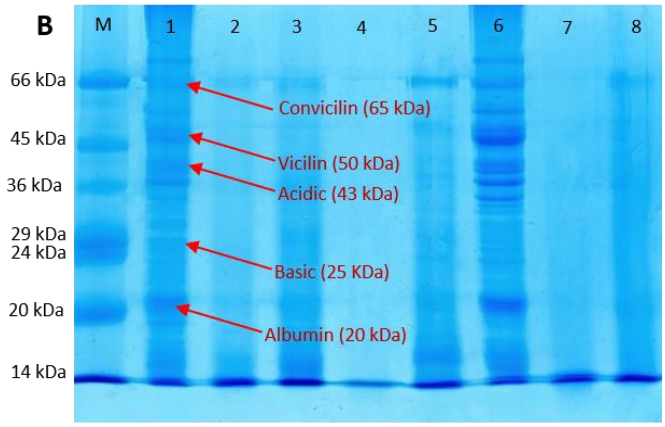


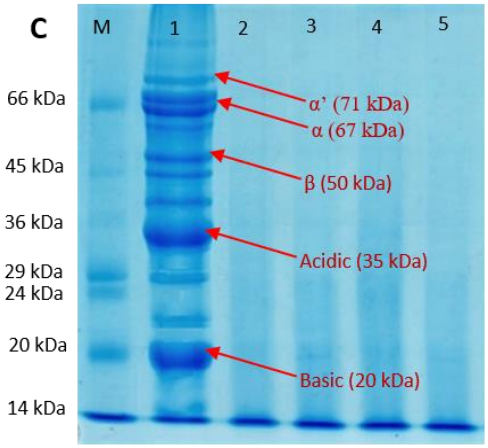
Figure 16 Trypsin inhibitory activity of physically processed fermented substrates: Top: Soybean; Middle: Green pea; Bottom: Lentil



Green pea protein: M-Marker, 1-Raw, 2-LPF, 3-LPF4, 4-PAF, 5-PAF4, 6-PC. raw, 7-PC. LP, 8-PC. PA



Lentil protein: M-Marker, 1-Raw, 2-LPF, 3-LPF4, 4-PAF, 5-PAF4, 6-PC. raw, 7-PC. LP, 8-PC. PA



Soybean protein: M-Marker, 1-Raw, 2-LPF, 3-LPF4, 4-PAF, 5-PAF4

Figure 17 Gel electrophoresis of physically processed and fermented substrate. (A) Green pea, (B) Lentil, (C) Soybean

Tables

Table 8 Proximate composition of plant substrate before physical modification

Substrate	Protein (%)	Ash (%)	Carbohydrate (%)	Moisture (5%)	Fat (%)	Fiber (%)
Green Pea	19.75	2.55	66.65	8.60	2.45	15.90
Precooked Green Pea	18.84	2.43	66.82	9.20	2.71	16.00
Lentil	24.31	2.62	62.15	8.70	2.20	19.30
Precooked Lentil	24.78	2.55	60.35	10.10	2.22	19.10
Soy flour	53.11	6.18	38.86	9.00	0.62	1.24

Table 9 Specific growth rates (μ), and doubling time (t_d) for microorganisms used to ferment physically processed substrates

Microorganism	Substrate	Sonication time (min)	μ (h^{-1})	t_d (h)
L. plantarum	Green Pea	0	0.70 ± 0.03[a]	1.00 ± 0.04
		2	0.34 ± 0.01[b]	2.07 ± 0.05
		4	0.28 ± 0.01[b]	2.46 ± 0.09
	Precooked Green Pea	0	0.72 ± 0.02[a]	0.96 ± 0.02
	Lentil	0	0.71 ± 0.03[a]	0.97 ± 0.05
		2	0.64 ± 0.03[a]	1.08 ± 0.05
		4	0.49 ± 0.04[b]	1.42 ± 0.12
	Precooked Lentil	0	0.73 ± 0.01[a]	0.95 ± 0.01
	Soybean	0	0.67 ± 0.03 [a]	1.03 ± 0.04
		2	0.95 ± 0.03[b]	0.73 ± 0.03
		4	0.76 ± 0.02[a]	0.91 ± 0.02
P. acidilactici	Green Pea	0	0.70 ± 0.01[a]	0.99 ± 0.02
		2	0.75 ± 0.00[b]	0.92 ± 0.00
		4	0.76 ± 0.00[b]	0.91 ± 0.00
	Precooked Green Pea	0	0.64 ± 0.01[c]	1.08 ± 0.02
	Lentil	0	0.71 ± 0.03[a]	0.97 ± 0.05
		2	0.78 ± 0.05[a]	0.92 ± 0.00
		4	0.76 ± 0.00[a]	0.91 ± 0.00
	Precooked Lentil	0	0.77 ± 0.03[a]	0.90 ± 0.04
	Soybean	0	0.70 ± 0.06[a]	0.99 ± 0.09
		2	0.76 ± 0.02[a]	0.91 ± 0.02
		4	0.77 ± 0.03[a]	0.91 ± 0.04

Values are mean ± standard deviations (n=2). Results having different superscript letters within each substrate are significantly different ($p < 0.05$) as determined by Tukey's test.

Table 10 Total phenolic compounds of physically processed fermented substrate

Substrates	Sonication time (min)	Microbes for fermentation	TPC (mg GAE/g)
Soybean	Raw	None	4.60 ± 0.22 [a]
	0	*L. plantarum*	2.80 ± 0.14 [b]
	2		2.65 ± 0.46 [b]
	4		2.38 ± 0.54 [b]
	0	*P. acidilactici*	2.71 ± 0.31 [b]
	2		3.19 ± 0.14 [ab]
	4		2.75 ± 0.61 [b]
Green pea	Raw	*None*	1.81 ± 0.32 [a]
	0	*L. plantarum*	0.70 ± 0.12 [b]
	2		0.49 ± 0.04 [bcd]
	4		0.21 ± 0.18 [bcd]
	0	*P. acidilactici*	0.58 ± 0.11 [bc]
	2		0.10 ± 0.05 [cd]
	4		0.01 ± 0.01 [d]
Precooked Green Pea	Raw	*None*	1.75 ± 0.02 [a]
	0	*L. plantarum*	0.71 ± 0.07 [b]
	0	*P. acidilactici*	0.58 ± 0.04 [bc]
Lentil	Raw	*None*	1.90 ± 0.09 [a]
	0	*L. plantarum*	1.61 ± 0.01 [ab]
	2		1.09 ± 0.01 [bcd]
	4		0.52 ± 0.17 [d]
	0	*P. acidilactici*	1.45 ± 0.45 [abc]
	2		0.94 ± 0.10 [cd]
	4		0.59 ± 0.13 [d]
Precooked Lentil	Raw	*None*	1.69 ± 0.01 [ab]
	0	*L. plantarum*	0.66 ± 0.03 [d]
	0	*P. acidilactici*	0.66 ± 0.16 [cd]

Values are mean ± standard deviations (n=2). Results having different superscript letters within each substrate are significantly different ($p < 0.05$) as determined by Tukey's test.

Table 11 Phytic acid content of physically processed fermented substrates

Substrates	Sonication time (min)	Microbes for fermentation	Phytic acid content (mg/100g)
Soybean	Raw	None	41.22 ± 0.57 [a]
	0	*L. plantarum*	35.60 ± 0.19 [b]
	2		32.65 ± 2.08 [cd]
	4		29.03 ± 0.76 [d]
	0	*P. acidilactici*	30.77 ± 1.33 [cd]
	2		33.85 ± 1.5 [bc]
	4		29.16 ± 3.22 [cd]
Green pea	Raw	None	2.79 ± 0.38 [a]
	0	*L. plantarum*	1.85 ± 0.57 [a]
	2		1.98 ± 1.51 [a]
	4		1.31 ± 0.57 [a]
	0	*P. acidilactici*	2.92 ± 0.19 [a]
	2		1.85 ± 0.95 [a]
	4		2.12 ± 0.95 [a]
Precooked Green Pea	Raw	None	2.52 ± 1.14 [a]
	0	*L. plantarum*	2.38 ± 0.57 [a]
	0	*P. acidilactici*	1.45 ± 1.14 [a]
Lentil	Raw	None	7.20 ± 1.70 [ab]
	0	*L. plantarum*	0.91 ± 0.76 [e]
	2		3.72 ± 0.95 [cd]
	4		7.47 ± 2.08 [a]
	0	*P. acidilactici*	2.92 ± 0.95 [cde]
	2		3.19 ± 0.19 [cd]
	4		5.33 ± 2.08 [ab]
Precooked Lentil	Raw	None	8.41 ± 0.38 [a]
	0	*L. plantarum*	4.53 ± 0.57 [bc]
	0	*P. acidilactici*	1.45 ± 0.0 [de]

Values are mean ± standard deviations (n=2). Results having different superscript letters within each substrate are significantly different ($p < 0.05$) as determined by Tukey's test.

CHAPTER 5: GENERAL CONCLUSIONS

Physical treatment using high power sonication was effective in breaking the bigger particle size of soy flakes, consequently leading to increased extraction yield of plant-based proteins. High power sonication of defatted soy flakes at power density 2.5 and 4.5 W/mL resulted in higher protein extraction yields compared to unsonicated soy flake. However, with chickpea flours, sonication resulted in a decrease in protein extraction yield, perhaps due to high carbohydrate and fat contents that hindered the accessibility of solvent to proteins in cell matrices. Protein subunit bands for all the substrates were not altered by sonication as indicated by protein electrophoretic band. However, significant changes in the secondary structure of kidney bean protein were determined by circular dichroism analyses. Also, an increase of the free sulfhydryl contents in sonicated kidney bean protein and chickpea protein suggests an alteration in the structure of native protein due to partial unfolding. Our study indicated that HPS has the potential to improve the extraction of various plant proteins with altered molecular structure. This will have an impact on how these proteins will be utilized in various food applications.

Legumes and pulse are a rich source of protein, carbohydrates, and minerals but have limited use because of several ANFs such as phytic acid, trypsin inhibitors as well as lower protein digestibility. In this study, soybean, lentil, and green pea flours were subjected to physical processing (sonication or precooking) and then fermented using *L. plantarum* or *P. acidilactici.*

All the non-modified and modified substrates supported the growth of these probiotic bacteria. The fermentation of these physically modified soybean flour, green pea flour, and lentil flour influenced the composition of a non-nutritive compound. The phytic acid contents were significantly reduced for soybean flour and lentil flour when modified and then fermented with

both probiotic bacteria. Similarly, TIA was also reduced for most of the physically modified and fermented substrates. However, the total phenolic content was reduced significantly when physically processed substrates were fermented due to the low pH resulting from an abstraction of hydride ions, which rearranged the structure of phenolic compounds. Physical modification combined with fermentation did not affect protein digestibility. This study shows the potential of physical modification such as sonication and extrusion to reduce the ANFs and thus enhance the nutritional quality of pulse-based ingredients.

Overall, physical processing as a pretreatment of substrates can be effective in extracting protein in preparation of ingredients. These pretreatments alter the physical, chemical, and structural property of the substrates, which in turn changes the functionality. These protein ingredients with improved functionality can be used in a wide range of food applications, thus increasing their utilization and value for the ingredient processing companies.

The preference for vegetarian and vegan diets has provided a plethora of opportunities for industries to identify alternative food, especially from plant origin. The protein-rich legume and pulse ingredients provide food industries, as well as consumers, a variety of food alternatives that offer functional, nutritional, and health benefits. The combination of physical processes, for example, sonication and further nutrition enhancement with fermentation will help create better quality ingredients for a wide range of applications such as pasta, noodles, baked goods. This research adds to the knowledge base for the pulse processing industry that will contribute towards creating nutritious and functional protein-rich ingredients and products.